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Multi-targeted docetaxel polymeric nanoparticles as delivery systems for chemotherapy to treat glioblastoma

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*To all that filled my PhD journey
with sparkles of joy.*

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Cláudia Martins

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ABSTRACT

Glioblastoma (GBM) is the most common primary brain tumor and one of the deadliest cancers to date. The standard of care is based on maximal surgical resection followed by radiotherapy and temozolomide (TMZ) oral chemotherapy. However, this treatment regimen only renders a 15-month median survival and less than 5% of 5-year survival rate. Thus, GBM is an unmet clinical need that urgently requires new treatment approaches to combat the lethal course of the disease. The development of new treatment approaches is often hindered by the presence of the blood-brain barrier (BBB), which protects the brain from harmful agents, but also prevents the most prospective systemic drugs from reaching the brain parenchyma. As an example, docetaxel (DTX) is a promising chemotherapeutic poorly explored in the field of GBM, with a 100,000-times lower IC_{50} compared with TMZ, but a very low BBB crossing capacity (<1% of the administered dose, compared with approximately 30% for TMZ). Moreover, DTX and most of the treatment approaches lack the ability to target tumor cells of the brain, hence resulting in poor therapeutic responses. These hurdles have shifted attention to treatment modalities with capacity to precisely and sequentially target (i) BBB cells, inducing blood-to-brain transport, and (ii) GBM cells, leading to a higher accumulation of therapeutic molecules at the tumor cells of the brain. This can be accomplished through the use of nanomedicine, which provides drug depot nanoparticle (NP) systems, easily surface-functionalized with targeting ligands that recognize specific biological barriers and cell receptors, such as from the BBB and GBM. The targeting ligands usually exploit the abnormal expression of certain cell transporters or surface molecules to induce intracellular trafficking at the BBB and GBM. Thus, besides improving formulation and pharmacokinetic constraints of loaded drugs, these multi-targeted nanomedicines have potential to significantly enhance therapeutic responses and reduce off-target toxicity.

In the first part of this work, aligned with the main aim of this doctoral thesis, polymeric NPs were dual-surface tailored with a (i) brain-targeted acid-cleavable Angiopep-2 moiety to trigger shuttling of the NPs across the BBB (via binding to the BBB-overexpressed low density lipoprotein receptor), followed by their structural rearrangement within BBB endosomal vesicles (acidic pH), and (ii) L-Histidine moiety to provide NP preferential accumulation into GBM tumor cells (via binding to the tumor-overexpressed L-type amino acid transporter 1). These stimuli-responsive NPs were loaded with DTX using a continuous-flow microfluidic technique. In a panel of GBM conventional cell lines and tumor invasive margin patient cells, the stimuli-responsive NPs targeted GBM cells, enhanced cell uptake by 12-fold, and induced 3-times higher

cytotoxicity in 2D and 3D cell models. Moreover, in a BBB *in vitro* model, the permeability of NPs was increased by 3-fold. A biodistribution *in vivo* trial confirmed a 3-fold enhancement of NP accumulation into mice brain, hence highlighting the ability of stimuli-responsive NPs to cross the intact BBB. The *in vivo* anti-tumor efficacy was further investigated in a GBM orthotopic mice model following either intratumoral or intravenous administrations at a low DTX dose (2 μg per brain and 2 mg kg^{-1} , respectively). The median survival of GBM-bearing mice, and number of long-term survivors, was significantly increased by around 20 days and more than 50%, respectively. Moreover, the treatment did not present histological, hematologic or blood biochemical signs of systemic toxicity.

An important issue in the field of nanomedicine translation is the fact that disease modeling toolboxes lack close-to-native *in vitro* models that emulate GBM microenvironment and bioarchitecture, thus poorly predicting clinical responses. In the second part of this work, aligned with the secondary aim of this doctoral thesis, a human GBM multicellular tumor microtissue (MCTM) constituted by three key cell populations of the GBM microenvironment, namely U-251 MG tumor cells, macrophages derived from blood peripheral monocytes, and brain microvascular primary endothelial cells, was proposed as a novel 3D *in vitro* tool to evaluate the efficacy of nano-therapeutics. The MCTM presented a tumor-like cell spatial organization, production of extracellular matrix, necrosis areas, and a predominant pro-tumor M2-like macrophage phenotype. The previously developed and reported L-Histidine functionalized NPs, targeted to GBM, were used to load DTX and assess the ability of the MCTM to respond to chemotherapy. The NPs provided 8- and 3-times higher cell uptake and anti-proliferative effect, respectively, compared with the non-targeted nanosystem and free DTX. Moreover, the NPs tended to reduce the expression of pro-tumor macrophage markers, while increasing the levels of expression of anti-tumor macrophage markers, suggesting a possible reeducation of the immune microenvironment. Accordingly, the NPs caused a reduction in the secretion of an array of cytokines with a role in GBM pro-tumor immunity (e.g. IL-1 β , IL-6, IL-8, TGF- β).

Overall, in the one hand, the first part of the work provided a preclinical proof of concept that supported the effectiveness of the developed stimuli-responsive NPs, sequentially targeted to the BBB and brain tumor, as a powerful anti-GBM multistage chemotherapeutic strategy with ability to respond to multiple features of the GBM microenvironment. On the other hand, in the second part of the work, a GBM MCTM was proposed as a novel GBM 3D *in vitro* technology, with capacity to better contribute to the recapitulation of the bioarchitecture and composition of the native tumor, dissect GBM therapeutic responses, and ameliorate the *in vivo* predictability.

RESUMO

Glioblastoma (GBM) é o tipo de tumor primário do cérebro mais comum e um dos tipos de cancro mais letais até à data. O tratamento padrão desta doença baseia-se na máxima remoção cirúrgica, seguida de radioterapia e quimioterapia oral com temozolomida (TMZ). Contudo, este tratamento providencia apenas uma média de sobrevivência de 15 meses e uma taxa de sobrevivência a 5 anos menor do que 5%. Assim sendo, o GBM é uma área clínica em que novos tratamentos são urgentemente necessários para combater o desfecho fatal da doença. O desenvolvimento de novos tratamentos é, muitas das vezes, dificultado pela presença da barreira hematoencefálica (BHE), que protege o cérebro de agentes patogénicos, mas também impede os fármacos sistémicos mais promissores de atingirem esta região anatómica. Por exemplo, o docetaxel (DTX) é um quimioterapêutico pouco explorado na área de GBM, com um IC_{50} 100.000 vezes inferior à TMZ, mas uma capacidade de permear a BHE muito baixa (<1% da dose administrada, comparativamente a aproximadamente 30% para a TMZ). Mais ainda, tanto o DTX como muitos outros tratamentos desenvolvidos apresentam uma falta de direcionamento para as células tumorais do cérebro, resultando numa baixa resposta terapêutica. Estes problemas têm atraído a atenção para modalidades de tratamento com capacidade de fazer um direcionamento preciso e sequencial para (i) a BBB, induzindo assim o transporte sangue-para-cérebro, e (ii) as células de GBM, conduzindo a um aumento da acumulação de moléculas de terapêuticos nas células tumorais do cérebro. Isto pode ser concretizado através da utilização de nanomedicinas, que consistem em nanopartículas (NPs) que funcionam como depósitos de fármacos, facilmente modificáveis ao nível da superfície com ligandos de direcionamento que reconhecem recetores de barreiras biológicas e células específicas, tais como da BHE e de GBM. Geralmente, estes ligandos de direcionamento focam-se na expressão anormal de certos transportadores e moléculas de superfície celulares para induzir o transporte intracelular na BHE e GBM. Logo, estas nanomedicinas multi-direcionadas, para além de melhorarem aspetos de formulação e farmacocinética do fármaco que encapsulam, também têm o potencial de aumentar significativamente a eficácia terapêutica e reduzir a toxicidade sistémica.

Na primeira parte do trabalho, associada ao objetivo principal desta tese de doutoramento, NPs poliméricas foram duplamente modificadas ao nível da superfície com (i) um ligando Angiopep-2 clivável em ambiente ácido e direcionado para o cérebro (através do reconhecimento do recetor de lipoproteínas de baixa densidade, sobreexpresso pelas células da BHE) para potenciar a passagem pela BBB, seguida de

um rearranjo estrutural das NPs nas vesículas endossomais (pH ácido) da barreira; e (ii) um ligando de L-Histidina para promover a acumulação das NPs preferencialmente nas células tumorais de GBM (através do reconhecimento do transportador de aminoácidos do tipo L 1, sobreexpresso pelas células tumorais). Estas NPs sensíveis a estímulos foram depois carregadas com DTX através de uma técnica de microfluídica em fluxo contínuo. Num painel de linhas celulares convencionais de GBM e células de GBM de pacientes, isoladas a partir da margem invasiva do tumor, as NPs sensíveis a estímulos apresentaram um direcionamento para as células de GBM, um aumento de 12 vezes na internalização celular e uma citotoxicidade aumentada em 3 vezes em modelos celulares 2D e 3D. Para além disto, num modelo *in vitro* de BHE, a permeabilidade das NPs através da barreira foi aumentada em 3 vezes. Num teste de biodistribuição *in vivo*, foi confirmado um aumento de 3 vezes na acumulação das NPs no cérebro de murganhos, enfatizando a capacidade das NPs sensíveis a estímulos de atravessar a BHE intacta. A eficácia anti-tumoral *in vivo* foi posteriormente analisada num modelo ortotópico de GBM em murganho, após administração intratumoral ou intravenosa de uma baixa dose de DTX (2 µg por cérebro e 2 mg kg⁻¹, respetivamente). A sobrevivência média dos animais com GBM foi significativamente aumentada em 20 dias, e o número de sobreviventes a longo prazo aumentado em mais de 50%. O tratamento não apresentou sinais histológicos, hematológicos ou bioquímicos (sangue) de toxicidade sistémica.

Um aspeto importante na translação de nanomedicinas é o facto de que existe uma falta de modelos *in vitro* que se assemelhem à estrutura nativa do tumor e mimetizem o microambiente e bioarquitetura tumorais, limitando a capacidade de prever as respostas clínicas. Na segunda parte do trabalho, associada ao objetivo secundário desta tese de doutoramento, um micro-tecido multi-celular tumoral (MTMC) de GBM, de origem humana, foi proposto como um novo modelo 3D *in vitro* para avaliar a eficácia de nanomedicinas. O MTMC foi composto por células tumorais de GBM U-251 MG, macrófagos derivados de monócitos periféricos e células endoteliais microvasculares primárias de origem cerebral. O MTMC apresentou uma organização espacial celular semelhante ao tumor, incluindo produção de matriz extracelular, áreas de necrose e um fenótipo predominante de macrófagos do tipo M2. As NPs com um ligando superficial de L-Histidina previamente desenvolvidas e reportadas, direcionadas para o GBM, foram carregadas com DTX e utilizadas para testar a capacidade do MTMC de responder à quimioterapia. As NPs apresentaram uma internalização celular 8 vezes superior, e um efeito anti-proliferativo 3 vezes superior, comparativamente às NPs sem direcionamento para o tumor e ao DTX na forma livre. Mais ainda, as NPs apresentaram uma tendência para reduzir a expressão de marcadores de macrófagos pró-tumorais, e

aumentar a expressão de marcadores de macrófagos anti-tumorais, sugerindo uma possível reeducação do microambiente imunitário. No seguimento disto, as NPs causaram uma redução na secreção de citocinas com um papel pró-tumoral em contexto de GBM (ex: IL-1 β , IL-6, IL-8, TGF- β).

Em resumo, por um lado, a primeira parte deste trabalho apresentou uma prova de conceito pré-clínica que demonstrou a eficiência das NPs sensíveis a estímulos desenvolvidas, com um direcionamento sequencial para a BBB e tumor, como uma estratégia quimioterapêutica faseada de elevada eficácia e com capacidade de reconhecer diferentes características do microambiente de GBM. Por outro lado, na segunda parte deste trabalho, um MTMC de GBM foi proposto como uma nova tecnologia *in vitro* 3D de GBM, com capacidade melhorada para representar a bioarquitetura e composição do tumor nativo, perceber mecanismos de resposta terapêutica e fazer uma previsão do efeito *in vivo*.

ACRONYMS AND ABBREVIATIONS LIST

¹H NMR: Proton nuclear magnetic resonance

5ALA: 5-aminolevulinic acid

ACUPA: S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid

AE: Association efficiency

AMT: Adsorptive-mediated transcytosis

ANG2: Angiopep-2 peptide

ANOVA: Analysis of variance

BBB: Blood-brain barrier

BBTB: Blood-brain tumor barrier

CHUSJ: Centro Hospitalar Universitário São João

CL-ANG2-NP: Nanoparticle with a surface acid-cleavable Angiopep-2 moiety

CNS: Central nervous system

CPP: Cell penetrating peptide

CSC: Cancer stem cell

DC: Dendritic cell

DLS: Dynamic light scattering

DMEM: Dulbecco's modified Eagle's medium

DOX: Doxorubicin

DTX: Docetaxel

EC: Endothelial cell

ECM: Extracellular matrix

EGFR: Epidermal growth factor receptor

EP-1: Epidermal growth factor receptor-targeting peptide

ETPN: European Technology Platform on Nanomedicine

FBS: Fetal bovine serum

FDA: Food and Drug Administration

FTIR: Fourier-transform infrared spectroscopy

GBM: Glioblastoma

ACRONYMS AND ABBREVIATIONS LIST

GIN: Glioblastoma invasive margin

GRP78: Glucose-regulated protein 78

H&E: Hematoxylin and eosin

H-CL-ANG2-NP: Nanoparticle with surface L-Histidine and acid-cleavable Angiopep-2 moieties

H-NCL-ANG2-NP: Nanoparticle with surface L-Histidine and non-cleavable Angiopep-2 moieties

H-NP: Nanoparticle with a surface L-Histidine moiety

HIF-1: Hypoxia inducible factor 1

HIF-2: Hypoxia inducible factor 1

His: L-Histidine amino acid

HPLC: High-performance liquid chromatography

i3S: Instituto de Investigação e Inovação em Saúde

IARC: International Agency for Research on Cancer

IHC: Immunohistochemistry

IL-13: Interleukin-13

KBr: Potassium bromide

LAT1: L-type amino acid transporter 1

LD: Loading degree

LDA: Laser Doppler anemometry

LDLR: Low-density lipoprotein receptor

MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MCTM: Multicellular tumor microtissue

MDSC: Myeloid-derived suppressor cell

MFI: Mean fluorescence intensity

MMP: Matrix metalloproteinase

MMP-2: Matrix metalloproteinase-2

Mn: Number average molecular weight

MON: Monocyte

mPEG NP: Non-targeted nanoparticle with surface polyethylene glycol

mPEG(2K) NP: Non-targeted nanoparticle with surface polyethylene glycol (2K molecular weight)

mPEG(5K) NP: Non-targeted nanoparticle with surface polyethylene glycol (5K molecular weight)

MRI: Magnetic resonance imaging

MW: Molecular weight

nAChR: Nicotine acetylcholine receptor

NCL-ANG2-NP: Nanoparticle with a surface non-cleavable Angiopep-2 moiety

NHA: Non-tumor astrocytes

NIH: National Institutes of Health

NK: Natural killer

NP: Nanoparticle

NPC: Neural progenitor cell

NRP1R: Neuropilin-1 receptor

OS: Overall survival

PBS: Phosphate buffered saline

PCL: Polycaprolactone

PD-1: Programmed death 1

PD-L1: Programmed death-ligand 1

PDI: Polydispersity index

PEG: Polyethylene glycol

Pen: Penetratin

PET: Positron emission tomography

PFA: Paraformaldehyde

PFS: Progression-free survival

pHA: P-hydroxybenzoic acid

PLA: Poly(lactic acid)

PLGA: Poly(lactic-co-glycolic acid)

PSMA: Prostate-specific membrane antigen

PTX: Paclitaxel

R: Regional

ACRONYMS AND ABBREVIATIONS LIST

RARE: Rapid acquisition with relaxation enhancement

RBT: $[\text{Ru}(\text{bpy})_2(\text{tip})]^{2+}$

RMT: Receptor-mediated transcytosis

ROI: Regions of interest

RT: Room temperature

SOC: Standard of care

STR: Single tandem repeat

SVZ: Subventricular zone

TAM: Tumor-associated macrophage

TAN: Tumor-associated neutrophil

TE_{eff}: Effective echo time

TEER: Transendothelial electrical resistance

T_{eff}: Effector T cells

TEM: Transmission electron microscopy

Tf: Transferrin

TfR: Transferrin receptor

TME: Tumor microenvironment

TMZ: Temozolomide

TPC: Total polymer content

TR: Repetition time

Treg: Regulatory T cells

TTF: Tumor treating field

WCA: Water contact angle

CHAPTER I. Literature review

This chapter was based on the following publications:

- **C Martins**, B Sarmiento. “Multi-ligand functionalized blood-to-tumor sequential targeting strategies in the field of glioblastoma nanomedicine” (manuscript under review)
- **C Martins**, C Pacheco, B Sarmiento. “The effect of glioblastoma microenvironment on therapeutic, diagnostic, or theranostic systems”, in New Insights into Glioblastoma: Diagnosis, Therapeutics and Theranostics, Ed. C Vitorino, C Cabral, C Balaña, under review, Elsevier, 2022
- **C Martins**, VM Chauhan, M Araújo, A Abouselo, C Barrias, JW Aylott, B Sarmiento. “Advanced polymeric nanotechnology to augment therapeutic delivery and disease diagnosis”. Nanomedicine, 15, 2287-2309 (2020)

ABSTRACT

Glioblastoma (GBM) is an unmet clinical need characterized by a standard of care (SOC) 5-year survival rate of only 5%, and a treatment mostly palliative. Significant hurdles in GBM therapies include an effective penetration of therapeutics through the brain protective barrier, namely the blood–brain barrier (BBB), and a successful therapeutic delivery to brain-invading tumor cells post-BBB crossing. These hurdles, along with the poor prognosis and critical heterogeneity of the disease, have shifted attention to treatment modalities with capacity to precisely and sequentially target (i) BBB cells, inducing blood-to-brain transport, and (ii) GBM cells, leading to a higher therapeutic accumulation at the tumor site. This sequential targeting allows therapeutic molecules to reach the brain parenchyma and compromise molecular processes that support tumor cell invasion. Besides improving formulation and pharmacokinetics constraints of drugs, nanomedicines offer the possibility of being surface functionalized with multiple possibilities of targeting ligands, while delivering the desired therapeutic cargos to the biological sites of interest. Targeting ligands exploit the site-specific expression or overexpression of specific molecules on BBB and GBM cells, triggering brain plus tumor transport. Since the efficacy of single-ligand functionalized nanomedicines is limited due to the GBM anatomical site (brain) and disease complexity, this review presents an overview of multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines reported in literature over the last 10 years. Moreover, in this chapter, GBM numbers, niches, key cell players and TME are presented, the role of the BBB and blood-brain tumor barrier (BBTB) in disease progression is discussed, GBM treatment options are debated, opportunities of targeted nanomedicine and possible BBB/neovasculature and tumor targets are detailed.

KEYWORDS

Blood-brain barrier; Brain drug delivery; Glioblastoma; Nanomedicine; Sequential targeting; Surface functionalization; Targeting ligands; Tumor microenvironment.

1. BACKGROUND

1.1. Glioblastoma – disease by the numbers

Cancer is a major public health problem and the second leading cause of death in the world, with rapidly growing incidence rates that are projected to double by 2040 [1, 2]. According to the estimates of cancer incidence and mortality of the International Agency for Research on Cancer (IARC) of the World Health Organization (GLOBOCAN), there were approximately 19 million new cases and 10 million deaths related with cancer worldwide in 2020, from which around 300,000 and 250,000, respectively, were due to brain and other central nervous system (CNS) cancers [3]. Among brain cancers, glioblastoma (GBM) is the most prevalent type, and accounts for nearly half of all CNS malignant tumors [4]. GBM is an aggressive type of brain cancer, with a 15-month median survival from the time of diagnosis.

1.2. The power of targeted nanomedicine

Nanomedicines have shown great promise in cancer therapy over the last decades. These systems are versatile nanoscale carriers that offer to cargos protection against harsh biological environments and modulation of their biodistribution, ultimately resulting in enhanced therapeutic outcomes along with lower side toxicity [5]. More than 50 nanomedicines are currently available in clinics to treat a variety of diseases [6]. Since nanomedicines were found to access solid tumors mainly through active transport mechanisms [7], active targeting-based systems have gained greater visibility in the field of anti-cancer therapies [8]. Active targeting strategies rely on the interaction between ligand (nanomedicine surface) and receptor (tissue of interest), promoting higher accumulation of cargos loaded into the nanosystems at the targeted site and, thus, an ameliorated therapeutic efficacy. Due to the privileged anatomical location of GBM, the brain, access of therapeutics is hindered by the presence of the most important of all gateways and difficult-to-permeate blood-brain barrier (BBB) [9]. Moreover, it is still a grand challenge to endow nanomedicines with the ability to target invading tumor cells in the brain, post-BBB crossing. Taking this into consideration, single-ligand functionalized nanomedicines, targeted to either the BBB or GBM, are not sufficient to simultaneously circumvent the BBB restrictive environment and the need to instruct drugs to accumulate at tumor cells [10]. This, along with the versatility of nanocarriers in regard to the multiple possibilities of surface functionalization, open avenues for the

exploitation of multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines.

1.3. Structure overview in brief

This literature review will be focused on multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines reported in literature over the last 10 years. Moreover, GBM biological key features will be presented (niches, cell players and tumor microenvironment (TME)), the role of the BBB in the progression of the disease will be discussed, and current treatment options will be analyzed. Lastly, BBB and GBM biological target opportunities, and their respective ligands, will be scrutinized and summarized.

2. Glioblastoma – disease niches, key cell players and tumor microenvironment

GBM is characterized by a high level of cell infiltration, migration and plasticity, as well as inter-patient heterogeneity [11]. The GBM cell origin is still characterized by lack of consensus among researchers, and grounded two distinct theories, namely the astrocytes de-differentiation and stem cell theory. The astrocytes de-differentiation theory claims that GBM originates from mature astrocytes that suffer driving mutations along the time and return to a more primitive and pluripotent state, with capacity to proliferate and give rise to heterogeneous progeny; on the other hand, the stem cell theory claims that GBM originates from stem cells of the subventricular zone (SVZ) that suffer malignant transformations and possess neurogenesis ability [12]. The TME of GBM is vast in regard to cell constituents, cell-cell/cell-extracellular matrix (ECM) interactions and function, thus leading to a tumoral organization into three typical niches – perivascular, hypoxic and invasive (**Figure 1**) [13]. Niches are considered strategic tumor areas of cancer stem cells (CSCs) residency and maximal influence of the TME on GBM malignant features such as angiogenesis [14].

The perivascular niche is home for resident or tumor-recruited non-neoplastic (e.g. pericytes, endothelial cells (ECs), macrophages derived from peripheral monocytes, microglia, infiltrating neural progenitor cells (NPCs), neutrophils and myeloid-derived suppressor cells (MDSCs)) and tumor cells, which work together to build an environment supportive of CSC maintenance, survival and growth [15]. Although macrophages can polarize into either a M1 (anti-tumor; pro-inflammatory) or M2 (pro-tumor; anti-

inflammatory) phenotype, or present a continuum of phenotypes, macrophages within the GBM TME are known to be mainly M2-like [16]. Macrophages, together with brain-intrinsic microglia, constitute the major non-neoplastic GBM cell population – tumor-associated macrophages (TAMs, 30-50% of the GBM bulk) – being this population key to support tumor phenomena such as stemness, proliferation, angiogenesis and immune suppression [17-19]. Within TAMs, and compared to microglia, macrophages of peripheral monocyte origin are more abundant ($CCR2^+/CX3CR1^-$) [20].

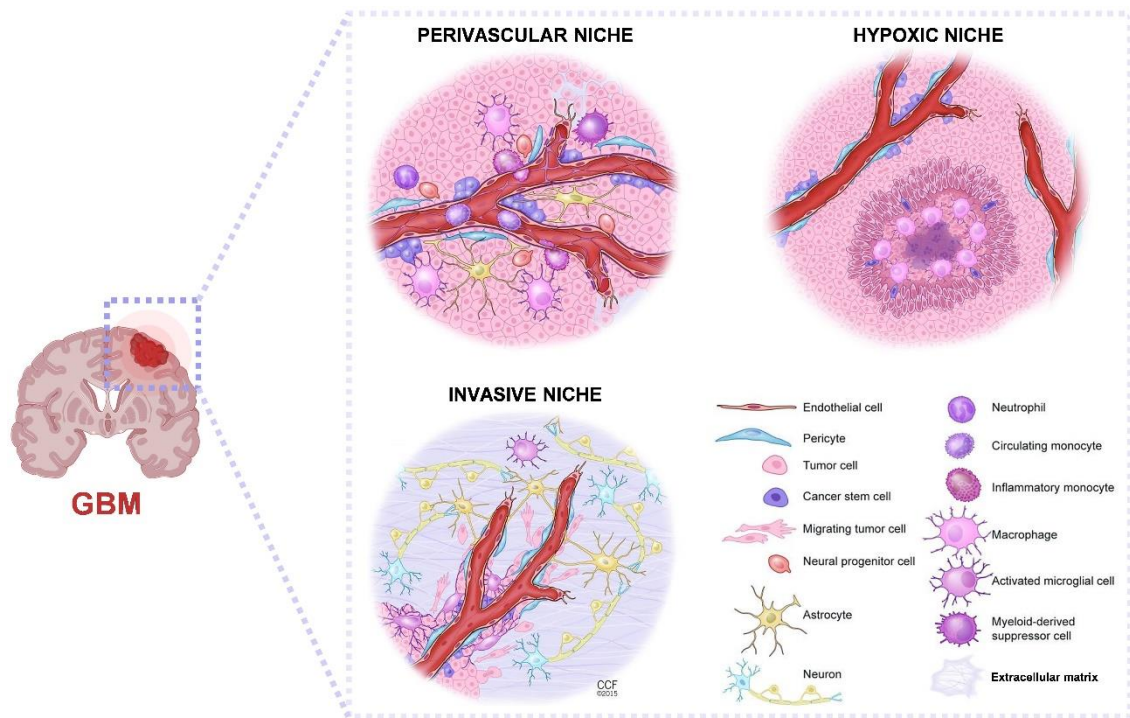


Figure 1 – GBM TME cell constituents, and inter-cellular and cell-ECM interactions, within each niche – perivascular, hypoxic and invasive. Adapted with permission from [13] and created with BioRender.com.

ECs are another example of a crucial TME non-neoplastic cell type, usually representing 5-30% of the tumor bulk in GBM patient biopsies [21]. The main role of ECs within the TME is directly related with tumor angiogenesis, which modulates tumor maintenance and progression, pro-tumor immune responses and therapeutic resistance [22]. The hypoxic niche of GBM originates when tumor growth exceeds neovascularization, and is characterized by a necrotic core and pseudopalisading areas [13]. Here, hypoxia inducible factor 1 and 2 (HIF-1 and HIF-2, respectively) are the environmental key players, being responsible for CSCs expansion, and recruitment of innate immune cells such as macrophages [13, 14]. The lack of nutrients and hypoxia lead to the formation

of new blood vessels, and adaptative processes such as cell anaerobic metabolism and quiescence [23]. New blood vessels are formed due to the hypoxic pressure on CSCs, which differentiate into endothelial progenitor cells and, later, endothelium structures. The newly formed blood vessels are typically abnormal, functionally immature and larger in diameter compared to their normal counterparts [24]. The invasive niche is characterized by tumor cells that migrate across blood vessels to invade the surrounding brain tissue [13]. Within this niche, major cell components are ECs and endothelium-supportive pericytes, microglia and neurons. GBM invasive margin cells are responsible for around 85% of tumor relapses, that usually take place within 2 cm³ of the infiltrative margin [25, 26]. Few examples of molecular features that have been reported for GBM invasive margin cells are the consistent upregulation, and expression by tumor core and rim region cells to a significantly lower extent, of the SERPINE1, FGF1 and ALDH1A1 genes, which are associated with primary mechanisms of GBM invasion by ECM degradation via plasmin, angiogenesis promotion and stem-like subpopulations, respectively [27, 28]. In the invasive niche, GBM cells ensure the blood supply needs by populating the areas of pre-existing blood vessels and causing the displacement of astrocytic endfeet from ECs, therefore promoting invasion through hijacking of the existing vasculature - process termed vessel co-option [29]. The ECM, in turn, is tailored by each cell component of the GBM TME, often resulting in conditions favorable to tumor progression [13]. 90% of all GBMs originates in the temporal, parietal or frontal lobe, while only 10% is found in the occipital lobe and, rarely, on the cerebellum, spinal cord or brainstem [30]. Primary GBM develops *de novo* (around 90% of the cases), affects mostly the elderly, and is characterized by epidermal growth factor receptor (EGFR) amplification (36% of cases), p16INK4a deletion (31% of cases), loss of heterozygosity 10q (70% of cases) and PTEN mutations (25% of cases). Whereas, secondary GBM originates from pre-existing low-grade astrocytomas, affects mostly younger patients, and is genetically characterized by TP53 mutations, which are the most common and earliest detectable alterations in approximately 60% of the low-grade precursor tumors [6, 31].

2.1 The blood-brain barrier and blood-brain tumor barrier roles

The brain is a privileged anatomical part of the body due to the presence of the most protective CNS gatekeeper, namely the BBB. First termed as “barrier” by Stern and Gautier in 1918, the BBB is mainly composed by ECs connected by tight and adherens junctions, surrounded by an ECM basal lamina embedding pericytes and astrocytic endfeet and, to a lower extent, microglia and neuron projections (**Figure 2A**) [32, 33].

The BBB presents a specialized network of transport channels that orchestrate the brain influx of molecules pivotal for CNS homeostasis and surveillance, and efflux of toxic byproducts [34]. This barrier is the richest network of blood capillaries in the body, characterized by 10 to 15 μm average distance between capillaries, and the ability to provide supply to brain cells in a ratio of about one capillary per neuron [35]. Compared to ECs of other anatomical sites, BBB ECs are non-fenestrated, due to a reduced number of pores in their membrane, and present limited pinocytic and intracellular vesicular trafficking [32]. Transcellular trafficking across the BBB (**Figure 2B**) can occur through: (i) carrier-mediated transport, or (ii) vesicle-mediated transcytosis via receptor (RMT)- or adsorptive (AMT)-mediated transcytosis [32]. Carrier-mediated transport does not involve a vesicular transport, uses either facilitated diffusion or active transport, and is responsible for the transport of molecules such as amino acids, glucose and vitamins [36, 37]. For RMT, characterized by active transport mechanisms, one of the possible BBB trafficking pathways involves clathrin-dependent endocytosis - herein, dynamin regulates the formation of the initial vesicle, the clathrin coat is further shed, and the vesicle fuses with the early endosome (sorting endosome). The endocytosed cargo is afterwards directed to late endosomes (and eventually to the lysosomal degradation pathway) or transcytotic vesicles [38]. Another trafficking pathway for RMT, shared with AMT, involves the endocytosis of molecules via caveosomes, which are uncoated vesicles based on lipid rafts stabilized by caveolin-1 that are speculated to avoid degradation pathways [36]. AMT is based on the interaction of polycationic molecules with negative components of the membrane of BBB ECs, hence triggering the delivery of the cargos into the brain [39]. Transferrin (Tf), lactoferrin, insulin, and lipoproteins are examples of molecules that undergo RMT across the BBB, while albumin and other plasma proteins are transported by AMT [40]. BBB trafficking through passive diffusion is practically negligible, since it only applies for highly lipophilic drugs with a molecular weight lower than 500 Da [41]. The transport of substances across the BBB is highly restrictive compared to other tissues and scrupulously controlled due to the brain-protecting nature of this barrier. However, despite restricting the brain permeability of harmful agents, the BBB also prevents 98% and nearly 100% of all small molecule and macromolecular drugs, respectively, to successfully reach the brain tissue [42]. In fact, the drug-impenetrable BBB is a major responsible for the 50% lower chances of CNS drug candidates to reach de market, compared to other therapeutic areas [43]. Therefore, the inability to successfully surpass the BBB is a major obstacle in the field of drug delivery to the brain.

The concept of the blood-brain tumor barrier (BBTB) arose to describe areas of physically altered BBB as a consequence of brain tumor damage and TME biological

pressures (**Figure 2C**). In the case of GBM, certain tumor areas are reported to possess a BBTB, characterized by a leaky and disrupted BBB [44]. This is associated with the impact of tumor events on the BBB structural integrity, leading to loss or displacement of astrocytic endfeet, lower expression of EC junction proteins, and an abnormal pericytic distribution [45-47]. However, it is known that the BBTB is localized at the tumor bulky core, while a gross region beyond the magnetic resonance imaging (MRI) contrast-enhancing tumor bulk, consisting of the peritumoral region and, specially, the tumor invasive margins, present an intact BBB [32, 48, 49]. The BBTB at the tumor bulky core is surrounded by areas of significant necrosis, where a leaky barrier and, consequently, higher permeation of therapeutics from blood, does not necessarily result in a therapeutic advantage [6, 50]. Altogether, the existence of a localized BBTB does not soften the fact that therapeutic delivery to GBM still poses a grand clinical challenge – the tumor invasive margins, encircled by an intact BBB, are highly correlated with tumor recurrence and disease progression. Thus, it is general consensus that drug distribution across an intact BBB is an important validation step in the development of novel therapies for GBM and other brain tumors, not placing too much hope on limited areas of BBTB [49].

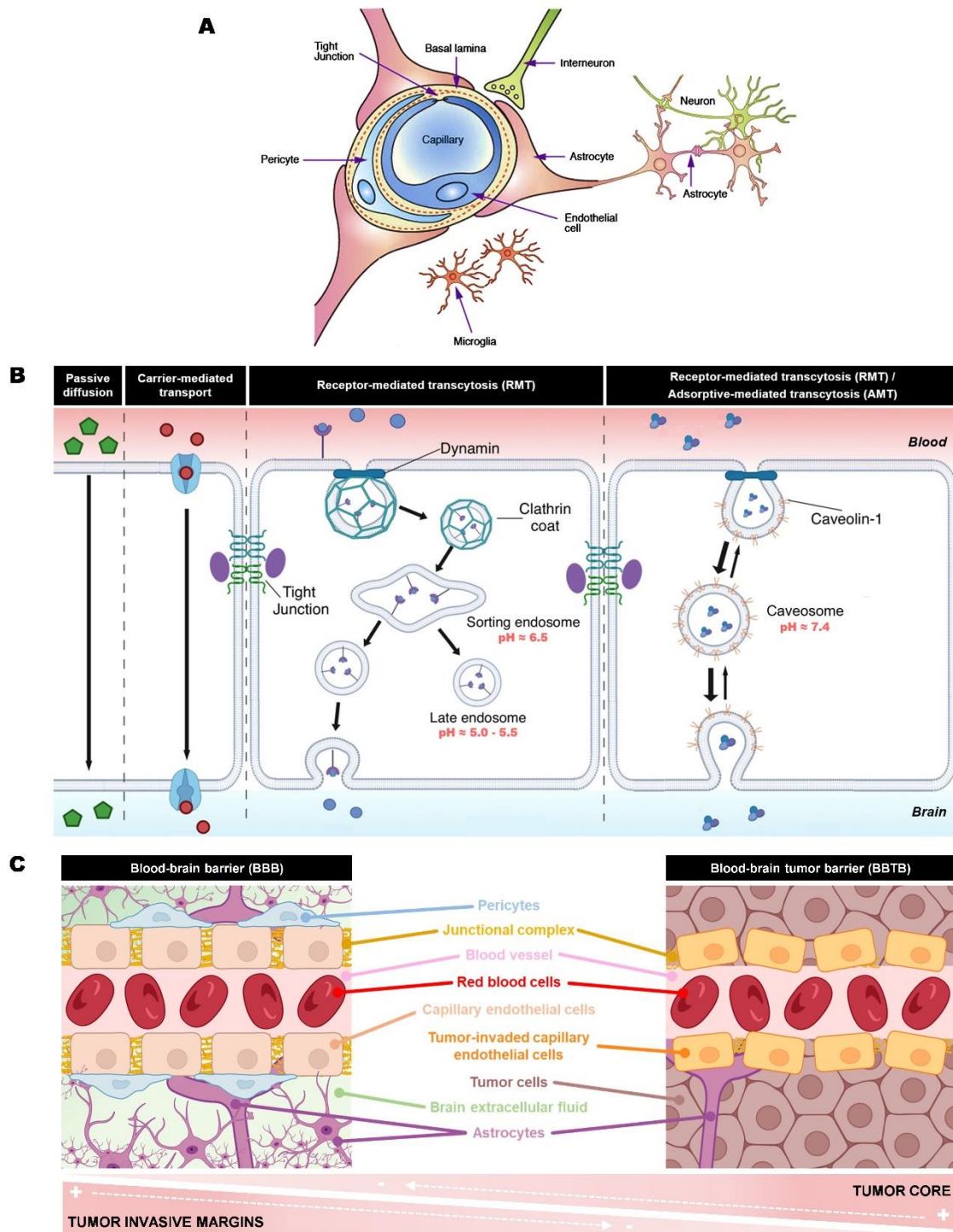


Figure 2 – A) Schematic representation of the BBB major components. Adapted with permission from [51]. B) Main intake transport routes of the BBB. Adapted with permission from [36, 52, 53]. C) Physiological differences between the GBM BBB (tumor invasive margins) and BBTB (tumor core). Created with BioRender.com.

3. Glioblastoma treatment options – standard of care, limitations and opportunities

The current standard of care (SOC) for GBM relies on maximally safe surgical resection, followed by (i) concomitant radiotherapy and temozolomide (TMZ) chemotherapy for 6 weeks, and (ii) adjuvant TMZ chemotherapy for 6 months [54]. This radiotherapy and TMZ pharmacotherapy regimen was proposed by Stupp *et al.* in 2005 – the so-called Stupp protocol - when findings from a randomized controlled trial revealed that radiotherapy plus TMZ significantly improve the overall survival (OS) and progression-free survival (PFS) of newly diagnosed GBM patients compared to radiotherapy alone (14.6 *versus* 12.1 months OS; 53.9% *versus* 36.4% PFS at 6 months) [55]. GBM surgical resection is often challenging since the boundaries of the tumor are not easily distinguishable from the surrounding brain tissue, ultimately leading to tumor relapse due to the invasive potential of residual tumor cells [56]. Apart from the SOC, four drugs and two medical devices are currently approved by the Food and Drug Administration (FDA) for the treatment of GBM (**Figure 3**): lomustine (1976), intravenous carmustine (1977), wafer implants of carmustine (Gliadel; 1996: recurrent GBM; 2003: newly diagnosed GBM), bevacizumab (2009), intratumoral thermotherapeutic iron oxide NPs (NanoTherm; 2010: recurrent GBM), and tumor treating fields (TTFs; Optune; 2011: recurrent GBM; 2015: newly diagnosed GBM) [5]. Despite some of them are approved for newly diagnosed GBM, these alternative FDA-approved treatments have been mainly used to manage recurrent GBM and disease symptoms [54]. Importantly, among these treatment alternatives, only TTFs have demonstrated ability to significantly improve OS and PFS of newly diagnosed GBM patients when combined with adjuvant TMZ chemotherapy (20.5 *versus* 15.6 OS; 56% *versus* 37% PFS at 6 months) [57]. However, following these findings, worldwide brain cancer experts decided to not yet add TTFs to the SOC due to marginal survival benefits, high costs, and patient compliance issues [58]. Nonetheless, over time, TTFs have gained great acceptance, not only because of the above-mentioned OS and PFS numbers, but also due to an increase in the 5-year survival of GBM patients from approximately 5% to 13% compared to the SOC arm [59]. Other treatment modalities, such as immunotherapy [9], gene therapy [60] and phototherapy [61] are emerging as promising therapeutic approaches for GBM.

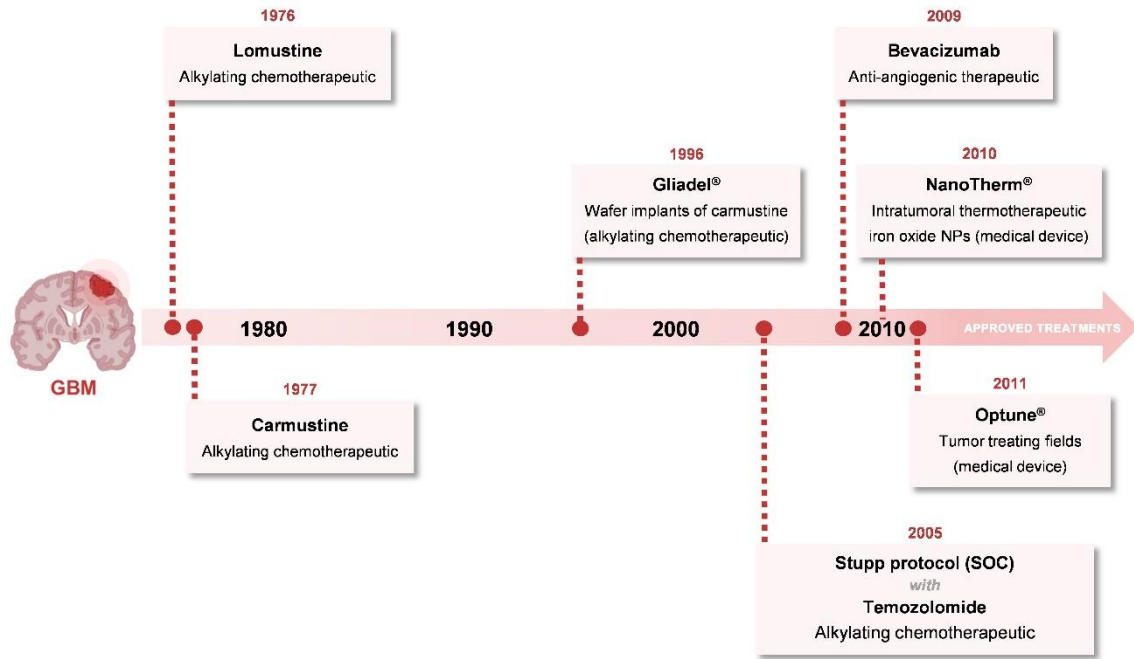


Figure 3 – Clinically approved drugs and medical devices for GBM treatment.

Although the Stupp protocol has remained the SOC for the past 20 years, great efforts have been invested in developing new and more effective therapeutic approaches to combat the lethal course of GBM. As already discussed in Section 3.1, a significant limitation is the presence of the BBB – the inability to surpass this barrier in therapeutically relevant concentrations limits the application of the most prospective drugs. Moreover, nearly 50% of the available anti-cancer drugs are characterized by poor water solubility, which challenges their formulation and compromises drug therapeutic concentration in biological fluids [62, 63]. Another challenge to the development of more effective therapeutic approaches is the short half-life of certain therapeutic molecules that are degraded or cleared from bloodstream minutes after their administration, thus requiring frequent dosing [64]. To circumvent this, chemical modification of these molecules with polyethylene glycol (PEG) might be a strategy to lengthen the therapeutics half-life, but it is nowadays known that free PEG moieties on drug-PEG conjugates lower their interaction with cancer cells [65]. Other modifications include the synthesis of cell-penetrating protein complexes and engineered fc-based proteins, but such direct derivatization of drug molecules deserves careful consideration as it might significantly alter anti-tumor efficacy [66]. Alongside with frequent drug dosing needs, the lack of tumor site targetability of anti-cancer therapeutics results in the emergence of severe side effects. This is particularly critical for chemotherapeutics, since they are able to trigger anti-proliferative mechanisms or cell cycle arrest in any type

of rapidly proliferating or dividing cell, independently of its healthy or tumor nature [67]. As an example, common TMZ side effects include nausea and vomiting, fatigue, headache and myelosuppression [68, 69]. Still in regard to GBM chemotherapy, TMZ presents an outstanding capacity of crossing the BBB (blood plasma to cerebrospinal fluid ratio of 0.2-0.3), but also well reported mechanisms of drug resistance and poor anti-cancer potency, as demonstrated by a 50% inhibitory concentration (IC_{50}) in U-87 MG GBM cells of 800 μ M [70, 71]. Therefore, this opens a room of opportunities for exploiting other chemotherapeutics, with a higher tumoricidal potency, such as docetaxel (DTX), paclitaxel (PTX), doxorubicin (DOX) or irinotecan - 0.041, 1.054, 0.061 and 10.916 μ M IC_{50} in U-87 MG GBM cells, respectively [70]. However, it is crucial to address key pharmaceutical constraints of these drugs, including low aqueous solubility, BBB permeation (blood plasma to cerebrospinal fluid ratio as low as 0.001) and GBM cell targeting post-BBB crossing (only 1% tumor uptake of the total administered dose) [72].

4. Nanomedicine to combat the limitations of glioblastoma therapeutics

Over the years, several nanomedicine strategies have been proposed to prevent, diagnose and treat a wide range of diseases. Nanomedicine gained popularity in the late 1990s and, according to the European Technology Platform on Nanomedicine (ETPN), its definition relies on "(...) the application of nanotechnology to health. It exploits the improved and often novel physical, chemical, and biological properties of materials at the nanometric scale." [73-75]. The US National Institutes of Health (NIH) standards, in turn, limit the definition of nanomedicine to nanotechnologies applied to medicine in the size range between 1 and 100 nm [76]. However, this definition is often considered too restrictive since several clinically approved nanomedicines exceed 100 nm, such as Abraxane (130 nm) and others, as reviewed elsewhere [77].

Nanomedicines are known to possess distinctive features that make them attractive for therapeutic delivery, including: (i) possibility of targeted delivery to a tissue, cell or organelle of interest, (ii) enhancement of formulation constraints of cargo molecules (e.g. aqueous solubility, *in vivo* half-life), (iii) amelioration of drug therapeutic indexes by increased efficacy and/or reduced toxicity, (iv) molecule release in a sustained or stimulus-triggered manner, (v) easing of delivery of biomacromolecule drugs, (vi) delivery of cocktails of cargos, (vii) facilitation of transcytosis of molecules across difficult-to-permeate biological barriers, and (viii) single-vehicle combination and delivery of therapeutic and imaging cargos [75]. There are more than 50 clinically-approved

nanomedicines currently available as prescription medicine [6] – **Table 1A of Appendix I** summarizes clinically-approved nanomedicines for cancer therapy (clinical advantages of these nanomedicines compared to the free drug reviewed in detail elsewhere) [5, 78, 79]. The progress of the nanomedicine field has generated diverse classes of systems with several different matrix compositions, shapes, mechanical properties, surface decorations, sizes, among others (**Figure 4A**) [80]. Polymeric nanoparticles (NPs), particularly solid particles, have been focus of considerable research due to their versatile physicochemical properties (e.g. hydrophobicity, size, charge) according to the manufacture protocol (e.g. matrix composition, precursors molecular weight, energy shearing), sustained release of cargo molecules over time, and possibility of encapsulation of cargo molecules with variable characteristics (e.g. from small molecule to biomacromolecule drugs, from practically water-insoluble to highly hydrophilic molecules) [81]. Poly(lactic-co-glycolic acid) (PLGA) is one of the most popular synthetic polymeric matrix used in drug delivery systems, and adds to the list of advantages of polymeric NPs an easy, reproducible and scalable production, as well as FDA approval for human use [82, 83]. Actually, PLGA entered the clinics in the early 1970s, as biodegradable surgical sutures, but its unique properties of biodegradability, biocompatibility and mechanical strength received the attention of pharmaceutical scientists, further leading to the emergence of drug delivery applications [84]. A gold standard modification of PLGA NPs relies on the combination of PLGA with PEG, thus endowing the system with multiple possibilities of surface modification by functionalization of the outer arms of PEG [78].

4.1 Targeted glioblastoma nanomedicine

Targeted nanomedicines, using active targeting strategies, are systems typically functionalized with surface moieties complementary to target sites of interest in the human body, such as tumor and vascular cells, intracellular organelles and ECM tags [72]. Active targeting strategies arose from the need to improve the sub-optimal biodistribution of nanomedicines, ultimately leading to the accumulation and premature release of therapeutic molecules in off-target healthy tissues and, consequently, a reduced therapeutic efficacy alongside with considerable side effects [78]. A strong basis for these targeted strategies is the antibody-drug conjugates already approved in clinics, whose antibody counterpart is often used to direct and deliver the conjugated drug to specific cancer cells - there are around 15 of these antibody-drug conjugates currently used in cancer therapy, as extensively reviewed elsewhere [85-87]. Some examples

include: (i) Mylotarg (first antibody-drug conjugate in the market, approved in 2000) containing gemtuzumab to target the leukemia patient-overexpressed CD33 antigen (90% of clinical cases) and deliver the N-acetyl gamma calicheamicin cytotoxic agent; (ii) Kadcyla (first antibody-drug conjugate in the market for solid tumors, approved in 2013) containing trastuzumab to target the breast cancer patient-overexpressed HER2 protein (25% of clinical cases) and deliver the mertansine/DM1 cytotoxic agent. Herein, the binding of trastuzumab to the HER2 protein also exerts a therapeutic effect *per se*, apart from targeting, based on the anti-tumor activation of the immune system and blocking of tumor cell growth by HER2 stimulation; (iii) Tivdak (antibody-drug conjugate in the market for solid tumors, approved in 2021) containing tisotumab to target the cervical cancer patient-overexpressed tissue factor (30% of clinical cases) and deliver the monomethyl auristatin E cytotoxic agent [85, 88-90]. It is also interesting to note that these clinically-approved antibody-drug conjugates already exploit mechanisms of stimuli-sensitive drug delivery. For instance, Mylotarg uses an acid-cleavable hydrazone linker between the antibody and drug counterparts, in order to profit from the hydrazone stability and rapid hydrolysis under neutral pH and lysosomal acidic conditions (4.5-5.0 pH), respectively. This triggers the release and delivery of the drug counterpart in the intracellular milieu of the CD33-targeted tumor cells. On the other hand, Tivdak uses an enzyme-cleavable valine-citrulline group between the antibody and drug counterparts that is recognized by cathepsin B, an enzyme overexpressed in the extracellular milieu of the targeted tissue factor-overexpressed cancer cells, and exclusively present in the intracellular lysosomal compartment of healthy mammals. This triggers the selective release and delivery of the drug counterpart only in the extracellular environment of the targeted tumor cells [86].

Although antibody-drug conjugates use antibody moieties to target the tumor, targeted cancer nanomedicines can present a diversity of other target-recognition surface molecules, including proteins, peptides, aptamers and amino acids, attached to the NP surface [80]. Although there is currently no clinically-approved targeted nanomedicine in the market [91], the field has experienced a research boom over the past years, and several candidates are undergoing clinical trials (examples presented in **Table 1B** of **Appendix I**). Perhaps the most appealing targeted cancer nanomedicine candidate over the past decade was BIND-014 (**Figure 4B**). It consisted of poly(lactic acid) (PLA) and PEG NPs encapsulating DTX, surface decorated with S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid (ACUPA), to target the prostate-specific membrane antigen (PSMA) overexpressed by prostate cancer cells [92]. BIND-014 pioneering nanotechnology resulted in the foundation of BIND Biosciences, and the initiation of Phase I clinical trials in 2007. In the patient cohort of Phase I trials

(NCT01300533), BIND-014 was well tolerated, demonstrated dose-dependent linear pharmacokinetics and an improved circulation time. However, in phase II trials (NCT02479178), the low response to treatment observed, along with manufacturer constraints, led to the termination of BIND-014 testing [93]. Careful analysis of this failure suggests that the inter-patient tumor heterogeneity and lack of patient stratification prior to treatment administration, as often performed for molecularly targeted therapeutics and antibody-drug conjugates, were responsible for the unmet endpoints of BIND-014 [8, 94].

4.1.1 Blood-brain barrier/neovasculature and tumor targets

The transferrin receptor (TfR) has been widely exploited for brain-targeted drug delivery since it is significantly overexpressed by ECs of the brain capillaries compared with other tissues [95, 96]. The transport of iron to the brain, to maintain iron homeostasis, is the major biological role of TfR and occurs through RMT in a clathrin-dependent pathway that involves the formation of endosomal vesicles [38]. Besides being overexpressed by BBB ECs, TfR is also overexpressed by tumor cells undergoing rapid proliferation such as GBM cells, therefore making this receptor a promising target for both BBB- and GBM-targeted therapies [97]. Ligands that recognize and bind TfR include the Tf protein [98-103] and the T7 peptide [104, 105]. Similarly to TfR, the low-density lipoprotein receptor (LDLR) has also been vastly exploited for the purpose of BBB- and GBM-targeted drug delivery, since it presents overexpression in both ECs of brain capillaries and tumor cells [106]. Contrary to brain capillaries, LDLR is often downregulated in large vessels, where its transcytosis rate is considerably lower compared with the brain tissue [107]. LDLR controls the brain homeostasis of cholesterol, and internalizes cargos through RMT in a clathrin- or caveolin-dependent pathway [38]. The Angiopep-2 peptide (ANG2) is the best-known LDLR-targeting ligand [108-112], but other ligands that recognize and bind the receptor include the Pep-22 peptide [113] and, indirectly, Polysorbate 80 [114]. Nicotine acetylcholine receptors (nAChRs) and choline transporters also present a very high expression at the brain due to their role in modulating the transport of acetylcholine or choline and, an aspect of particular interest, working as a gateway for the entrance of certain viruses in the brain such as the rabies virus [38]. Although further research is required to better understand the trafficking mechanisms behind these transporters, the nAChR-targeted D8 peptide ligand [115] and choline analogues [116] have shown a promising BBB-targeting ability. Focus of great attention in the fields of GBM neovasculature and cell targets have been the $\alpha\beta 3$ integrins. These molecules are poorly expressed by quiescent ECs, but highly expressed by ECs of newly formed vascular structures such as the tumor neovasculature [117]. Moreover, $\alpha\beta 3$ integrins

are overexpressed by glioma cells compared with normal brain cells [118]. A well-studied ligand for $\alpha v \beta 3$ integrins in the context of neovasculature- and tumor-targeted delivery is the RGD peptide [103, 113, 119-122]. The RGD peptide is also reported to bind the neuropilin-1 receptor (NRP1R) of cancer cells [119]. The folate receptor, in turn, can also be used for both BBB/neovasculature and GBM targeting since it is overexpressed by glioma cells, tumor-associated vasculature and the intact BBB [123]. Thus, folic acid-based NP surface moieties have been exploited to shuttle nanosystems across the BBB and target brain tumor cells [102, 114]. Other classes of receptors that have been studied for brain- or brain- and tumor-targeted delivery, respectively, are the dopamine and sigma receptors. These receptors present a prominent expression in most parts of the CNS such as the BBB and, for sigma receptors only, an overexpression by brain tumor cells [124]. Heparan sulfate residues have also been considered as a potential brain-targeting strategy in brain tumor scenarios since their expression is augmented in neovascular ECs [125, 126]. For GBM cell targeting, the interleukin-13 (IL-13) $\alpha 2$ receptor is one of the few receptors that is specifically overexpressed by GBM cells but undetectable in normal brain cells, thus making it an attractive target for GBM-targeted therapies with high selectivity for tumor cells along with low toxicity for healthy brain resident cells [127]. However, in order to understand the failure in clinical trials of therapies molecularly targeted to the IL-13 $\alpha 2$ receptor, several patient specimens were analyzed and it was concluded that the percentage of clinical GBMs that present IL-13 $\alpha 2$ receptor overexpression is actually lower than 50% [128]. Nevertheless, if the strategy is preceded by proper patient stratification to maximize the targeting effect, the Pep-1 peptide [125] or IL-13 peptide [121, 122] are promising choices as IL-13 $\alpha 2$ receptor-targeted ligands. Similarly to the IL-13 $\alpha 2$ receptor, the EGFR might be used as a GBM target, since the receptor is overexpressed by GBM cancer cells and is involved in the process of tumorigenesis [129]. However, it should be taken into consideration that only approximately 36% of clinical GBMs present EGFR amplification, thus, EGFR-targeted therapies require a patient pre-selection [6, 31]. The EP-1 peptide is an example of an EGFR-targeted moiety [108]. The glucose-regulated protein 78 (GRP78) is another option for GBM cell targeting since it is highly expressed in clinical GBMs in comparison with normal brain specimens [130]. The expression of GRP78 is induced by TME key features, including glucose starvation, oxidative stress and hypoxia [131]. The RI-VAP peptide has been used as a NP surface ligand for GBM GRP78 targeting [115]. Nucleolin is a cell surface protein abnormally expressed in GBM cells, promoting the binding of signaling molecules that stimulate cancer proliferation [132]. An aptamer with high affinity for nucleolin, AS1411, has been used for GBM targeting purposes [101, 109, 133, 134]. The CD13 receptor has also been used as a GBM cell target, since it is overexpressed

by glioma cells and, moreover, neovascular ECs [135]. The NGR peptide has been reported to target the CD13 receptor [104, 136]. Amino acid transporters such as the L-type amino acid transporter 1 (LAT1) also offer great promise as GBM targets. LAT1 fulfils the high metabolic demands of GBM cells through amino acid uptake [137], hence evidencing amino acids as potential NP surface moieties to target the transporter. LAT1 presents overexpression by GBM cells, lower expression in low-grade gliomas, and basal expression in the normal cerebral cortex [137-139]. A different class of NP surface moieties that potentiate GBM tissue penetration consist of cell penetrating peptides (CPPs). These peptides are usually based on cationic or amphipathic sequences that possess ability to cross cellular membranes, although they cannot be considered tumor-selective [140]. Examples of CPPs that have been used for GBM cell accumulation include the R8 [98, 112], penetratin (Pen) [99, 100] and TAT [105, 110] peptides. Following the same rationale, despite not common, the tLyp-1 peptide has been used as a CPP for BBB cell targeting and accumulation [141]. Another approach for GBM targeting consists of using ligands directed to TME components, including the programmed death 1 (PD-1) protein on cytotoxic T cells [116], and the NKG2A receptor on T and natural killer (NK) cells [141].

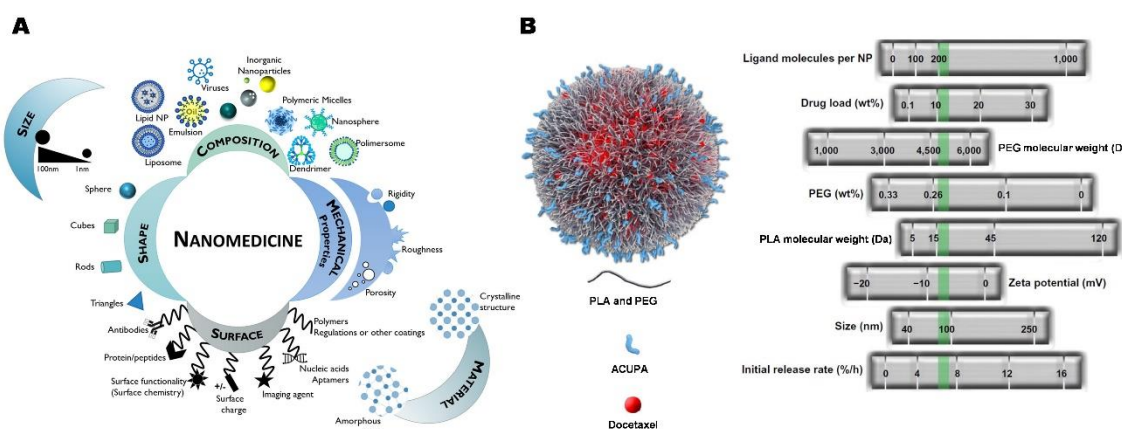


Figure 4 – A) Diverse classes of systems resulting from the progress of the nanomedicine field over recent years, leading to the report of several different matrix compositions, shapes, mechanical properties, surface decorations, sizes, among others. Reproduced with permission from [80]. Copyright 2022 American Chemical Society. B) Formulation composition, key parameters and physicochemical characteristics (green range) of the targeted cancer nanomedicine candidate BIND-014. Adapted with permission from [8].

4.2 Multi-ligand functionalized blood-to-glioblastoma sequentially targeted nanomedicines

The need to overcome two physiological barriers in the context of GBM intravenous therapies, namely the BBB and tumor barrier, opens avenues for the development of novel BBB and GBM dual-targeted nanosystems presenting multi-ligand surface functionalization. These nanosystems are designed to perform sequential biological roles, as the following: (i) BBB targeting and consequent blood-to-brain transport, and (ii) tumor cell targeting once arrival at the brain parenchyma.

Gao *et al.* proposed a DTX-loaded polymeric NP system of polycaprolactone (PCL) and PEG, targeted to the BBB LDLR by an ANG2 peptide moiety, and further decorated with a TME matrix metalloproteinase-2 (MMP-2)-activable R8 CPP for enhanced GBM cell accumulation [112]. In an *in vivo* therapeutic efficacy trial, the dual-ligand NP group demonstrated an increase in the median survival of a C6 GBM orthotopic mouse model (32 days), compared to the untreated group (19 days), non-functionalized NPs (20 days), NPs presenting only the MMP-2-activable R8 CPP (26 days), and NPs presenting only the ANG2 peptide moiety (27 days) – of important note, non-nanoparticulate DTX was not included as a control group in the study. Other authors developed DTX-loaded dithiolane trimethylene carbonate and PEG micelles, using ANG2 peptide for both LDLR BBB and glioma cell targeting, along with a TAT CPP for enhanced glioma cell penetration [110]. It was observed an improved median survival in a U-87 MG GBM orthotopic mouse model (53 days), compared with the untreated group (24 days), free DTX (30 days), non-functionalized NPs (33 days), and NPs presenting only the ANG2 peptide moiety (44 days). In another study, liposomes loaded with DOX and erlotinib were decorated with a Tf protein moiety for BBB TfR targeting, and a Pen CPP for enhanced GBM cell accumulation (**Figure 5**) [99]. *In vivo*, the median survival of a U-87 MG GBM orthotopic mice model increased to 36 days compared with the untreated group (22 days), free drugs cocktail (25 days), non-functionalized NPs (25.5 days), liposomes presenting only the Tf moiety (30 days), and liposomes presenting only the Pen CPP moiety (27.5 days). The same authors previously proposed a similar liposome system for 5-fluorouracil loading, which presented a 20-30% cell viability decrease in a 3D *in vitro* tumor model in comparison with the free drug, non-functionalized liposomes, Tf-functionalized liposomes and Pen CPP-functionalized liposomes [100].

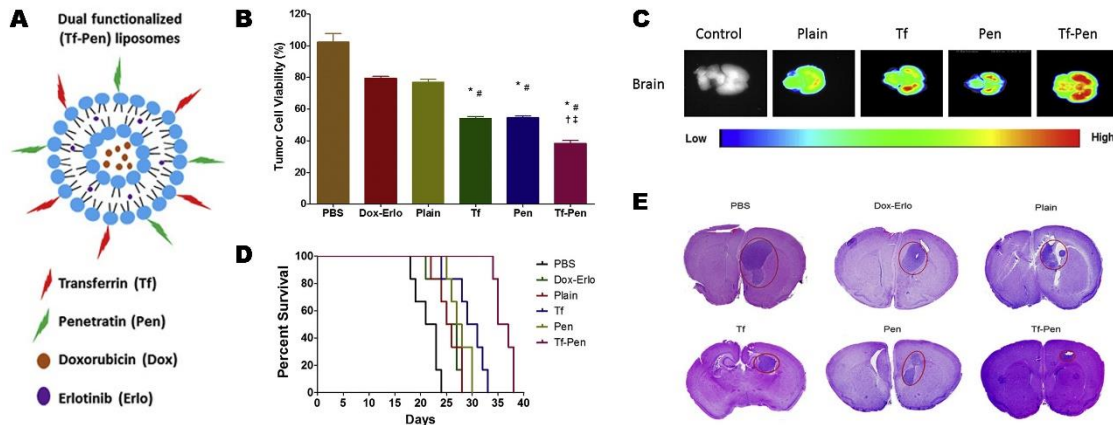


Figure 5 – Multi-ligand, BBB and GBM dual-targeted nanosystem based on liposomes loaded with DOX and erlotinib, surface decorated with Tf (BBB targeting) and Pen CPP (GBM targeting) moieties. A) Nanosystem design. B) *In vitro* inhibition of tumor cell viability. C) *In vivo* brain accumulation of the different formulations. D) Kaplan-Meier survival curves of mice after treatment. E) Histological sections of brain displaying tumor regression (red circle) after treatment. The images were taken at 20× magnification. PBS, dox-erlo, plain, Tf, Pen and Tf-Pen denote the untreated group, free drugs cocktail (DOX and erlotinib), non-functionalized liposomes, liposomes presenting only the Tf moiety, liposomes presenting only the Pen CPP moiety, and liposomes presenting both the Tf and Pen CPP moieties, respectively. Adapted with permission from [99].

Wang *et al.* exploited the dual-functionalization of PLGA NPs with the BBB LDLR-targeted ANG2 peptide, and an AS1411 aptamer targeted to the cancer cell-overexpressed nucleolin, in order to deliver DOX to GBM [109]. It was found that the dual-functionalized NP system was not able to ameliorate the BBB *in vitro* crossing capacity compared with NPs functionalized with only ANG2, providing a permeability advantage only compared with the free drug, non-functionalized NPs and AS1411-functionalized NPs. In a different approach, other authors functionalized DOX-loaded dendrimer nanocarriers with ANG2 and an EGFR-targeting peptide (EP-1) for BBB and GBM cell targeting, respectively [108]. The system was tested in a U-87 MG GBM orthotopic mice model and provided 34 days median survival, which represented an improvement compared with the untreated group, free DOX, non-functionalized NPs, NPs functionalized with only ANG2, and NPs functionalized with only EP-1 (16, 19, 24, 31 and 27 days, respectively). A more advanced approach focused on the development of a polymeric NP system, surface modified with a BBB choline transporter-targeted molecule and a tumor programmed death-ligand 1 (PD-L1) acid-cleavable antibody [116]. The BBB-targeted molecule was expected to shuttle the NPs to the brain, while

the PD-L1 antibody was attached to the NPs via an acid-cleavable strategy to promote its release from the carrier upon encountering the TME acidic pH – the release from the carrier was expected to allow the antibody to exert its therapeutic role through binding to the PD-1 protein on cytotoxic T cells, ultimately resulting in a T cell-mediated anti-tumor immunity. In a LCPN GBM orthotopic mice model, the median survival of animals was increased to 19 days compared with the untreated group (8 days), free PD-L1 antibody (8 days) and non-functionalized NPs (12 days), respectively - herein, it would have been interesting to unravel the therapeutic benefit of the pH-sensitive properties by testing the control based on BBB-targeted NPs presenting a non-cleavable PD-L1 antibody moiety [116]. In what concerns highly advanced systems, Fu et al. proposed vincristine-loaded, erythrocyte membrane-enveloped lipid NPs (longer circulation half-life and lower immunogenicity), surface modified with the BBB TfR- and GBM CD13-targeted T7 and NGR peptides, respectively (**Figure 6**) [104]. In a C6 orthotopic GBM mice model, the system provided an increased median survival of animals (36 days) in comparison with the untreated group, free drug, non-functionalized NPs, NPs presenting only the T7 moiety, and NPs presenting only the NGR moiety (18, 19, 20, 29 and 21 days, respectively). Of important note, despite the selection of a nude mice model, the tumor was based on a rat-based GBM xenograft, instead of the conventional human-based xenografts (e.g. U-87 MG GBM). In the same GBM rodent model, Lv et al. assessed the efficacy of PLGA and PEG polymeric NPs, encapsulating PTX, surface decorated with CGKRK and Pep-1 peptides to target neovascular ECs (heparan sulfate) and GBM cells (IL-13 $\alpha 2$ receptor), respectively [125]. It was observed a promising improvement in the median survival of animals treated with the developed NPs (61 days) compared with the untreated group, free PTX, non-functionalized NPs, NPs decorated with only CGKRK, and NPs decorated with only Pep-1 (17, 22, 24, 34 and 32 days, respectively).

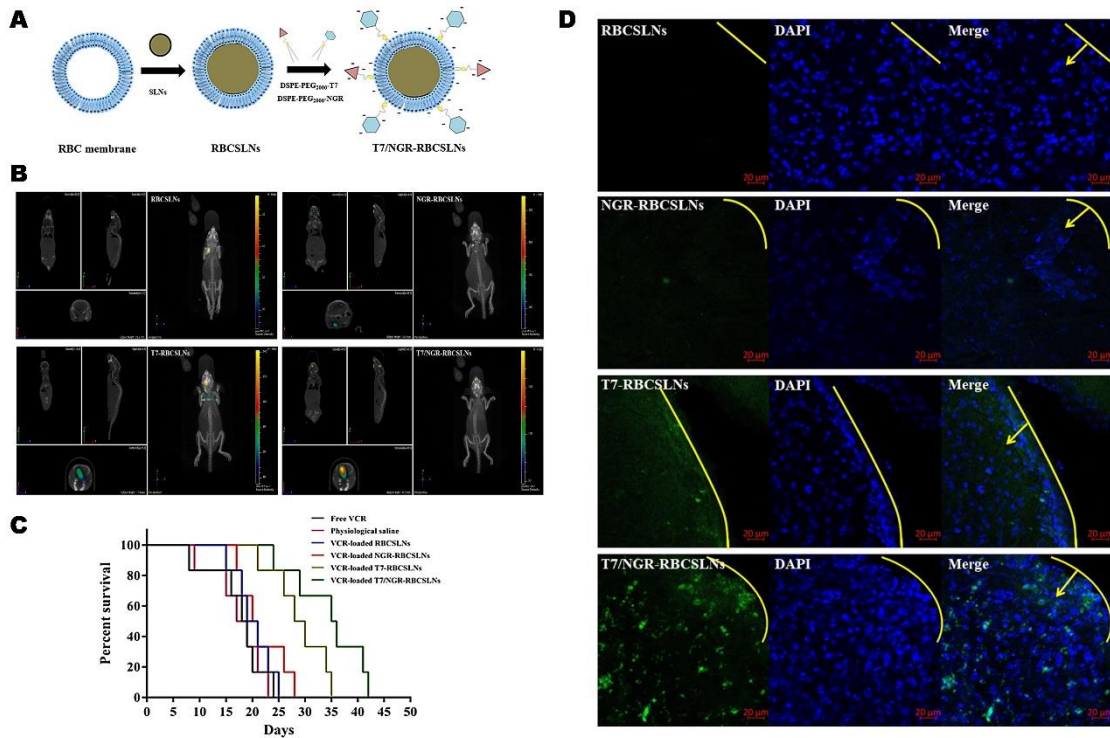


Figure 6 – Multi-ligand, BBB and GBM dual-targeted nanosystem based on vincristine-loaded, erythrocyte membrane-enveloped lipid NPs, surface modified with the BBB TfR- and GBM CD13-targeted T7 and NGR peptides, respectively. A) Nanosystem design. B) Biodistribution of formulations in mice-bearing intracranial C6 GBM. C) Kaplan-Meier survival curves of mice after treatment. D) Distribution of NPs in mice brain determined by confocal laser scanning microscopy. Formulations were labeled with cyanine5.5 (red), and cell nuclei with DAPI (blue). The yellow line shows the margin of the intracranial glioma, and the arrow points the location of glioma cells. RBC, SLN, DSPE-PEG₂₀₀₀-T7, DSPE-PEG₂₀₀₀-NGR, T7/NGR-RBCSLNs, RBCSLNs, NGR-RBCSLNs, T7-RBCSLNs and VCR denote red blood cells, solid lipid NPs, NP lipid modified with the T7 peptide, NP lipid modified with the NGR peptide, T7 and NGR NPs, non-targeted NPs enveloped by erythrocyte membranes, NGR-functionalized NPs, T7-functionalized NPs, and vincristine, respectively. Adapted with permission from [104].

Gao *et al.* exploited the use of polymeric NPs to target the GBM IL-13 $\alpha 2$ receptor (IL-13 peptide surface moiety), but using a brain targeting strategy focusing on the BBB $\alpha v \beta 3$ integrins (RGD peptide surface moiety) [122]. In a C6 orthotopic GBM mice model, the NPs loaded with DTX provided a superior animal median survival (35 days), compared with the untreated group (17 days), free DTX (20 days), non-functionalized NPs (22 days), RGD peptide-functionalized NPs (27 days) and IL-13 peptide-functionalized NPs (26 days). In a different approach, PEGylated liposomes loaded with DOX were surface

decorated with a cyclic RGD peptide to target both neovasculature and GBM cells ($\alpha v \beta 3$ integrins), while a second surface decoration based on p-hydroxybenzoic acid (pHA) served the purpose of BBB targeting (dopamine receptors) [120]. In a U-87 MG GBM orthotopic mice model, the dual-functionalized liposomes provided the longest median survival, 35 days, compared with the other treatment controls (20, 23, 26.5, 30 and 28.5 days for the untreated group, free DOX, non-functionalized NPs, pHA-functionalized NPs and cyclic RGD-functionalized NPs, respectively). Farshbaf *et al.* proposed bortezomib-loaded lipid NPs, surface functionalized with the D8 and RI-VAP peptides to target the BBB nAChRs and GBM cell GRP78 surface marker, respectively (**Figure 7**) [115]. In a GL261 GBM orthotopic mice model, the authors found out a promising increase in the median survival of animals treated with the proposed dual-targeted system (46 days), compared with the untreated group (16 days), free drug (26 days), non-functionalized NPs (30 days), NPs functionalized with only the D8 peptide (36 days), and NPs functionalized with only the RI-VAP peptide (29 days). Bruun and colleagues proposed lipid NPs for siRNA model delivery, surface modified with the ANG2 peptide for both BBB and GBM targeting, and TME matrix metalloproteinase (MMP)-activable glutamic acid residues [111]. These glutamic acid residues were expected to cause negative to positive charge switch at the tumor site, favoring GBM cell endocytosis and endosomal escape. The *in vitro* uptake in U-87 MG GBM cells and bEnd.3 BBB cells was significantly enhanced compared with non-functionalized NPs, but no relevant differences were found between the proposed formulation and the control formulation without ANG2, suggesting the poor effect of ANG2 in regard to BBB and GBM cell targeting [111]. In the field of biologic-responsive nanosystems, other authors developed mesoporous ruthenium inorganic NPs attached with $[\text{Ru}(\text{bpy})_2(\text{tip})]^{2+}$ (RBT) via tumor glutathione-cleavable bonds, surface functionalized with Tf and the AS1411 aptamer to target the BBB TfR and GBM nucleolin, respectively [101]. The median survival of a U-87 MG GBM orthotopic mice model was increased to 40 days, which was longer than the untreated group (22 days), free drug (26 days) and NPs presenting only the Tf moiety (34 days) – the effect of NPs functionalized with only the AS1411 aptamer was not assessed. As an adjuvant therapeutic effect, the median survival suffered a proportional increase across all treatment types under laser irradiation, since the irradiated RBT provided photodynamic therapy by the local production of reactive oxygen species that augmented tumor cell apoptosis [101].

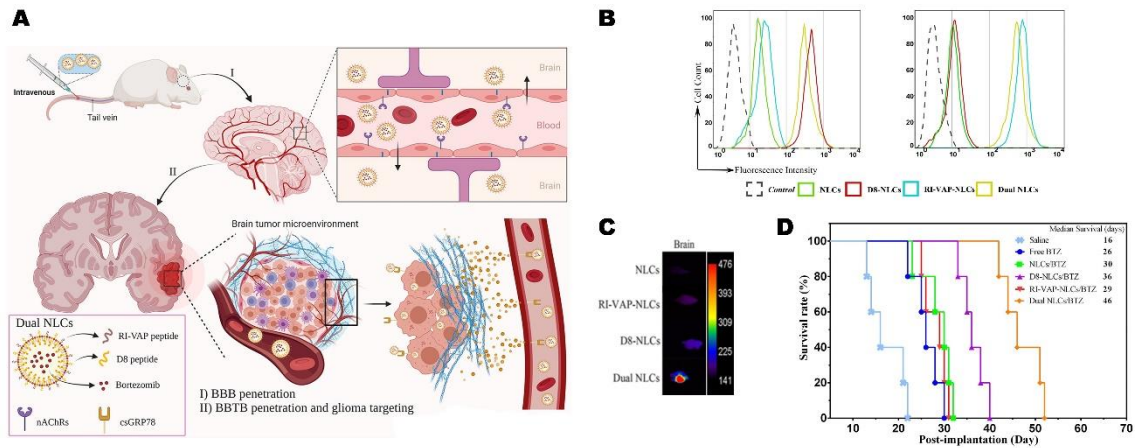


Figure 7 – Multi-ligand, BBB and GBM dual-targeted nanosystem based on bortezomib-loaded lipid NPs, surface functionalized with the D8 and RI-VAP peptides to target the BBB nAChRs and GBM cell GRP78 surface marker, respectively. A) Nanosystem design and proposed biological mechanism. B) Cellular uptake of NP formulations by hCMEC/D3 BBB cells (left) and GL261 GBM cells (right). C) *Ex vivo* fluorescence imaging of brain of intracranial glioma-bearing mice harvested 24 h after NPs administration. D) Kaplan-Meier survival curves of mice after treatment. NLCs, csGRP78, D8-NLCs, RI-VAP-NLCs, dual NLCs and BTZ denote nanostructured lipid NPs, cell surface GRP78, D8 peptide-functionalized NPs, RI-VAP peptide-functionalized NPs, NPs functionalized with both D8 and RI-VAP peptides, and bortezomib, respectively. Adapted with permission from [115].

Table 2 of **Appendix I** summarizes the multi-ligand functionalized, BBB- and GBM-dual targeted nanomedicines reported in literature data. A scheme of the main biological targets exploited in the development of multi-ligand functionalized, BBB and tumor dual-targeted nanomedicines for GBM therapies over the past 10 years is presented in **Figure 8**.

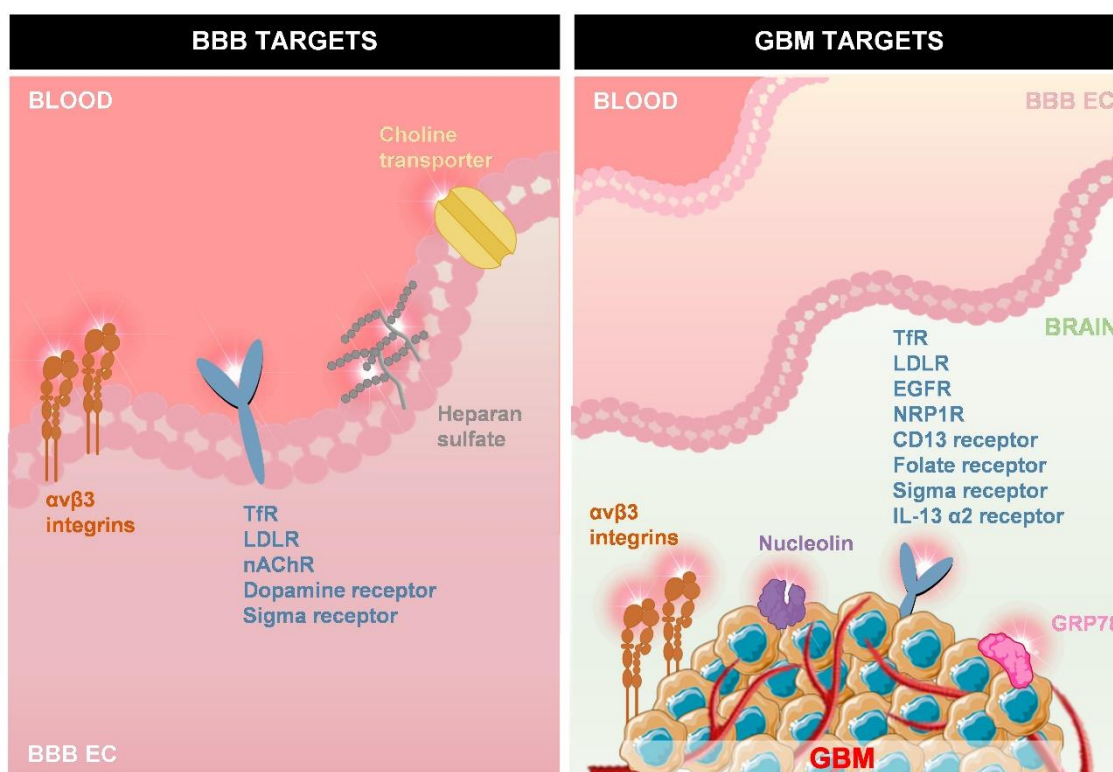


Figure 8 – Main biological targets exploited in the development of multi-ligand functionalized, BBB and tumor dual-targeted nanomedicines for GBM therapies over the past 10 years. Created with BioRender.com.

5. Remarks and considerations

Multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines have shown promising capacity to precisely target (i) BBB cells, inducing transport from blood to brain, and (ii) GBM cells, leading to a higher tumor therapeutic accumulation. The BBB and GBM sequentially- and dual-targeted nanomedicines herein revised demonstrated the ability to successfully penetrate the BBB and lower tumor cell viability in different *in vivo* testing models that emulate the GBM clinical scenario. Moreover, the anti-GBM therapeutic outcomes of these nanomedicines were generally superior compared with each of the single-ligand nanosystem control counterparts (targeted to either the BBB or GBM) and the free cargo controls. However, there is significant room for improvement, and the road to translation is still long and winding. Since GBM is characterized by a high level of molecular heterogeneity, thus leading to dynamic variations in the percentage of expression of the envisaged targets, it is necessary to ensure patient stratification prior to the treatment administration in order to make sure that the dual-targeting properties of the nanosystems are maintained. In

addition, priority should be given to receptor or transporter targets that are less prone to saturation and rapidly recycled at the cell surface, which will lead to higher rates of transport across the BBB and tumor barriers. The development of the nanocarriers should be based on a careful design since the presence of more than one surface targeting ligand might cause phenomena of inter-steric hindrance, thus compromising the individual targetability of each one of the ligands. Herein, it should be of interest to play with polymer outer arms (e.g. PEG) to endow the nanocarrier surface with different length layers of each ligand or, as another alternative, develop stimulus-sensitive systems that eliminate the BBB-targeting ligand during blood-to-brain transport, in order to surface expose further ligands responsible for GBM targeting without hindrance constraints. Lastly, it is foreseen that future clinical approval of the first targeted nanomedicines will help to speed up the process of translation of more complex systems, such as these multi-ligand functionalized, dual-targeted nanomedicines.

References

- [1] M. Puyol, J. Seoane, E. Aguilar, L.B. Voza, I. Orbe, K.H. Crawford, A. Fernández, F. Bray, S.E. Johnson, S. Gopal, World Cancer Research Day: A call to action for a coordinated international research effort to prevent, diagnose, and treat cancer, *Clin Cancer Res*, 27 (2021) 963-966.
- [2] A. Salam, A. Woodman, A. Chu, L.H. Al-Jamea, M. Islam, M. Sagher, M. Sager, M. Akhtar, Effect of post-diagnosis exercise on depression symptoms, physical functioning and mortality in breast cancer survivors: A systematic review and meta-analysis of randomized control trials, *Cancer Epidemiol*, 77 (2022) 102111.
- [3] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J Clin*, 71 (2021) 209-249.
- [4] Q.T. Ostrom, G. Cioffi, H. Gittleman, N. Patil, K. Waite, C. Kruchko, J.S. Barnholtz-Sloan, CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2012-2016, *Neuro Oncol*, 21 (2019) 1-100.
- [5] I. de Lázaro, D.J. Mooney, Obstacles and opportunities in a forward vision for cancer nanomedicine, *Nat Mater*, 20 (2021) 1469-1479.
- [6] S. Quader, K. Kataoka, H. Cabral, Nanomedicine for brain cancer, *Adv Drug Deliv Rev*, 182 (2022) 114115.
- [7] S. Sindhwani, A.M. Syed, J. Ngai, B.R. Kingston, L. Maiorino, J. Rothschild, P. MacMillan, Y. Zhang, N.U. Rajesh, T. Hoang, J.L.Y. Wu, S. Wilhelm, A. Zilman, S. Gadde, A. Sulaiman, B. Ouyang, Z. Lin, L. Wang, M. Egeblad, W.C.W. Chan, The entry of nanoparticles into solid tumours, *Nat Mater*, 19 (2020) 566-575.
- [8] W. Gu, F. Meng, R. Haag, Z. Zhong, Actively targeted nanomedicines for precision cancer therapy: Concept, construction, challenges and clinical translation, *J Control Release*, 329 (2021) 676-695.
- [9] Y. Ma, Z. Yang, K. Huntoon, W. Jiang, B.Y.S. Kim, Advanced immunotherapy approaches for glioblastoma, *Adv Ther*, 4 (2021) 2100046.
- [10] Y. Zhu, J. Feijen, Z. Zhong, Dual-targeted nanomedicines for enhanced tumor treatment, *Nano Today*, 18 (2018) 65-85.
- [11] N. Pandey, P. Anastasiadis, C.P. Carney, P.P. Kanvinde, G.F. Woodworth, J.A. Winkles, A.J. Kim, Nanotherapeutic treatment of the invasive glioblastoma tumor microenvironment, *Adv Drug Deliv Rev*, 188 (2022) 114415.
- [12] A. Habib, M. Hoppe, J. Beiriger, C.V. Kodavali, L. Edwards, P.O. Zinn, Letter: Glioblastoma cell of origin, *Stem Cell Rev Rep*, 18 (2022) 691-693.

- [13] D. Hambardzumyan, G. Bergers, Glioblastoma: Defining tumor niches, *Trends Cancer*, 1 (2015) 252-265.
- [14] D. Schiffer, M. Mellai, E. Bovio, I. Bisogno, C. Casalone, L. Annovazzi, Glioblastoma niches: From the concept to the phenotypical reality, *Neurol Sci*, 39 (2018) 1161-1168.
- [15] N. Charles, E.C. Holland, The perivascular niche microenvironment in brain tumor progression, *Cell Cycle*, 9 (2010) 3012-3021.
- [16] R.S. Andersen, A. Anand, D.S.L. Harwood, B.W. Kristensen, Tumor-associated microglia and macrophages in the glioblastoma microenvironment and their implications for therapy, *Cancers*, 13 (2021) 4255.
- [17] D. Hambardzumyan, D.H. Gutmann, H. Kettenmann, The role of microglia and macrophages in glioma maintenance and progression, *Nat Neurosci*, 19 (2016) 20-27.
- [18] A.P. Landry, M. Balas, S. Alli, J. Spears, Z. Zador, Distinct regional ontogeny and activation of tumor associated macrophages in human glioblastoma, *Sci Rep*, 10 (2020) 19542.
- [19] R.A. Morantz, G.W. Wood, M. Foster, M. Clark, K. Gollahon, Macrophages in experimental and human brain tumors. Part 1: Studies of the macrophage content of experimental rat brain tumors of varying immunogenicity, *J Neurosurg*, 50 (1979) 298-304.
- [20] W. Zhou, S.Q. Ke, Z. Huang, W. Flavahan, X. Fang, J. Paul, L. Wu, A.E. Sloan, R.E. McLendon, X. Li, J.N. Rich, S. Bao, Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth, *Nat Cell Biol*, 17 (2015) 170-182.
- [21] A. Golebiewska, S. Bougnaud, D. Stieber, N.H. Brons, L. Vallar, F. Hertel, B. Klink, E. Schröck, R. Bjerkvig, S.P. Niclou, Side population in human glioblastoma is non-tumorigenic and characterizes brain endothelial cells, *Brain*, 136 (2013) 1462-1475.
- [22] M.L. Broekman, S.L.N. Maas, E.R. Abels, T.R. Mempel, A.M. Krichevsky, X.O. Breakefield, Multidimensional communication in the microenvirons of glioblastoma, *Nat Rev Neurol*, 14 (2018) 482-495.
- [23] A. Mosteiro, L. Pedrosa, A. Ferrés, D. Diao, À. Sierra, J.J. González, The vascular microenvironment in glioblastoma: A comprehensive review, *Biomedicines*, 10 (2022).
- [24] B.K. Ahir, H.H. Engelhard, S.S. Lakka, Tumor development and angiogenesis in adult brain tumor: Glioblastoma, *Mol Neurobiol*, 57 (2020) 2461-2478.
- [25] K. Petrecca, M.-C. Guiot, V. Panet-Raymond, L. Souhami, Failure pattern following complete resection plus radiotherapy and temozolomide is at the resection margin in patients with glioblastoma, *J Neuro-Oncol*, 111 (2013) 19-23.
- [26] C.E. Vasey, R.J. Cavanagh, V. Taresco, C. Moloney, S. Smith, R. Rahman, C. Alexander, Polymer pro-drug nanoparticles for sustained release of cytotoxic drugs

evaluated in patient-derived glioblastoma cell lines and in situ gelling formulations, *Pharmaceutics*, 13 (2021).

[27] S.J. Smith, J. Rowlinson, M. Estevez-Cebrero, D. Onion, A. Ritchie, P. Clarke, K. Wood, M. Diksin, A. Lourdasamy, R.G. Grundy, R. Rahman, Metabolism-based isolation of invasive glioblastoma cells with specific gene signatures and tumorigenic potential, *Neurooncol Adv*, 2 (2020) vdaa087.

[28] S.J. Smith, M. Diksin, S. Chhaya, S. Sairam, M.A. Estevez-Cebrero, R. Rahman, The invasive region of glioblastoma defined by 5ALA guided surgery has an altered cancer stem cell marker profile compared to central tumour, *Int J Mol Sci*, 18 (2017).

[29] D. Ribatti, The chick embryo chorioallantoic membrane as an experimental model to study in vivo angiogenesis in glioblastoma multiforme, *Brain Res Bull*, 182 (2022) 26-29.

[30] K.S. Natsis, A. Kalyvas, E. Theochari, E. Papamichalis, A. Gerkou, Area postrema syndrome in a patient with brainstem glioblastoma, *Acta Neurol Belg*, 121 (2021) 1087-1088.

[31] A.A. Balachandran, L.M. Larcher, S. Chen, R.N. Veedu, Therapeutically significant microRNAs in primary and metastatic brain malignancies, *Cancers*, 12 (2020) 2534.

[32] C.D. Arvanitis, G.B. Ferraro, R.K. Jain, The blood–brain barrier and blood–tumour barrier in brain tumours and metastases, *Nat Rev Cancer*, 20 (2020) 26-41.

[33] N.R. Saunders, J.J. Dreifuss, K.M. Dziegielewska, P.A. Johansson, M.D. Habgood, K. Møllgård, H.C. Bauer, The rights and wrongs of blood-brain barrier permeability studies: A walk through 100 years of history, *Front Neurosci*, 8 (2014) 404.

[34] R. Pandit, L. Chen, J. Götz, The blood-brain barrier: Physiology and strategies for drug delivery, *Adv Drug Deliv Rev*, 165-166 (2020) 1-14.

[35] X. Tian, D.M. Leite, E. Scarpa, S. Nyberg, G. Fullstone, J. Forth, D. Matias, A. Apriceno, A. Poma, A. Duro-Castano, M. Vuyyuru, L. Harker-Kirschneck, A. Šarić, Z. Zhang, P. Xiang, B. Fang, Y. Tian, L. Luo, L. Rizzello, G. Battaglia, On the shuttling across the blood-brain barrier via tubule formation: Mechanism and cargo avidity bias, *Sci Adv*, 6 (2020) eabc4397.

[36] A.G. Sorets, J.C. Rosch, C.L. Duvall, E.S. Lippmann, Caveolae-mediated transport at the injured blood–brain barrier as an underexplored pathway for central nervous system drug delivery, *Curr Opin Chem Eng*, 30 (2020) 86-95.

[37] N.U. Khan, T. Miao, X. Ju, Q. Guo, L. Han, 6 - Carrier-mediated transportation through BBB, in: H. Gao, X. Gao (Eds.) *Brain Targeted Drug Delivery System*, Academic Press, 2019, pp. 129-158.

- [38] R.P. Moura, C. Martins, S. Pinto, F. Sousa, B. Sarmento, Blood-brain barrier receptors and transporters: An insight on their function and how to exploit them through nanotechnology, *Expert Opin Drug Deliv*, 16 (2019) 271-285.
- [39] X. Zhu, K. Jin, Y. Huang, Z. Pang, 7 - Brain drug delivery by adsorption-mediated transcytosis, in: H. Gao, X. Gao (Eds.) *Brain Targeted Drug Delivery System*, Academic Press, 2019, pp. 159-183.
- [40] F. Gosselet, R.A. Loiola, A. Roig, A. Rosell, M. Culot, Central nervous system delivery of molecules across the blood-brain barrier, *Neurochem Int*, 144 (2021) 104952.
- [41] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Adv Drug Deliv Rev*, 23 (1997) 3-25.
- [42] M. Liang, J. Li, L. Han, Receptor-mediated cascade targeting strategies for the application to medical diagnoses and therapeutics of glioma, *J Nanopart Res*, 24 (2022) 106.
- [43] A.S. Kesselheim, T.J. Hwang, J.M. Franklin, Two decades of new drug development for central nervous system disorders, *Nat Rev Drug Discov*, 14 (2015) 815-816.
- [44] S.-S. Kim, J.B. Harford, K.F. Pirollo, E.H. Chang, Effective treatment of glioblastoma requires crossing the blood–brain barrier and targeting tumors including cancer stem cells: The promise of nanomedicine, *Biochem Biophys Res Commun*, 468 (2015) 485-489.
- [45] A.S. Achrol, R.C. Rennert, C. Anders, R. Soffietti, M.S. Ahluwalia, L. Nayak, S. Peters, N.D. Arvold, G.R. Harsh, P.S. Steeg, S.D. Chang, Brain metastases, *Nat Rev Dis Primers*, 5 (2019) 5.
- [46] L.G. Dubois, L. Campanati, C. Righy, I. D'Andrea-Meira, T.C. Spohr, I. Porto-Carreiro, C.M. Pereira, J. Balça-Silva, S.A. Kahn, M.F. DosSantos, A. Oliveira Mde, A. Ximenes-da-Silva, M.C. Lopes, E. Faveret, E.L. Gasparetto, V. Moura-Neto, Gliomas and the vascular fragility of the blood brain barrier, *Front Cell Neurosci*, 8 (2014) 418.
- [47] S. Watkins, S. Robel, I.F. Kimbrough, S.M. Robert, G. Ellis-Davies, H. Sontheimer, Disruption of astrocyte-vascular coupling and the blood-brain barrier by invading glioma cells, *Nat Commun*, 5 (2014) 4196.
- [48] A.T. Kessler, A.A. Bhatt, Brain tumour post-treatment imaging and treatment-related complications, *Insights Imaging*, 9 (2018) 1057-1075.
- [49] J.N. Sarkaria, L.S. Hu, I.F. Parney, D.H. Pafundi, D.H. Brinkmann, N.N. Laack, C. Giannini, T.C. Burns, S.H. Kizilbash, J.K. Laramy, K.R. Swanson, T.J. Kaufmann, P.D. Brown, N.Y.R. Agar, E. Galanis, J.C. Buckner, W.F. Elmquist, Is the blood-brain barrier really disrupted in all glioblastomas? A critical assessment of existing clinical data, *Neuro-oncology*, 20 (2018) 184-191.

- [50] C. Kim, J.-Y. Suh, C. Heo, C.K. Lee, W.H. Shim, B.W. Park, G. Cho, D.-W. Lee, D.-C. Woo, S.-Y. Kim, Y.J. Kim, D.-J. Bae, J.K. Kim, Spatiotemporal heterogeneity of tumor vasculature during tumor growth and antiangiogenic treatment: MRI assessment using permeability and blood volume parameters, *Cancer Med*, 7 (2018) 3921-3934.
- [51] Y. Chen, L. Liu, Modern methods for delivery of drugs across the blood–brain barrier, *Adv Drug Deliv Rev*, 64 (2012) 640-665.
- [52] T. Tashima, Brain Cancer Chemotherapy through a Delivery System across the Blood-Brain Barrier into the Brain Based on Receptor-Mediated Transcytosis Using Monoclonal Antibody Conjugates, *Biomedicines*, 10 (2022).
- [53] S. Naqvi, A. Panghal, S.J.S. Flora, Nanotechnology: A promising approach for delivery of neuroprotective drugs, *Front Neurosci*, 14 (2020) 494-494.
- [54] J.P. Fisher, D.C. Adamson, Current FDA-approved therapies for high-grade malignant gliomas, *Biomedicines*, 9 (2021) 324.
- [55] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, *N Engl J Med*, 352 (2005) 987-996.
- [56] A.H.A. Sales, J. Beck, O. Schnell, C. Fung, B. Meyer, J. Gempt, Surgical treatment of glioblastoma: State-of-the-art and future trends, *J Clin Med*, 11 (2022).
- [57] R. Stupp, S. Taillibert, A.A. Kanner, S. Kesari, D.M. Steinberg, S.A. Toms, L.P. Taylor, F. Lieberman, A. Silvani, K.L. Fink, G.H. Barnett, J.-J. Zhu, J.W. Henson, H.H. Engelhard, T.C. Chen, D.D. Tran, J. Sroubek, N.D. Tran, A.F. Hottinger, J. Landolfi, R. Desai, M. Caroli, Y. Kew, J. Honnorat, A. Idbaih, E.D. Kirson, U. Weinberg, Y. Palti, M.E. Hegi, Z. Ram, Maintenance therapy with tumor-treating fields plus temozolomide vs temozolomide alone for glioblastoma: A randomized clinical trial, *JAMA*, 314 (2015) 2535-2543.
- [58] M. Mehta, P. Wen, R. Nishikawa, D. Reardon, K. Peters, Critical review of the addition of tumor treating fields (TTFields) to the existing standard of care for newly diagnosed glioblastoma patients, *Crit Rev Oncol Hematol*, 111 (2017) 60-65.
- [59] R. Stupp, S. Taillibert, A. Kanner, W. Read, D.M. Steinberg, B. Lhermitte, S. Toms, A. Idbaih, M.S. Ahluwalia, K. Fink, F. Di Meco, F. Lieberman, J.-J. Zhu, G. Stragliotto, D.D. Tran, S. Brem, A.F. Hottinger, E.D. Kirson, G. Lavy-Shahaf, U. Weinberg, C.-Y. Kim, S.-H. Paek, G. Nicholas, J. Bruna, H. Hirte, M. Weller, Y. Palti, M.E. Hegi, Z. Ram, Effect of tumor-treating fields plus maintenance temozolomide vs maintenance temozolomide alone on survival in patients with glioblastoma: A randomized clinical trial, *JAMA*, 318 (2017) 2306-2316.

- [60] J.Y. Yoo, M. Yeh, B. Kaur, T.J. Lee, Targeted delivery of small noncoding RNA for glioblastoma, *Cancer Lett*, 500 (2021) 274-280.
- [61] X. Li, X. Geng, Z. Chen, Z. Yuan, Recent advances in glioma microenvironment-response nanoplatforms for phototherapy and sonotherapy, *Pharmacol Res*, 179 (2022) 106218.
- [62] X. Ren, J. Qi, W. Wu, Z. Yin, T. Li, Y. Lu, Development of carrier-free nanocrystals of poorly water-soluble drugs by exploring metastable zone of nucleation, *Acta Pharm Sin B*, 9 (2019) 118-127.
- [63] J. Wong, A. Brugger, A. Khare, M. Chaubal, P. Papadopoulos, B. Rabinow, J. Kipp, J. Ning, Suspensions for intravenous (IV) injection: A review of development, preclinical and clinical aspects, *Adv Drug Deliv Rev*, 60 (2008) 939-954.
- [64] S. Marqus, E. Pirogova, T.J. Piva, Evaluation of the use of therapeutic peptides for cancer treatment, *J Biomed Sci*, 24 (2017) 21.
- [65] J.J.F. Verhoef, T.J. Anchordoquy, Questioning the use of PEGylation for drug delivery, *Drug Deliv Transl Res*, 3 (2013) 499-503.
- [66] M.K.L. Fung, G.C.-F. Chan, Drug-induced amino acid deprivation as strategy for cancer therapy, *J Hematol Oncol*, 10 (2017) 144.
- [67] R. Mahato, W. Tai, K. Cheng, Prodrugs for improving tumor targetability and efficiency, *Adv Drug Deliv Rev*, 63 (2011) 659-670.
- [68] W.K. Yung, M.D. Prados, R. Yaya-Tur, S.S. Rosenfeld, M. Brada, H.S. Friedman, R. Albright, J. Olson, S.M. Chang, A.M. O'Neill, A.H. Friedman, J. Bruner, N. Yue, M. Dugan, S. Zaknoen, V.A. Levin, Multicenter phase II trial of temozolomide in patients with anaplastic astrocytoma or anaplastic oligoastrocytoma at first relapse. Temodal Brain Tumor Group, *J Clin Oncol*, 17 (1999) 2762-2771.
- [69] M.H. Cohen, J.R. Johnson, R. Pazdur, Food and Drug Administration Drug approval summary: Temozolomide plus radiation therapy for the treatment of newly diagnosed glioblastoma multiforme, *Clin Cancer Res*, 11 (2005) 6767-6771.
- [70] W. Yang, J. Soares, P. Greninger, E.J. Edelman, H. Lightfoot, S. Forbes, N. Bindal, D. Beare, J.A. Smith, I.R. Thompson, S. Ramaswamy, P.A. Futreal, D.A. Haber, M.R. Stratton, C. Benes, U. McDermott, M.J. Garnett, Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells, *Nucleic Acids Research*, 41 (2012) D955-D961.
- [71] S. Ostermann, C. Csajka, T. Buclin, S. Leyvraz, F. Lejeune, L.A. Decosterd, R. Stupp, Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients, *Clin Cancer Res*, 10 (2004) 3728-3736.
- [72] S. Wilhelm, A.J. Tavares, Q. Dai, S. Ohta, J. Audet, H.F. Dvorak, W.C.W. Chan, Analysis of nanoparticle delivery to tumours, *Nat Rev Mater*, 1 (2016) 16014.

- [73] P. Boisseau, B. Loubaton, Nanomedicine, nanotechnology in medicine, *Comptes Rendus Physique*, 12 (2011) 620-636.
- [74] R. Freitas, Nanomedicine, Volume I: Basic Capabilities, Landes Bioscience, 1999.
- [75] J. Shi, P.W. Kantoff, R. Wooster, O.C. Farokhzad, Cancer nanomedicine: progress, challenges and opportunities, *Nat Rev Cancer*, 17 (2017) 20-37.
- [76] P. Dolez, Nanoengineering: Global Approaches to Health and Safety Issues, 1st ed., Elsevier, Amsterdam, 2015.
- [77] R.T. Stiepel, E. Duggan, C.J. Batty, K.M. Ainslie, Micro and nanotechnologies: The little formulations that could, *Bioeng Transl Med*, In press (2022) e10421.
- [78] M. Souiri, M. Soltani, F. Moradi Kashkooli, M. Kiani Shahvandi, M. Chiani, F.S. Shariati, M.R. Mehrabi, L.L. Munn, Towards principled design of cancer nanomedicine to accelerate clinical translation, *Mater Today Bio*, 13 (2022) 100208.
- [79] H. He, L. Liu, E.E. Morin, M. Liu, A. Schwendeman, Survey of clinical translation of cancer nanomedicines-lessons learned from successes and failures, *Acc Chem Res*, 52 (2019) 2445-2461.
- [80] C. Domingues, A. Santos, C. Alvarez-Lorenzo, A. Concheiro, I. Jarak, F. Veiga, I. Barbosa, M. Dourado, A. Figueiras, Where is nano today and where is it headed? A review of nanomedicine and the dilemma of nanotoxicology, *ACS Nano*, 16 (2022) 9994-10041.
- [81] D. Wibowo, S.H.T. Jorritsma, Z.J. Gonzaga, B. Evert, S. Chen, B.H.A. Rehm, Polymeric nanoparticle vaccines to combat emerging and pandemic threats, *Biomaterials*, 268 (2021) 120597.
- [82] C. Martins, F. Sousa, F. Araújo, B. Sarmento, Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications, *Adv Healthc Mater*, 7 (2018) 1701035.
- [83] B. Wan, J.V. Andhariya, Q. Bao, Y. Wang, Y. Zou, D.J. Burgess, Effect of polymer source on in vitro drug release from PLGA microspheres, *Int J Pharm*, 607 (2021) 120907.
- [84] P. Blasi, Poly(lactic acid)/poly(lactic-co-glycolic acid)-based microparticles: An overview, *J Pharm Investig*, 49 (2019) 337-346.
- [85] Z. Fu, S. Li, S. Han, C. Shi, Y. Zhang, Antibody drug conjugate: The “biological missile” for targeted cancer therapy, *Signal Transduct Target Ther*, 7 (2022) 93.
- [86] J.T.W. Tong, P.W.R. Harris, M.A. Brimble, I. Kavianinia, An insight into FDA approved antibody-drug conjugates for cancer therapy, *Molecules* 26 (2021) 5847.
- [87] J. Schwach, M. Abdellatif, A. Stengl, More than toxins -Current prospects in designing the next generation of antibody drug conjugates, *Front Biosci* 27 (2022) 240.

- [88] X. Zhao, C. Cheng, J. Gou, T. Yi, Y. Qian, X. Du, X. Zhao, Expression of tissue factor in human cervical carcinoma tissue, *Exp Ther Med*, 16 (2018) 4075-4081.
- [89] J. Carlsson, H. Nordgren, J. Sjöström, K. Wester, K. Villman, N.O. Bengtsson, B. Ostenstad, H. Lundqvist, C. Blomqvist, HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review, *Br J Cancer*, 90 (2004) 2344-2348.
- [90] O. Legrand, J.Y. Perrot, M. Baudard, A. Cordier, R. Lautier, G. Simonin, R. Zittoun, N. Casadevall, J.P. Marie, The immunophenotype of 177 adults with acute myeloid leukemia: Proposal of a prognostic score, *Blood*, 96 (2000) 870-877.
- [91] L. Woythe, N.B. Tito, L. Albertazzi, A quantitative view on multivalent nanomedicine targeting, *Adv Drug Deliv Rev*, 169 (2021) 1-21.
- [92] J. Hrkach, D. Von Hoff, M. Mukkaram Ali, E. Andrianova, J. Auer, T. Campbell, D. De Witt, M. Figa, M. Figueiredo, A. Horhota, S. Low, K. McDonnell, E. Peeke, B. Retnarajan, A. Sabnis, E. Schnipper, J.J. Song, Y.H. Song, J. Summa, D. Tompsett, G. Troiano, T. Van Geen Hoven, J. Wright, P. LoRusso, P.W. Kantoff, N.H. Bander, C. Sweeney, O.C. Farokhzad, R. Langer, S. Zale, Preclinical development and clinical translation of a PSMA-targeted docetaxel nanoparticle with a differentiated pharmacological profile, *Sci Transl Med*, 4 (2012) 128ra139.
- [93] M. Jurczyk, J. Kasperczyk, D. Wrześniok, A. Beberok, K. Jelonek, Nanoparticles loaded with docetaxel and resveratrol as an advanced tool for cancer therapy, *Biomedicines*, 10 (2022).
- [94] V. Sanna, M. Sechi, Therapeutic potential of targeted nanoparticles and perspective on nanotherapies, *ACS Med Chem Lett*, 11 (2020) 1069-1073.
- [95] W.A. Jefferies, M.R. Brandon, S.V. Hunt, A.F. Williams, K.C. Gatter, D.Y. Mason, Transferrin receptor on endothelium of brain capillaries, *Nature*, 312 (1984) 162-163.
- [96] E.M. Taylor, E.H. Morgan, Developmental changes in transferrin and iron uptake by the brain in the rat, *Brain Res Dev Brain Res*, 55 (1990) 35-42.
- [97] B. Voth, D.T. Nagasawa, P.E. Pelargos, L.K. Chung, N. Ung, Q. Gopen, S. Tenn, D.T. Kamei, I. Yang, Transferrin receptors and glioblastoma multiforme: Current findings and potential for treatment, *J Clin Neurosci*, 22 (2015) 1071-1076.
- [98] X. Wang, Y. Zhao, S. Dong, R.J. Lee, D. Yang, H. Zhang, L. Teng, Cell-penetrating peptide and transferrin co-modified liposomes for targeted therapy of glioma, *Molecules*, 24 (2019) 3540.
- [99] S. Lakkadwala, B. dos Santos Rodrigues, C. Sun, J. Singh, Dual functionalized liposomes for efficient co-delivery of anti-cancer chemotherapeutics for the treatment of glioblastoma, *J Control Release*, 307 (2019) 247-260.

- [100] S. Lakkadwala, J. Singh, Dual functionalized 5-fluorouracil liposomes as highly efficient nanomedicine for glioblastoma treatment as assessed in an in vitro brain tumor model, *J Pharm Sci*, 107 (2018) 2902-2913.
- [101] X. Zhu, H. Zhou, Y. Liu, Y. Wen, C. Wei, Q. Yu, J. Liu, Transferrin/aptamer conjugated mesoporous ruthenium nanosystem for redox-controlled and targeted chemo-photodynamic therapy of glioma, *Acta Biomater*, 82 (2018) 143-157.
- [102] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, Glioma targeting and blood-brain barrier penetration by dual-targeting doxorubicin liposomes, *Biomaterials*, 34 (2013) 5628-5639.
- [103] P. Zhang, L. Hu, Q. Yin, L. Feng, Y. Li, Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy, *Mol Pharm*, 9 (2012) 1590-1598.
- [104] S. Fu, M. Liang, Y. Wang, L. Cui, C. Gao, X. Chu, Q. Liu, Y. Feng, W. Gong, M. Yang, Z. Li, C. Yang, X. Xie, Y. Yang, C. Gao, Dual-modified novel biomimetic nanocarriers improve targeting and therapeutic efficacy in glioma, *ACS Appl Mater Interfaces*, 11 (2019) 1841-1854.
- [105] T. Zong, L. Mei, H. Gao, W. Cai, P. Zhu, K. Shi, J. Chen, Y. Wang, F. Gao, Q. He, Synergistic dual-ligand doxorubicin liposomes improve targeting and therapeutic efficacy of brain glioma in animals, *Mol Pharm*, 11 (2014) 2346-2357.
- [106] L. Di, A. Maisseyeu, Low-density lipoprotein nanomedicines: Mechanisms of targeting, biology, and theranostic potential, *Drug deliv*, 28 (2021) 408-421.
- [107] B. Dehouck, M.P. Dehouck, J.C. Fruchart, R. Cecchelli, Upregulation of the low density lipoprotein receptor at the blood-brain barrier: Intercommunications between brain capillary endothelial cells and astrocytes, *J Cell Biol*, 126 (1994) 465-473.
- [108] C. Liu, Z. Zhao, H. Gao, I. Rostami, Q. You, X. Jia, C. Wang, L. Zhu, Y. Yang, Enhanced blood-brain-barrier penetrability and tumor-targeting efficiency by peptide-functionalized poly(amidoamine) dendrimer for the therapy of gliomas, *Nanotheranostics*, 3 (2019) 311-330.
- [109] S. Wang, C. Zhao, P. Liu, Z. Wang, J. Ding, W. Zhou, Facile construction of dual-targeting delivery system by using lipid capped polymer nanoparticles for anti-glioma therapy, *RSC Adv*, 8 (2018) 444-453.
- [110] Y. Zhu, Y. Jiang, F. Meng, C. Deng, R. Cheng, J. Zhang, J. Feijen, Z. Zhong, Highly efficacious and specific anti-glioma chemotherapy by tandem nanomicelles co-functionalized with brain tumor-targeting and cell-penetrating peptides, *J Control Release*, 278 (2018) 1-8.

- [111] J. Bruun, T.B. Larsen, R.I. Jøllck, R. Eliassen, R. Holm, T. Gjetting, T.L. Andresen, Investigation of enzyme-sensitive lipid nanoparticles for delivery of siRNA to blood-brain barrier and glioma cells, *Int J Nanomed*, 10 (2015) 5995-6008.
- [112] H. Gao, S. Zhang, S. Cao, Z. Yang, Z. Pang, X. Jiang, Angiopep-2 and activatable cell-penetrating peptide dual-functionalized nanoparticles for systemic glioma-targeting delivery, *Mol Pharm*, 11 (2014) 2755-2763.
- [113] C. Chen, Z. Duan, Y. Yuan, R. Li, L. Pang, J. Liang, X. Xu, J. Wang, Peptide-22 and cyclic RGD functionalized liposomes for glioma targeting drug delivery overcoming BBB and BBTB, *ACS Appl Mater Interfaces*, 9 (2017) 5864-5873.
- [114] C. Yang, Z. Yang, S. Wang, J. Chen, Q. Liu, H. Tianle, L. Hai, R. Lu, Y. Wu, Berberine and folic acid co-modified pH-sensitive cascade-targeted PTX-liposomes coated with Tween 80 for treating glioma, *Bioorg Med Chem*, 69 (2022) 116893.
- [115] M. Farshbaf, S. Mojarad-Jabali, S. Hemmati, A.Y. Khosroushahi, H. Motasadizadeh, A. Zarebkohan, H. Valizadeh, Enhanced BBB and BBTB penetration and improved anti-glioma behavior of bortezomib through dual-targeting nanostructured lipid carriers, *J Control Release*, 345 (2022) 371-384.
- [116] H. Wang, Y. Chao, H. Zhao, X. Zhou, F. Zhang, Z. Zhang, Z. Li, J. Pan, J. Wang, Q. Chen, Z. Liu, Smart nanomedicine to enable crossing blood–brain barrier delivery of checkpoint blockade antibody for immunotherapy of glioma, *ACS Nano*, 16 (2022) 664-674.
- [117] W. Echavidre, V. Picco, M. Faraggi, C. Montemagno, Integrin- $\alpha v \beta 3$ as a therapeutic target in glioblastoma: Back to the future?, *Pharmaceutics*, 14 (2022).
- [118] M. Paolillo, M.C. Galiazzo, A. Daga, E. Ciusani, M. Serra, L. Colombo, S. Schinelli, An RGD small-molecule integrin antagonist induces detachment-mediated anoikis in glioma cancer stem cells, *Int J Oncol*, 53 (2018) 2683-2694.
- [119] X. Shi, R. Ma, Y. Lu, Y. Cheng, X. Fan, J. Zou, H. Zheng, F. Li, J.-G. Piao, iRGD and TGN co-modified PAMAM for multi-targeted delivery of ATO to gliomas, *Biochem Biophys Res Commun*, 527 (2020) 117-123.
- [120] Z. Belhadj, C. Zhan, M. Ying, X. Wei, C. Xie, Z. Yan, W. Lu, Multifunctional targeted liposomal drug delivery for efficient glioblastoma treatment, *Oncotarget*, 8 (2017) 66889-66900.
- [121] H. Gao, Y. Xiong, S. Zhang, Z. Yang, S. Cao, X. Jiang, RGD and interleukin-13 peptide functionalized nanoparticles for enhanced glioblastoma cells and neovasculature dual targeting delivery and elevated tumor penetration, *Mol Pharm*, 11 (2014) 1042-1052.

- [122] H. Gao, Z. Yang, S. Cao, Y. Xiong, S. Zhang, Z. Pang, X. Jiang, Tumor cells and neovasculature dual targeting delivery for glioblastoma treatment, *Biomaterials*, 35 (2014) 2374-2382.
- [123] E. McCord, S. Pawar, T. Koneru, K. Tatiparti, S. Sau, A.K. Iyer, Folate receptors' expression in gliomas may possess potential nanoparticle-based drug delivery opportunities, *ACS Omega*, 6 (2021) 4111-4118.
- [124] J. Lu, R. Li, B. Mu, Y. Peng, Y. Zhao, Y. Shi, L. Guo, L. Hai, Y. Wu, Multiple targeted doxorubicin-lonidamine liposomes modified with p-hydroxybenzoic acid and triphenylphosphonium to synergistically treat glioma, *Eur J Med Chem*, 230 (2022) 114093.
- [125] L. Lv, Y. Jiang, X. Liu, B. Wang, W. Lv, Y. Zhao, H. Shi, Q. Hu, H. Xin, Q. Xu, Z. Gu, Enhanced antiglioblastoma efficacy of neovasculature and glioma cells dual targeted nanoparticles, *Mol Pharm*, 13 (2016) 3506-3517.
- [126] T.A. Järvinen, E. Ruoslahti, Molecular changes in the vasculature of injured tissues, *Am J Pathol*, 171 (2007) 702-711.
- [127] J.P. Newman, G.Y. Wang, K. Arima, S.P. Guan, M.R. Waters, W.K. Cavenee, E. Pan, E. Aliwarga, S.T. Chong, C.Y.L. Kok, B.B. Endaya, A.A. Habib, T. Horibe, W.H. Ng, I.A.W. Ho, K.M. Hui, T. Kordula, P.Y.P. Lam, Interleukin-13 receptor alpha 2 cooperates with EGFRvIII signaling to promote glioblastoma multiforme, *Nat Commun*, 8 (2017) 1913.
- [128] J.S. Jarboe, K.R. Johnson, Y. Choi, R.R. Lonser, J.K. Park, Expression of interleukin-13 receptor $\alpha 2$ in glioblastoma multiforme: Implications for targeted therapies, *Cancer Res*, 67 (2007) 7983-7986.
- [129] F. Liu, Gary C. Hon, Genaro R. Villa, Kristen M. Turner, S. Ikegami, H. Yang, Z. Ye, B. Li, S. Kuan, Ah Y. Lee, C. Zanca, B. Wei, G. Lucey, D. Jenkins, W. Zhang, Cathy L. Barr, Frank B. Furnari, Timothy F. Cloughesy, William H. Yong, Timothy C. Gahman, Andrew K. Shiau, Webster K. Cavenee, B. Ren, Paul S. Mischel, EGFR mutation promotes glioblastoma through epigenome and transcription factor network remodeling, *Mol Cell*, 60 (2015) 307-318.
- [130] H.K. Lee, C. Xiang, S. Cazacu, S. Finniss, G. Kazimirsky, N. Lemke, N.L. Lehman, S.A. Rempel, T. Mikkelsen, C. Brodie, GRP78 is overexpressed in glioblastomas and regulates glioma cell growth and apoptosis, *Neuro Oncol*, 10 (2008) 236-243.
- [131] J. Li, A.S. Lee, Stress induction of GRP78/BiP and its role in cancer, *Curr Mol Med*, 6 (2006) 45-54.
- [132] A. Sathiyaseelan, K. Saravanakumar, A.V.A. Mariadoss, M.-H. Wang, pH-controlled nucleolin targeted release of dual drug from chitosan-gold based aptamer

functionalized nano drug delivery system for improved glioblastoma treatment, *Carbohydr Polym*, 262 (2021) 117907.

[133] H. Gao, Z. Yang, S. Zhang, Z. Pang, Q. Liu, X. Jiang, Study and evaluation of mechanisms of dual targeting drug delivery system with tumor microenvironment assays compared with normal assays, *Acta Biomater*, 10 (2014) 858-867.

[134] H. Gao, J. Qian, S. Cao, Z. Yang, Z. Pang, S. Pan, L. Fan, Z. Xi, X. Jiang, Q. Zhang, Precise glioma targeting of and penetration by aptamer and peptide dual-functioned nanoparticles, *Biomaterials*, 33 (2012) 5115-5123.

[135] N. Huang, S. Cheng, X. Zhang, Q. Tian, J. Pi, J. Tang, Q. Huang, F. Wang, J. Chen, Z. Xie, Z. Xu, W. Chen, H. Zheng, Y. Cheng, Efficacy of NGR peptide-modified PEGylated quantum dots for crossing the blood–brain barrier and targeted fluorescence imaging of glioma and tumor vasculature, *Nanomedicine*, 13 (2017) 83-93.

[136] Y. Cui, J. Sun, W. Hao, M. Chen, Y. Wang, F. Xu, C. Gao, Dual-target peptide-modified erythrocyte membrane-enveloped PLGA nanoparticles for the treatment of glioma, *Front Oncol*, 10 (2020) 563938.

[137] P. Häfliger, R.-P. Charles, The L-Type amino acid transporter LAT1 - an emerging target in cancer, *Int J Mol Sci*, 20 (2019) 2428.

[138] Z. Haining, N. Kawai, K. Miyake, M. Okada, S. Okubo, X. Zhang, Z. Fei, T. Tamiya, Relation of LAT1/4F2hc expression with pathological grade, proliferation and angiogenesis in human gliomas, *BMC Clin Pathol*, 12 (2012) 4.

[139] H. Nawashiro, N. Otani, N. Shinomiya, S. Fukui, H. Ooigawa, K. Shima, H. Matsuo, Y. Kanai, H. Endou, L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors, *Int J Cancer*, 119 (2006) 484-492.

[140] W. Zhang, A. Mehta, Z. Tong, L. Esser, N.H. Voelcker, Development of polymeric nanoparticles for blood–brain barrier transfer—strategies and challenges, *Adv Sci*, 8 (2021) 2003937.

[141] Z. Jin, L. Piao, G. Sun, C. Lv, Y. Jing, R. Jin, Dual functional nanoparticles efficiently across the blood–brain barrier to combat glioblastoma via simultaneously inhibit the PI3K pathway and NKG2A axis, *J Drug Target*, 29 (2021) 323-335.

CHAPTER II. Overview, motivation, aims and tasks

1. OVERVIEW AND MOTIVATION

Glioblastoma (GBM) is the most common primary malignant brain tumor and presents a dismal median survival of approximately 15 months from diagnosis [1]. The current standard of care (SOC) relies on maximally safe surgical resection followed by radiotherapy plus concomitant and adjuvant temozolomide (TMZ) oral chemotherapy [2]. Despite such a heavy treatment setting, GBM recurrence is often inevitable due to the development of further satellite tumors that form at distant locations in the brain and arise from remaining and infiltrative non-targeted tumor cells. Several late phase clinical trials have failed to demonstrate a meaningful anti-GBM therapeutic benefit [3], with deprived drug delivery to the brain and systemic toxicity as major causes of these fails [4]. Poor drug delivery to the brain is necessarily associated with the presence of the blood-brain barrier (BBB) – a brain-protective barrier that also prevents most systemic therapeutic molecules from penetrating the brain parenchyma, resulting in low efficacy of brain disease treatments [5]. Altogether, the only first-line drug available for GBM treatment is still TMZ, which was approved more than 15 years ago [2] - TMZ presents an unique capacity to surpass the BBB (blood plasma to cerebrospinal fluid ratio of 0.2-0.3 [6]) but, on the less positive side, well reported mechanisms of resistance and a poor tumoricidal potency highlighted by an IC_{50} of approximately 1 mM in GBM cells [7]. Therefore, GBM is considered an area of unmet clinical need where new therapeutic approaches are urgently required. Strategic directions for the development of these new therapeutic approaches might include the exploitation of alternative chemotherapeutics with a higher tumoricidal potency – this is the case of docetaxel (DTX) that presents an IC_{50} in GBM cells approximately 100,000-times lower than TMZ [7]. However, key challenges regarding the use of DTX in anti-GBM therapies reside on circumventing the principal pharmaceutical constraints of this drug, including (i) a suboptimal aqueous solubility, (ii) very poor BBB permeation (blood plasma to cerebrospinal fluid ratio of 0.001-0.09 [8]), and lack of tumor cell targeting (only 1% of tumor tissue uptake of the total dose [9]). Poor drug delivery to the brain and systemic toxicity, which are responsible for the failure of the most prospective anti-cancer therapeutics in clinical trials, can be overcome through the use of targeted nanomedicine. Nanomedicines are attractive systems for therapeutic delivery since they are able to (i) enhance the formulation constraints of cargo molecules (e.g. low aqueous solubility), (ii) provide cargo release in a sustained manner, and (iii) promote transcytosis of cargo molecules across difficult-to-permeate biological barriers such as the BBB and tumor barriers [10]. Among nanomedicine systems, nanoparticles (NP) of the poly(lactic-co-glycolic acid) (PLGA) polymer are focus of great interest due to their biodegradable, biocompatible and versatile matrix, as well

as reproducible and scalable production [11, 12]. A gold standard modification of PLGA NPs relies on the surface decoration with polyethylene glycol (PEG), thus endowing them with multiple possibilities of surface modification by functionalization of the PEG outer arms [13]. Surface modification has been key to modulate the responsiveness of PLGA and PEG NPs to certain biologic stimuli [14]. Several studies have attempted the surface functionalization of NPs with targeting ligands that recognize and bind specific cell transporters usually overexpressed or preferentially present at the site of interest [11, 15, 16]. These targeted nanomedicines promote higher accumulation and/or probability of intracellular transport of therapeutic NPs at the target site, therefore ameliorating drug therapeutic indices and reducing off-target side effects [17]. Surface functionalization with ligands targeted to BBB or GBM cells has been exploited to shuttle therapeutic NPs from blood to brain, or provide tumor cell intracellular transport of drug cargos, respectively [18]. However, single-ligand functionalized NPs, targeted to either the BBB or GBM, are not sufficient to simultaneously circumvent the BBB restrictive environment and the need to instruct drugs to accumulate at tumor cells [19]. Thus, the need to simultaneously and sequentially target the privileged anatomical location of GBM (BBB-protected brain) and the invading GBM cells, opens avenues for the exploitation of multi-ligand functionalized and multi-targeted nanomedicines.

2. AIMS AND OBJECTIVES

2.1 Main aim (CHAPTER III)

The main aim of this doctoral thesis was to develop an intravenous multi-stage blood-to-tumor polymeric NP platform to deliver DTX to GBM. Here, NPs of PLGA and PEG encapsulating DTX are proposed for the composition of the NP platform. **The main aim can be divided into two sub-aims, including:**

- i. **Endow this NP platform with tumor-accumulating properties (transport towards tumor cells of the brain):** NPs will present a PLGA core along with a short-length PEG shielding coupled with a first targeting ligand, L-Histidine amino acid (His), to target the GBM-overexpressed L-type amino acid transporter 1 (LAT1) and trigger transport into tumor cells. LAT1 is a promising target for GBM therapies, since it is extensively reported to be overexpressed by GBM cells, whilst expressed only in basal levels in the normal cerebral cortex [20-22].

- ii. **Endow this NP platform with BBB-targeting properties (blood-to-brain transport):** Apart from the previous His surface functionalization, PLGA core NPs will present an acid-cleavable long-length PEG shielding coupled with a second targeting ligand, Angiopep-2 peptide (ANG2), to target the BBB-overexpressed low-density lipoprotein receptor (LDLR) and trigger blood-to-brain endocytosis. ANG2 has been one of the most exploited LDLR-ligands in the field of brain-targeted nanomedicine due to its proven capability of shuttling NP systems across the BBB [5, 23]. The acid-cleavable properties will ensure that the PEG-ANG2 moiety separates from the PLGA core of the NPs after they encounter the acidic pH of endosomes during BBB endocytosis, in order to lower ANG2-LDLR avidity and increase the efficiency of the process of NP trafficking from blood to brain [24, 25]. The acid-cleavable properties, along with the difference of length of the PEG shielding used for His (low) and ANG2 (high) attachment, will assure that, after separation of the PEG-ANG2 moiety from the PLGA core of the NPs, surface His become free of steric hindrance and able to perform further tumor targeting.

This is the first time that a multi-ligand functionalized NP platform is proposed to sequentially target the BBB and GBM through exploitation of a BBB-responsive transport mechanism. Over the past 10 years, other authors exploited multi-ligand functionalization of therapeutic NPs for BBB and GBM targeting [26-35], but none of the proposed systems presented BBB-stimulus responsiveness to boost NP permeation across the BBB [24, 25], paving the way to allow tumor-targeted loaded cargos to exert an enhanced anti-GBM effect.

Specific tasks for the main aim of this doctoral thesis include:

- a. Chemical synthesis of the His and acid-cleavable ANG2 polymeric conjugates that will constitute the NP matrix.
- b. Manufacturing and characterization of DTX-loaded NPs assembled from the polymeric conjugates synthesized in (a).
- c. *In vitro* validation of the NPs regarding tumor uptake, cytotoxicity and BBB permeability.
- d. *In vivo* validation of the NPs in a GBM rodent model regarding biodistribution, therapeutic efficacy and systemic toxicity.

The work related with the main aim of this doctoral thesis will be presented in **CHAPTER III.**

2.2 Secondary aim (CHAPTER IV)

The secondary aim of this doctoral thesis was to develop and validate a novel GBM 3D *in vitro* model as an evaluation tool of anti-cancer nano-therapeutics.

The lack of appropriate GBM *in vitro* models that recapitulate the *in vivo* complexity of the disease, and accurately predict the therapeutic response in a clinical setting, is one of the challenges faced by anti-GBM nanomedicines in what concerns clinical translation [36, 37]. Here, a multicellular tumor microtissue (MCTM), based on a heterotypic tumor spheroid-like structure, is proposed to emulate the tumor microenvironment (TME) of GBM. The GBM MCTM will include: a GBM tumor cell line, macrophages differentiated from healthy blood donors' peripheral monocytes, and brain microvascular primary endothelial cells.

This is the first time that a GBM 3D *in vitro* model combines GBM cells with both primary macrophages and endothelial cells of human and brain origin, respectively, instead of only GBM cells and macrophages (as cell line) or endothelial cells [38-41]. This is expected to increase the fidelity of the proposed *in vitro* model for preclinical testing.

Specific tasks for the secondary aim of this doctoral thesis include:

- a. High-throughput production of GBM MCTMs using agarose micro-molding technology.
- b. Morphological and histological characterization of the GBM MCTMs.
- c. Validation of the ability of GBM MCTMs to respond to therapy using a model nano-therapeutic, namely the DTX-loaded His-functionalized NPs produced in (2.1i) for GBM targeting via LAT1. The validation involves the investigation of MCTM cell uptake, cytotoxicity, macrophage polarization and biomolecular signature post-treatment.

The work related with the secondary aim of this doctoral thesis will be presented in **CHAPTER IV**.

2.3 Symbiosis of supervision institutions

The main focuses of the Nanomedicines & Translational Drug Delivery group of Instituto de Investigação e Inovação em Saúde (i3S) are the development and characterization

of (i) multifunctional nanoformulations with preventive, diagnostic or therapeutic application in the pharmaceutical and biomedical fields, such as in the field of cancer, and (iii) biorelevant *in vitro* cell models of excellent *in vitro/in vivo* correlation to predict drug bioavailability and understanding of the cell-nanomaterial interactions. This group has a vast expertise in tailoring a wide range of nanoformulations, such as polymer-based, to target specific tissues and surpass difficult-to-permeate biological barriers (including a great focus on the BBB), ultimately enhancing the therapeutic effect of their payloads for treatment of high-burden diseases such as cancer.

The Advanced Drug Delivery and Biomaterials group of University of Louvain is specialized in the use of nanomedicines for cancer applications such as GBM treatment, and pioneered important advancements in the field of anti-cancer controlled release nanosystems to induce long-term survival in GBM-bearing animal models. This institution counts with up-to-date technologies for *in vivo* testing, as well as an extensive know-how in GBM rodent models. Specialized technologies for *in vivo* testing, biophysical, bioimaging and analytical characterization of nanomedicines will be provided.

The Advanced Materials and Healthcare Technologies division of the School of Pharmacy of University of Nottingham provides state-of-the-art facilities for the manufacture and characterization of translationable nanoformulations. The research activities of this institution align with the needs of this doctoral thesis and focus on the manufacture and implementation of new dosage formulations for targeted healthcare (precision medicine), with key expertise in the manufacture, functionalization, characterization, delivery and application of nanoformulations to biological targets.

REFERENCES

- [1] R. Stupp, M. Brada, M.J. van den Bent, J.C. Tonn, G. Pentheroudakis, High-grade glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up, *Ann Oncol*, 25 (2014) iii93-101.
- [2] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, *N Engl J Med*, 352 (2005) 987-996.
- [3] W. Wu, J.L. Klockow, M. Zhang, F. Lafortune, E. Chang, L. Jin, Y. Wu, H.E. Daldrup-Link, Glioblastoma multiforme (GBM): An overview of current therapies and mechanisms of resistance, *Pharm Res*, 171 (2021) 105780.
- [4] B. Pająk, Looking for the holy grail-drug candidates for glioblastoma multiforme chemotherapy, *Biomedicines*, 10 (2022) 1001.
- [5] W. Zhang, A. Mehta, Z. Tong, L. Esser, N.H. Voelcker, Development of polymeric nanoparticles for blood–brain barrier transfer—strategies and challenges, *Adv Sci*, 8 (2021) 2003937.
- [6] S. Ostermann, C. Csajka, T. Buclin, S. Leyvraz, F. Lejeune, L.A. Decosterd, R. Stupp, Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients, *Clin Cancer Res*, 10 (2004) 3728-3736.
- [7] W. Yang, J. Soares, P. Greninger, E.J. Edelman, H. Lightfoot, S. Forbes, N. Bindal, D. Beare, J.A. Smith, I.R. Thompson, S. Ramaswamy, P.A. Futreal, D.A. Haber, M.R. Stratton, C. Benes, U. McDermott, M.J. Garnett, Genomics of Drug Sensitivity in Cancer (GDSC): A resource for therapeutic biomarker discovery in cancer cells, *Nucleic Acids Res* 41 (2012) D955-D961.
- [8] A.J. ten Tije, W.J. Loos, M. Zhao, S.D. Baker, R.H. Enting, H. van der Meulen, J. Verweij, A. Sparreboom, Limited cerebrospinal fluid penetration of docetaxel, *Anticancer Drugs*, 15 (2004) 715-718.
- [9] S. Wilhelm, A.J. Tavares, Q. Dai, S. Ohta, J. Audet, H.F. Dvorak, W.C.W. Chan, Analysis of nanoparticle delivery to tumours, *Nat Rev Mater*, 1 (2016) 16014.
- [10] J. Shi, P.W. Kantoff, R. Wooster, O.C. Farokhzad, Cancer nanomedicine: progress, challenges and opportunities, *Nat Rev Cancer*, 17 (2017) 20-37.
- [11] C. Martins, F. Sousa, F. Araújo, B. Sarmiento, Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications, *Adv Healthc Mater*, 7 (2018) 1701035.

- [12] B. Wan, J.V. Andhariya, Q. Bao, Y. Wang, Y. Zou, D.J. Burgess, Effect of polymer source on in vitro drug release from PLGA microspheres, *Int J Pharm*, 607 (2021) 120907.
- [13] M. Souri, M. Soltani, F. Moradi Kashkooli, M. Kiani Shahvandi, M. Chiani, F.S. Shariati, M.R. Mehrabi, L.L. Munn, Towards principled design of cancer nanomedicine to accelerate clinical translation, *Mater Today Bio*, 13 (2022) 100208.
- [14] M. Elsabahy, K.L. Wooley, Design of polymeric nanoparticles for biomedical delivery applications, *Chem Soc Rev*, 41 (2012) 2545-2561.
- [15] R. Mout, D.F. Moyano, S. Rana, V.M. Rotello, Surface functionalization of nanoparticles for nanomedicine, *Chem Soc Rev*, 41 (2012) 2539-2544.
- [16] L. Feng, C. Zhu, H. Yuan, L. Liu, F. Lv, S. Wang, Conjugated polymer nanoparticles: preparation, properties, functionalization and biological applications, *Chem Soc Rev*, 42 (2013) 6620-6633.
- [17] J. Shi, Z. Xiao, N. Kamaly, O.C. Farokhzad, Self-assembled targeted nanoparticles: evolution of technologies and bench to bedside translation, *Acc Chem Res*, 44 (2011) 1123-1134.
- [18] S. Quader, K. Kataoka, H. Cabral, Nanomedicine for brain cancer, *Adv Drug Deliv Rev*, 182 (2022) 114115.
- [19] Y. Zhu, J. Feijen, Z. Zhong, Dual-targeted nanomedicines for enhanced tumor treatment, *Nano Today*, 18 (2018) 65-85.
- [20] Z. Haining, N. Kawai, K. Miyake, M. Okada, S. Okubo, X. Zhang, Z. Fei, T. Tamiya, Relation of LAT1/4F2hc expression with pathological grade, proliferation and angiogenesis in human gliomas, *BMC Clin Pathol*, 12 (2012) 4.
- [21] H. Nawashiro, N. Otani, N. Shinomiya, S. Fukui, H. Ooigawa, K. Shima, H. Matsuo, Y. Kanai, H. Endou, L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors, *Int J Cancer*, 119 (2006) 484-492.
- [22] P. Häfliger, R.-P. Charles, The L-Type amino acid transporter LAT1 - an emerging target in cancer, *Int J Mol Sci*, 20 (2019) 2428.
- [23] A. Duro-Castano, D. Moreira Leite, J. Forth, Y. Deng, D. Matias, C. Noble Jesus, G. Battaglia, Designing peptide nanoparticles for efficient brain delivery, *Adv Drug Deliv Rev*, 160 (2020) 52-77.
- [24] X. Tian, D.M. Leite, E. Scarpa, S. Nyberg, G. Fullstone, J. Forth, D. Matias, A. Apriceno, A. Poma, A. Duro-Castano, M. Vuyyuru, L. Harker-Kirschneck, A. Šarić, Z. Zhang, P. Xiang, B. Fang, Y. Tian, L. Luo, L. Rizzello, G. Battaglia, On the shuttling across the blood-brain barrier via tubule formation: Mechanism and cargo avidity bias, *Sci Adv*, 6 (2020) eabc4397.

- [25] A.J. Clark, M.E. Davis, Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core, *Proc Natl Acad Sci USA*, 112 (2015) 12486-12491.
- [26] Z. Jin, L. Piao, G. Sun, C. Lv, Y. Jing, R. Jin, Dual functional nanoparticles efficiently across the blood–brain barrier to combat glioblastoma via simultaneously inhibit the PI3K pathway and NKG2A axis, *J Drug Target*, 29 (2021) 323-335.
- [27] X. Wang, Y. Zhao, S. Dong, R.J. Lee, D. Yang, H. Zhang, L. Teng, Cell-penetrating peptide and transferrin co-modified liposomes for targeted therapy of glioma, *Molecules*, 24 (2019) 3540.
- [28] S. Lakkadwala, B. dos Santos Rodrigues, C. Sun, J. Singh, Dual functionalized liposomes for efficient co-delivery of anti-cancer chemotherapeutics for the treatment of glioblastoma, *J Control Release*, 307 (2019) 247-260.
- [29] C. Liu, Z. Zhao, H. Gao, I. Rostami, Q. You, X. Jia, C. Wang, L. Zhu, Y. Yang, Enhanced blood-brain-barrier penetrability and tumor-targeting efficiency by peptide-functionalized poly(amidoamine) dendrimer for the therapy of gliomas, *Nanotheranostics*, 3 (2019) 311-330.
- [30] Z. Belhadj, C. Zhan, M. Ying, X. Wei, C. Xie, Z. Yan, W. Lu, Multifunctional targeted liposomal drug delivery for efficient glioblastoma treatment, *Oncotarget*, 8 (2017) 66889-66900.
- [31] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, Glioma targeting and blood–brain barrier penetration by dual-targeting doxorubicin liposomes, *Biomaterials*, 34 (2013) 5628-5639.
- [32] X. Zhu, H. Zhou, Y. Liu, Y. Wen, C. Wei, Q. Yu, J. Liu, Transferrin/aptamer conjugated mesoporous ruthenium nanosystem for redox-controlled and targeted chemo-photodynamic therapy of glioma, *Acta Biomater*, 82 (2018) 143-157.
- [33] C. Chen, Z. Duan, Y. Yuan, R. Li, L. Pang, J. Liang, X. Xu, J. Wang, Peptide-22 and cyclic RGD functionalized liposomes for glioma targeting drug delivery overcoming BBB and BBTB, *ACS Appl Mater Interfaces*, 9 (2017) 5864-5873.
- [34] P. Zhang, L. Hu, Q. Yin, L. Feng, Y. Li, Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy, *Mol Pharm*, 9 (2012) 1590-1598.
- [35] Y. Zhu, Y. Jiang, F. Meng, C. Deng, R. Cheng, J. Zhang, J. Feijen, Z. Zhong, Highly efficacious and specific anti-glioma chemotherapy by tandem nanomicelles co-functionalized with brain tumor-targeting and cell-penetrating peptides, *J Control Release*, 278 (2018) 1-8.

- [36] F. Madani, S.S. Esnaashari, T.J. Webster, M. Khosravani, M. Adabi, Polymeric nanoparticles for drug delivery in glioblastoma: State of the art and future perspectives, *J Control Release*, 349 (2022) 649-661.
- [37] S. Đorđević, M.M. Gonzalez, I. Conejos-Sánchez, B. Carreira, S. Pozzi, R.C. Acúrcio, R. Satchi-Fainaro, H.F. Florindo, M.J. Vicent, Current hurdles to the translation of nanomedicines from bench to the clinic, *Drug Deliv Transl Res*, 12 (2022) 500-525.
- [38] M.J. Gattas, I.G. Estecho, M.A. Lago Huvelle, A.E. Errasti, E.A. Carrera Silva, M. Simian, A heterotypic tridimensional model to study the interaction of macrophages and glioblastoma in vitro, *Int J Mol Sci*, 22 (2021).
- [39] M. Tang, S.K. Tiwari, K. Agrawal, M. Tan, J. Dang, T. Tam, J. Tian, X. Wan, J. Schimelman, S. You, Q. Xia, T.M. Rana, S. Chen, Rapid 3D bioprinting of glioblastoma model mimicking native biophysical heterogeneity, *Small*, 17 (2021) 2006050.
- [40] M.A. Heinrich, R. Bansal, T. Lammers, Y.S. Zhang, R. Michel Schiffelers, J. Prakash, 3D-bioprinted mini-brain: A glioblastoma model to study cellular interactions and therapeutics, *Adv Mater*, 31 (2019) e1806590.
- [41] E.A. Akins, M.K. Aghi, S. Kumar, Incorporating tumor-associated macrophages into engineered models of glioma, *iScience*, 23 (2020) 101770.

CHAPTER III. Stimuli-responsive multifunctional nanomedicine for enhanced glioblastoma chemotherapy

This chapter was based on the following publications:

- **C Martins**, M Araújo, A Malfanti, C Pacheco, SJ Smith, B Ucakar, R Rahman, JW Aylott, V Pr  at, B Sarmiento. "Stimuli-responsive multifunctional nanomedicine for enhanced glioblastoma chemotherapy augments multistage blood-to-brain trafficking and tumor targeting" (manuscript under review)
- **C Martins**, B Sarmiento. "Cell interplay model to assess the impact of glioma cells on blood–brain barrier permeability", in Methods in Molecular Biology, Ed. N Stone, in press, Springer, 2022
- **C Martins**, B Sarmiento. "Microfluidic-manufacturing of multi-targeted PLGA/PEG nanoparticles for delivery of taxane chemotherapeutics", in Methods in Molecular Biology, Ed. K Jain, 2059:213-224, Springer, 2020

ABSTRACT

Minimal therapeutic advances have been achieved over the past two decades for glioblastoma (GBM), which remains an unmet clinical need. Here, hypothesis driven stimuli-responsive nanoparticles (NPs) for docetaxel (DTX) delivery to GBM are reported, with multifunctional features that circumvent insufficient blood-brain barrier (BBB) trafficking and lack of GBM targeting – two major hurdles for anti-GBM therapies. NPs were dual-surface tailored with a (i) brain-targeted acid-responsive Angiopep-2 moiety that triggers NP structural rearrangement within BBB endosomal vesicles, and (ii) L-Histidine moiety that provides NP preferential accumulation into GBM cells post-BBB crossing. In tumor invasive margin patient cells, the stimuli-responsive multifunctional NPs targeted GBM cells, enhanced cell uptake by 12-fold, and induced 3-times higher cytotoxicity in 2D and 3D cell models. Moreover, the *in vitro* BBB permeability was increased by 3-fold. A biodistribution *in vivo* trial confirmed a 3-fold enhancement of NP accumulation into the brain. Lastly, the *in vivo* anti-tumor efficacy was validated in GBM orthotopic models following either intratumoral or intravenous administration. Median survival and number of long-term survivors was increased by 50%. Histological, hematologic or blood biochemical systemic toxicity was not detected. Altogether, a preclinical proof of concept supported these stimuli-responsive multifunctional NPs as an effective anti-GBM multistage chemotherapeutic strategy, with ability to respond to multiple fronts of the GBM microenvironment.

KEYWORDS

Blood-brain barrier; Drug delivery; Dual-ligand functionalization; Glioblastoma; Nanomedicine; Stimuli-responsiveness; Targeting.

1. INTRODUCTION

According to the Global Cancer Observatory 2020, the incidence of brain cancers is currently averaging 300,000 new cases per year, associated with 250,000 deaths worldwide [1]. From this, glioblastoma (GBM) is the most prevalent and lethal type of brain cancer, with less than 15-month median survival [2]. The current standard of care relies on surgical resection, followed by radiotherapy and temozolomide (TMZ) chemotherapy. TMZ presents an unique capacity to cross the blood-brain barrier (BBB), but also well reported mechanisms of resistance and a poor tumoricidal potency highlighted by an IC_{50} of approximately 800 μM (U-87 MG GBM cell line) [3]. Therefore, avenues are open for exploiting more tumoricidal chemotherapeutics such as docetaxel (DTX), which presents an IC_{50} of only 0.04 μM (U-87 MG GBM cell line) [3]. However, key challenges to address reside on circumventing the principal pharmaceutical constraints of DTX, which include a (i) suboptimal aqueous solubility, (ii) poor BBB permeation - blood plasma to cerebrospinal fluid ratio of 0.001-0.09 *versus* 0.2-0.3 for TMZ [4, 5] -, and lack of GBM cell accumulation post-BBB crossing - 1% tumor tissue uptake of the total dose [6].

Over recent years, targeted cancer nanomedicine has provided multi-component nanoparticle (NP) systems with the ultimate goal of improving the biological performance of difficult-to-deliver cargos, such as DTX. NP nanomedicines are able to counterattack the principal pharmaceutical constraints of DTX by providing (i) safekeeping depot systems that enhance drug solubilization, (ii) shuttling vehicles across biological barriers that improve drug bioavailability at the site of interest, and (iii) a navigation function for tissue targeting and/or accumulation enhancement purposes [7]. In the field of cancer nanomedicine, the biocompatible, biodegradable and Food and Drug Administration (FDA)-approved poly(lactic-co-glycolic acid) (PLGA) polymer has been widely explored as a matrix for NP assembly [8]. One of the gold standard modifications for PLGA NP systems relies on its combination with polyethylene glycol (PEG), which endows PLGA NPs with a plethora of surface tailoring options by functionalization of the PEG outer arms [9].

Surface tailoring has been key to modulate the responsiveness of nanomedicines, such as PLGA/PEG NPs, to specific biologic stimuli. Ligand moieties are typically attached to the NP surface to recognize strategic cell transporters preferentially present or overexpressed at the target tissue, leading to higher drug accumulation at the target site, improved therapeutic responses and reduced off-target effects. On the other hand, nanomedicines have been surface-tailored to take advantage of pH gradients naturally

inherent to certain microenvironments, such as cell machinery compartments or tumors. Focusing on cell machinery compartments in light of the interest of this work, nanomedicines with acid-cleavable features have been proved promising to stimuli-triggered drug delivery since early and late endosomes are characterized by a pH 4.5–6.5 [10]. An optimal NP design relies on systems with sufficient stability under near neutral physiological conditions, and capable of a triggered response upon endosomal trafficking through the use of acid-cleavable chemical bonds. As an example, this could cause NP structural rearrangement in order to cleave and consequently remove a first surface layer, and expose a second surface layer, hence escaping the setback of steric hindrance. Acid-cleavable chemical bonds used for this purpose include acetals and hydrazones, typically hydrolyzed under pH 5 and 5.5 in approximately 6 min and 3 h, respectively [11].

Herein, we propose a novel intravenous therapy for GBM based on a stimuli-responsive multifunctional PLGA/PEG NP platform encapsulating DTX and fostering the perfect symbiosis between BBB targeting and endocytosis, structural rearrangement within the acidic pH of BBB endosomes, and enhancement of GBM cell accumulation post-BBB trafficking. The proposed NPs will present a PLGA core alongside with (i) an acid-cleavable long-length PEG shielding coupled with a first targeting ligand, Angiopep-2 peptide, to target the BBB-overexpressed low-density lipoprotein receptor (LDLR) and trigger blood-to-brain endocytosis [12, 13], and (ii) a short-length PEG shielding coupled with a second targeting ligand, L-Histidine (termed His), to target the tumor-overexpressed L-type amino acid transporter 1 (LAT1) [14-16] and trigger BBB-to-GBM transport. Angiopep-2 has been one of the most exploited LDLR-ligands in the field of brain-targeted nanomedicine due to its proven capability of shuttling nanosystems across the BBB via LDLR and, consequently, increase the endocytosis and/or transcytosis rates by several-fold [12, 13]. Whereas, LAT1 is a promising target for GBM therapies, extensively reported to be overexpressed by GBM cells [14-16] - analysis of clinical samples confirmed that LAT1 is significantly overexpressed in high-grade gliomas, expressed in lower levels in low-grade gliomas, and expressed only in basal levels in the normal cerebral cortex (**Figure S1**). The NP acid-cleavable properties will assure that the long-length PEG-Angiopep-2 separates from the NP structure after encountering the acid pH of endosomes during BBB trafficking to facilitate endosomal escape and NP expulsion towards the brain [17]. On the other hand, the difference of PEG length will assure that, after separation of the long-length PEG shielding from the NP structure, the second targeting ligand, His, coupled with a PEG shielding of short-length, is surface exposed due to steric de-protection. This is the first time that a multi-ligand functionalized NP system is proposed to sequentially target the BBB and GBM

through exploitation of a BBB-responsive transport mechanism. Over the last 10 years, other authors exploited multi-ligand functionalization of therapeutic NPs for BBB and GBM targeting [18-27], but none of these systems presented BBB-stimulus responsiveness to boost NP permeation across the BBB [17, 28], paving the way to allow tumor-targeted loaded cargos to exert an enhanced anti-GBM effect. In this work, targeting polymeric conjugates with Angiopep-2 and His moieties were chemically synthesized to assemble the stimuli-responsive multifunctional NPs, which were further evaluated regarding cell uptake and cytotoxicity in 2D and 3D models of conventional cell lines and GBM invasive margin (GIN) patient cells, and BBB permeability. Moreover, *in vivo* biodistribution and therapeutic efficacy studies following intratumoral and intravenous administration were performed to provide a preclinical proof of concept. A schematic illustration is displayed in **Figure 1A**.

2. MATERIALS AND METHODS

2.1 Materials

PLGA-COOH was kindly provided by Corbion (Netherlands). Hydrocortisone, ascorbic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, basic fibroblast growth factor, resazurin, acetic acid, dimethylformamide, N-hydroxysuccinimide, N,N-dicyclohexylcarbodiimide, N,N-diisopropylethylamine, paraformaldehyde, uranyl acetate, Poloxamer 407, diethyl ether, methanol, dichloromethane, tris(2-carboxyethyl)phosphine, potassium bromide, acetonitrile, α -Cyano-4-hydroxycinnamic acid, deuterated dichloromethane, deuterated dimethyl sulfoxide, trypsin, paraformaldehyde, COOH-L-Histidine-BOC, isoflurane and hematoxylin and eosin (H&E) staining solution were acquired from Sigma-Aldrich (USA); DTX from LC Laboratories (USA); endothelial basal medium-2, high glucose Dulbecco's Modified Eagle medium and SeaKem[®] LE agarose from Lonza (Switzerland); acetonitrile, Coomassie Reagent, Versene, 10x TrypLE[™] Select and human fibronectin from Thermo Fisher Scientific (USA); Triton X-100 from Spi-Chem (USA); fetal bovine serum, penicillin-streptomycin, chemically defined lipid concentrate, Hank's Balanced Salt Solution, rat tail collagen type I and acetic acid from Gibco (USA); Cy7.5 carboxylic acid from Lumiprobe (Germany); Alexa Fluor[®] 546 Phalloidin from Life Technologies (USA). Transwell[®] cell culture inserts (transparent polyester membrane, 12-well plate format, 1 μ m pore size) were purchased from Corning (USA). PLGA-PEG(2K)-NH₂, PLGA-acetal-PEG(5K)-Mal, PLGA-PEG(5K)-Mal, mPEG(2K)-PLGA and mPEG(5K)-PLGA were

acquired from Ruixibio (China). PLGA-FKR648 and PLGA-FITC were acquired from Akina (USA). Angiopep-2 modified with a N-terminal cysteine was purchased from GenScript (USA). Rabbit cleaved caspase-3 antibody was purchased from Cell Signaling Technology (USA), and rabbit Ki67 antibody was purchased from Abcam (UK). DAB staining was purchased from Vector Labs (USA). The X-junction microfluidic chip (Quartz Droplet Junction Chip, 190 μm etch depth) was acquired from Dolomite (UK). Amicon Ultra-15 centrifugal filter units (molecular weight cutoff of 100K) were acquired from Merck Millipore (USA). U-87 MG, U-251-MG and U-373 MG cells were purchased from Biochrom (Germany); a non-tumor astrocyte cell line from ATCC[®] (USA); hCMEC/D3 cells from Cedarlane (Canada).

2.2 Chemical synthesis of the L-Histidine functionalized targeting polymeric conjugate

A solution of COOH-L-Histidine-BOC (COOH-His-BOC, molecular weight (MW) $\sim$ 0.255 kDa, 30.6 mg, 120 μmol), N-hydroxysuccinimide (41.4 mg, 360 μmol) and N,N-dicyclohexylcarbodiimide (74.3 mg, 360 μmol) in 9 ml anhydrous dimethylformamide was treated with PLGA-PEG(2K)-NH₂ (number average molecular weight (Mn) $\sim$ 17 kDa, 360 mg, 22 μmol) and N,N-diisopropylethylamine (37.8 mg, 293 μmol) in 5 ml anhydrous dimethylformamide. The reaction was stirred for 24 h at room temperature (RT) under inert atmosphere. The resultant mixture was poured into cold diethyl ether (80 mL), washed 3-times with methanol (40 mL) and dried under vacuum overnight. The polymeric conjugate was re-dissolved and filtered in 15 mL anhydrous dichloromethane, and dried under vacuum overnight. The obtained polymeric conjugate, termed PLGA-PEG(2K)-His-BOC, was obtained with 70% yield. A selective carbamate hydrolysis was further employed to remove the -BOC protecting group, giving PLGA-PEG(2K)-His, as the following: trifluoroacetic acid (455 μL , 5.9 mmol) was added to PLGA-PEG(2K)-His-BOC (150 mg, 0.009 mmol) dissolved in anhydrous dichloromethane (16 mL), and the reaction mixture was stirred overnight at RT under inert atmosphere. The reaction mixture was then extracted and neutralized 3-times with a 1 M NaOH aqueous solution (8.5 mL). The organic layer was dried under vacuum overnight, washed with cold diethyl ether (10 mL) and dried again. The obtained polymeric conjugate, termed PLGA-PEG(2K)-His, was obtained at a yield of around 70%.

2.3 Chemical synthesis of the cleavable and non-cleavable Angiopep-2 functionalized targeting polymeric conjugates

Angiopep-2 containing a N-terminal cysteine (MW ~ 2.405 kDa, 15.45 mg, 6.4 μmol) was treated with tris(2-carboxyethyl)phosphine (3.8 mg, 13.2 μmol) in 8 mL anhydrous dimethylformamide and stirred for 1 h at RT under inert atmosphere. 150 mg of either PLGA-acetal-PEG(5K)-Mal (acid-cleavable, M_n ~ 35 kDa, 4.3 μmol) or PLGA-PEG(5K)-Mal (non-cleavable, M_n ~ 35 kDa, 4.3 μmol) dissolved in 2 mL anhydrous dimethylformamide were then added to the mixture, and allowed to react for 24 h at 4 °C under inert atmosphere. The reaction mixture was poured into cold diethyl ether (40 mL), washed 3-times with methanol (30 mL) and dried under vacuum overnight, giving the cleavable PLGA-acetal-PEG(5K)-ANG2, or the non-cleavable PLGA-PEG(5K)-ANG2, with 85% yield.

2.4 Characterization of the targeting polymeric conjugates

FTIR spectra were recorded with a FTIR-RAMAN Perkin Elmer 2000 (Perkin Elmer, USA) spectrometer using the potassium bromide (KBr) pellet method. Each pellet was prepared by blending 2 mg of the polymeric conjugate with 200 mg KBr. After a 5-min purge of the sample chamber with N_2 , IR spectra were immediately recorded by accumulation of 200 interferograms at a 4 cm^{-1} spectral resolution over the range from 400 to 8,000 cm^{-1} with background subtraction. MALDI-TOF MS was carried out on a Bruker Autoflex III with NdYAG laser (Bruker Corporation, USA), operating in reflector positive mode. Samples were dissolved in acetonitrile at 2.5 mg mL^{-1} and mixed with the matrix (α -Cyano-4-hydroxycinnamic acid, 10 mg mL^{-1} in 50% v/v acetonitrile) in a 1:2 matrix:sample proportion. ^1H NMR measurements were performed with a BRUKER AVANCE III 400 MHz (Bruker Corporation, USA) or Bruker Avance III HD 600 MHz (Bruker Corporation, USA) spectrometers at 25 °C in deuterated dichloromethane or dimethyl sulfoxide. Chemical shifts are reported in ppm (δ units) and were referenced to the residual solvent signal. For water contact angle (WCA) characterization, polymeric conjugate solutions at 1 mg mL^{-1} in dimethylformamide were prepared to coat glass coverslips using a spin-coater at 4500 rpm for 1 min (15 μL sample, WS-650-23, Laurell Technologies Corporation, USA). A contact angle measuring system from Data Physics, model OCA 15, equipped with a video CCD-camera and SCA 20 software, was used with sessile drop method. Drops of type I water (4 μL) were deposited onto each sample surface. Contact angles were measured using Laplace-Young method with brightness of

96 and contrast of 511, at 15 s after the fall of the drop. Results are the average of six independent samples. Bradford assay was used to quantify the amount of Angiopep-2 in the reaction supernatants. Coomassie Reagent (150 μ L) was added to the same volume of each sample in a 96-well plate, and shaken during 30 seconds. Then, the plate was incubated for 10 minutes at RT, and the absorbance was measured at 595 nm in a SynergyMx™ MultiMode microplate reader (BioTek™, USA). Angiopep-2 standards were prepared. For testing the acid-cleavable properties of PLGA-acetal-PEG(5K)-Mal, 40 mg of the polymer were suspended in an acetate buffer solution (1 M, pH 5.5), vortexed and left overnight under vigorous stirring. Afterwards, the polymer was recovered by centrifugation, washed 3-times with water and freeze dried (FreeZone 6, Labconco, USA). The dried polymer was dissolved in dichloromethane (2 mL), the solution poured into cold diethyl ether (10 mL), washed 3-times with methanol (10 mL) and dried under vacuum overnight. The polymeric conjugate was analyzed by ^1H NMR in deuterated dimethylsulfoxide.

2.5 Cell culturing

U-87 MG, U-251-MG, U-373 MG, non-tumor astrocytes (NHA) and GIN cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (FBS, 10% v/v) and penicillin-streptomycin (1% v/v). GIN cells were derived from the infiltrative margin of GBM adult patients undergoing 5-aminolevulinic acid (5ALA, Gliolan™) fluorescence guided neurosurgical resection at the Queen's Medical Centre (Nottingham, UK), and obtained from Dr Ruman Rahman. The full protocol for cell isolation [29, 30] and cell authentication details [31] are reported elsewhere. GIN-8 cells were isolated from a 54y female, wild-type IDH (primary GBM), intact ATRX, 0% MGMT promoter methylation, 90% resection plus Gliadel wafers; treatment 60Gy radiotherapy, concurrent and adjuvant TMZ; patient died 5 months after surgery. GIN-27 cells were isolated from a 71y male, wild-type IDH (primary GBM), intact ATRX, 0% MGMT promoter methylation, 99% resection; no adjuvant therapy (patient choice); patient died 3 months after surgery. GIN-31 cells were isolated from a 62y female, wild-type IDH (primary GBM), intact ATRX, 0% MGMT promoter methylation, 100% resection; treatment 60Gy radiotherapy, concurrent and adjuvant TMZ; patient died 16.1 months after surgery. Single tandem repeat (STR) genotyping data for GIN cells are detailed in Supporting Information (**Table S1**). hCMEC/D3 cells were maintained in endothelial basal medium-2 supplemented with FBS (5% v/v), penicillin-streptomycin (1% v/v), hydrocortisone (1.4 μ M), ascorbic acid (5 μ g mL $^{-1}$), chemically

defined lipid concentrate (1/100 v/v), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (10 mM) and basic fibroblast growth factor (1 ng mL⁻¹). This last supplement was added extemporaneously in the culture medium. Cell cultures were kept in an incubator (CellCulture CO₂ incubator, ESCO GB Ltd., UK) at 37 °C with 5% CO₂ and 95% relative humidity. Cell culture medium was changed every 2–3 days.

2.6 Nanoparticle manufacture

The microfluidic protocol for NP manufacture was adapted from elsewhere [32-34]. Briefly, the organic phase consisted of a polymer solution at 40 mg mL⁻¹ (total polymer content (TPC) based on PLGA-COOH, Mn ~ 35 kDa, and each targeting polymeric conjugate at the desired percentage) and DTX at 2 mg mL⁻¹ in acetonitrile, whereas the aqueous phase consisted of a 1% (w/v) Poloxamer 407 aqueous solution. Both phases were injected into a X-junction microfluidic chip with a three-inlet layout, in which the organic (0.75 mL min⁻¹) and aqueous (2.25 mL min⁻¹) phases were injected through the central and the two outer inlets, respectively. Each experiment was conducted until 20 mg of NPs were produced (equivalent to a 40 s run time). NPs were washed 3-times with water and recovered by ultrafiltration using Amicon Ultra-15 centrifugal filter units. For the obtention of fluorescent labeled NPs, 10% of the polymer content of the organic phase consisted of either PLGA-FKR648 (Mn ~ 20 kDa) or PLGA-FITC (Mn ~ 25 kDa), or DTX in the organic phase was replaced by Cy7.5 carboxylic acid at 0.1 mg mL⁻¹ in acetonitrile. To manufacture PEGylated but non-targeted NP controls, mPEG(2K)-PLGA (Mn ~ 17 kDa) or mPEG(5K)-PLGA (Mn ~ 35 kDa) were included in the polymer content of the organic phase, giving NP formulations termed mPEG(2K) NPs and mPEG(5K) NPs, respectively.

2.7 Nanoparticle characterization

NPs were characterized for their average size and polydispersity index (PDI) by dynamic light scattering (DLS), and zeta-potential through laser Doppler anemometry (LDA), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). For these measurements, samples were diluted (1:100) in an ionic solution of 10 mM sodium chloride. The morphological features of the NPs were analyzed by transmission electron microscopy (TEM) with a JEOL JEM 1400 microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 120 kV. Images were digitally recorded using a Gatan SC 1100 ORIUS CCD camera (Gatan, Warrendale, PA). Samples were

prepared by dropping 10 μL of NPs suspension onto a 300-mesh nickel grid, and stained with uranyl acetate. The amount of loaded DTX was directly quantified by high-performance liquid chromatography (HPLC) in order to determine the association efficiency (AE) and loading degree (LD) of the developed formulations by dissolving the NPs in 2 mL dimethylformamide. The calculation of AE and DL followed Equation (1) and (2), respectively.

$$\text{AE (\%)} = \frac{\text{Quantified loaded mass of cargo}}{\text{Initial mass of cargo}} \times 100 \quad (1)$$

$$\text{LD (\%)} = \frac{\text{Quantified loaded mass of cargo}}{\text{Total mass of cargo}} \times 100 \quad (2)$$

The quantification of DTX was performed by reversed-phase HPLC with ultraviolet detection using a Merck-Hitachi LaChrom[®] HPLC system (Merck, USA). A Symmetry C18 column (5 μm , 125 $\times$ 4 mm; Milford, USA) with a LiChrospher 100 RP-18 guard column (Merck, USA) was used as stationary phase. Chromatographic runs were performed using a gradient method at a flow rate of 1 mL/min. Initial stage was 10% acetonitrile and 90% water from 0 to 5 min; from 5 to 14 min, the conditions were defined as 60% acetonitrile and 40% water; final stage returned to 10% acetonitrile and 90% water from 14 to 17 min. The injection volume was 50 μL per sample, analyzed at a detection wavelength of 227 nm. For the NPs loaded with Cy7.5, the amount of loaded Cy7.5 was quantified by mixing 5 μL of the final NP suspension with 200 μL acetonitrile, followed by immediate measurement of the fluorescent signal (excitation 788 nm, emission 808 nm, SpectraMax plate reader, Molecular Devices, USA).

2.8 *In vitro* drug release

For the release study, DTX-loaded NPs (corresponding to 273 μg of drug) were added to 30 mL of 0.5% (w/v) Poloxamer 407 in PBS solution, pH 7.4, and then placed into an orbital shaker incubator, at 100 rpm and 37 $^{\circ}\text{C}$, during 24 h (sink conditions were assured). Aliquots of 150 μL were collected at specific time points during the assay, and the withdrawn volume was replaced with pre-heated 0.5% (w/v) Poloxamer 407 in PBS solution. Aliquots were centrifuged at 10,000 g for 15 min and the supernatant was used for HPLC analysis, in order to quantify the DTX released from NPs over time.

2.9 Flow cytometry analysis

0.25x10⁶, 0.3x10⁶ or 0.5x10⁶ cells per well were seeded for U-87 MG/U-251 MG/U-373 MG (6-well plate) cells, GIN primary cells (6-well plate) and hCMEC/D3 cells (24-well plate) [35], respectively. The cells were allowed to attach overnight, washed with phosphate buffered saline (PBS), and PLGA-FKR648 or PLGA-FITC labeled NPs in medium (25 µg mL⁻¹ or 300 µg mL⁻¹, respectively, 1 mL) were added for 4 h at 37 °C. Afterwards, cells were washed twice with PBS, detached with trypsin (50/250 µL), and 150/500 µL media were added to each well. Cells were subsequently washed with PBS, fixed with 2% paraformaldehyde (PFA) for 20 min at RT, washed again, and placed into cytometer tubes for further analysis. Cells were analyzed using an Accuri C6 cytometer (BD Biosciences, USA). Mean fluorescence intensity (MFI) was measured for at least 10,000 viable plus single cell gated events per sample, and all data was processed with FlowJo software (Tree Star, USA). Experiments were performed at least in triplicate.

2.10 Confocal microscopy analysis

GIN cells were seeded in 13 mm diameter glass cover slips placed into 24-well plates (0.06x10⁶ cells per coverslip, 400 µL medium), and allowed to attach overnight. The coverslips were pre-coated with human fibronectin at 2 µg cm⁻² for 24 h at 37 °C. The cells were washed with PBS, and PLGA-FKR648 labeled NPs in medium (25 µg mL⁻¹, 1 mL) were added and incubated for 4 h at 37 °C. After incubation, the cells were washed twice with PBS, fixed with 2% PFA for 20 min at RT, and washed again. The cells were then permeabilized with 0.25% Triton-X100 (400 µL) for 10 min at RT, and rinsed with PBS. The cells were blocked in 10% FBS in PBS for 30 min at RT. The solution was then replaced by 400 µL of 10% FBS in PBS containing Alexa Fluor 546 labelled-phalloidin (1:200 dilution) and incubated for 20 min at RT. The cells were rinsed 3-times with PBS, the coverslips mounted on glass microscope slides with mounting media and dried overnight at RT in the dark. Cells were imaged with a SP5 confocal microscope (Leica Microsystems, Germany) and image analysis was performed with the LAS AF Lite software (Leica). The images are shown as maximum projections of 10–20 Z-stacks with a distance of 1-2 µm between them.

2.11 Evaluation of cell metabolic activity (2D)

For 2D cultures, 0.002x10⁶ GIN cells per well were seeded in 96-well plates and allowed

to attach during 48 h, in order to reach a starting confluence level of around 20-30%. For U-87 MG, U-251 MG or U-373 MG cells, 0.010×10^6 cells per well were seeded in 96-well plates and allowed to attach during 24 h. After that, the medium was removed, cells were washed 3-times with PBS, and different concentrations of drug or NP samples (0.00045 to $4.5 \mu\text{M}$, in regard to the drug) in medium (200 μL) were incubated up to 48 h or 96 h, at 37°C . For dynamic-like boost treatments, the same procedure was followed, but $4.5 \mu\text{M}$ and $45 \mu\text{M}$ concentrations of drug or NP samples in medium were added for 4 h, cells washed 3-times with PBS and incubated with fresh medium up to 96 h. At each time-point, the medium was removed and 200 μL resazurin (20%, v/v) in media were added to each well and incubated for 4 h at 37°C in the dark. After, fluorescence was measured at the excitation and emission wavelengths of 530 nm and 590 nm, respectively, using a SynergyMx™ MultiMode microplate reader (BioTek™, USA). Experiments were performed at least in quadruplicate, and all data was normalized in regard to the negative (1% Triton X-100) and positive (medium) controls, which were considered 0% and 100% metabolic activity, respectively. Graphical data for cell metabolic activity was referenced to a threshold of 70% cell viability according to the ISO 10993–5 standard [36].

2.12 Evaluation of cell metabolic activity (3D)

GIN spheroids were formed using commercially available micro-molds (3D Petri Dish®, MicroTissues, USA). Agarose was dissolved at 2% (w/v) in 0.9% NaCl (w/v) and casted into 3D Petri Dish® molds to form hydrogel micro-molds with 81 homogenous circular recesses. 0.0025×10^6 cells per spheroid was added to the micro-molds and allowed to settle for 30 min, before adding media (2 mL) to each well. Media was replaced every two days. Spheroids size was assessed using a ZOE™ Fluorescent Cell Imager (Bio-Rad Laboratories, USA), and considered the average of two diameter measurements randomly evaluated in three different spheroids per micro-mold core. Two days after seeding, medium was removed, micro-molds were washed with PBS, and different concentrations of drug or NP samples (0.00045 to $4.5 \mu\text{M}$, in regard to the drug) in medium (190 μL per micro-molds and 2 mL per well) were added and incubated up to 48 h and 96 h, at 37°C . For dynamic-like boost treatments, the same procedure was followed, but $4.5 \mu\text{M}$ and $45 \mu\text{M}$ concentrations of drug or NP samples in medium were added for 4 h or 12 h, micro-molds washed 3-times with PBS and incubated with fresh medium up to 96 h. At each time-point, the medium was removed and the spheroids transferred to a 15 mL plastic tube. Per tube, spheroids were incubated with Versene

(300 μ L) for 10 min and 1x TrypLE™ Select (200 μ L) for 15 min, followed by vigorous pipetting to dissociate the spheroids into single cells. Versene and TrypLE™ Select were used to promote weakness of cell-cell adhesion bonds and foster the preservation of surface transporters during dissociation, respectively. The cells were afterwards washed 3-times with PBS and resuspended in 200 μ L media, from which 30 μ L were transferred to 96-well plates and allowed to attach overnight. The following day, 200 μ L resazurin (20%, v/v) in media were added to each well and incubated for 4 h at 37 °C in the dark. After, fluorescence was measured at the excitation and emission wavelengths of 530 nm and 590 nm, respectively, using a SynergyMx™ MultiMode microplate reader (BioTek™, USA). Experiments were performed at least in quadruplicate, and all data was normalized in regard to the positive control (medium), which was considered 100% metabolic activity. Graphical data for cell metabolic activity was referenced to a threshold of 70% cell viability according to the ISO 10993–5 standard [36].

2.13 Assessment of blood-brain barrier permeability

The protocol to set up a BBB *in vitro* model was followed from elsewhere [37, 38]. 0.025×10^6 cells/cm² hCMEC/D3 cells in media (500 μ L) were seeded in the apical side on 12-Transwell® cell culture inserts. Previously to the seeding, inserts were coated with 90 μ L of 50 μ g mL⁻¹ rat tail collagen type I in acetic acid (0.02 M) for 1 h, at 37 °C, and then washed twice with PBS. The basolateral side was filled with 1.5 mL medium. The model was kept at 37 °C during 8 days, and medium changed every 2 days. The hCMEC/D3 cell monolayer became confluent at day 8, which was the day selected to initiate the permeability study. The integrity of the cell monolayer was checked every 2 days by monitoring the TEER using an endothelial Volt–Ohm meter (Millicell® ER S-2; Merck Millipore, USA). The resistance value of an empty filter was subtracted from each measurement. The permeability experiment was performed in the apical-to-basolateral direction. The apical compartment was filled with FITC labeled NPs in Hanks' Balanced Salt Solution (300 μ g mL⁻¹, 500 μ L) [35]. Then, the assay was conducted at 37 °C using an orbital shaker incubator (100 rpm). At 24 h, 200 μ L samples were taken from the basolateral side and the fluorescence signal measured at the excitation and emission wavelengths of 490 nm and 520 nm, respectively, using a SynergyMx™ MultiMode microplate reader (BioTek™, USA). Experiments were performed in triplicate.

2.14 *In vivo* studies

All experiments were performed following the Belgian national regulations guidelines as well as in accordance with EU Directive 2010/63/EU, and were approved by the ethical committee for animal care of the Université catholique de Louvain (2019/UCL/MD/004). Seven-week-old female NMRI nude mice (Janvier Labs, France) were given free access to water and food.

2.15 *In vivo* biodistribution

To assess Cy7.5 labeled NP biodistribution, mice were intravenously injected with a NP dose equivalent to 10 μg Cy7.5 per animal (110-130 μL injection in PBS, $n = 3$). Biodistribution was visually observed using a Xenogen IVIS 50 imaging system (PerkinElmer, Belgium) at 2 h, 6 h and 24 h after injection. At 24 h, mice were sacrificed, and major organs (heart, lungs, liver, kidneys, spleen) excised for *ex vivo* analysis. Regions of interest (ROI) were drawn over the signals and area-normalized average radiant efficiency was quantified in [$\text{p s}^{-1} \text{cm}^{-2} \text{sr}^{-1}/\mu\text{W cm}^{-2}$]. Results are represented as floating bar plots using minimum and maximum values with a line at the mean.

2.16 *In vivo* therapeutic efficacy

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100/13 mg kg^{-1} , respectively) and fixed in a stereotactic frame. A hole was drilled using a surgical high-speed drill (Velleman, Belgium) and 2 μL of DMEM containing 3×10^4 U-87 MG cells were injected into the right hemisphere using an infusion syringe pump (Harvard Apparatus, USA) mounted with a Hamilton syringe (26S gauge needle) as previously described [39, 40]. The injection coordinates were 0.5 mm anterior or posterior, 2.1 mm lateral from the bregma and 2.5–3 mm deep from the outer border of the cranium, respectively. The presence, volume and location of the tumors were determined by magnetic resonance imaging (MRI, 11.7T Bruker Biospec, Bruker, Germany) at day 8 post U-87 MG cell inoculation. The tumor volume was assessed using the rapid acquisition with relaxation enhancement (RARE) sequence (TR=2500 ms; effective echo time (TE_{eff})=30 ms; RARE factor=8; FOV=2×2 cm; matrix 256×256; twenty-five contiguous slices of 0.3 mm, NA=4). At day 10 post U-87 MG cell inoculation, treatments were initiated. For intratumoral treatment, the same coordinates of U-87 MG cell inoculation were used, and 2 μg DTX in 7.5 μL were injected per brain ($n = 8$) - Group 1: untreated; Group 2: free

DTX (solubilized in 3-times diluted Taxotere® vehicle); Group 3: non-functionalized NPs; Group 4: H-NPs. For intravenous treatment, 2 mg kg⁻¹ DTX in 150 µL were injected per animal ($n = 6$), every 3 days, for a total of 5 injections - Group 1: untreated; Group 2: free DTX (solubilized in Taxotere® vehicle); Group 3: non-functionalized NPs; Group 4: H-NPs; Group 5: H-NCL-ANG2-NPs; Group 6: H-CL-ANG2-NPs.

2.17 Histological analysis of brain sections

48 h after the previously detailed intravenous treatment, for GBM bearing mice assigned to each treatment group ($n = 3$), brains were harvested for H&E stainings and immunohistochemistry (IHC) studies. Mice were sacrificed by cervical dislocation, and brains fixed in 10% neutral buffered formalin, embedded in paraffin blocks and frozen at 4 °C before 3-5 µm sections were cut. Sections were used for H&E staining, or IHC was performed to detect cleaved caspase-3 and Ki67 (antibodies used at 1:200 dilution). Visualization of stained nuclei was performed using DAB staining (Vector Labs, USA). An Olympus optical microscope (Tokyo, Japan) was used to observe the stained slices.

2.18 *In vivo* toxicity

48 h after the previously detailed intravenous treatment, for GBM bearing mice assigned to each treatment group ($n = 3$), animals were anesthetized by intraperitoneal injection of ketamine/xylazine (100/13 mg kg⁻¹, respectively) and blood was collected through the retro orbital plexus region in lithium heparin tubes (Microvette® 500 LH, Sarstedt, Germany). After a 1:5,000 dilution in PBS, blood cell counts were determined by direct microscopic counting in a Bürker cell counting chamber (Fisher Scientific, USA). Blood samples were then centrifuged at 1,500 g for 10 min to obtain the serum. Blood urea nitrogen, creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, total bilirubin and total protein levels were assessed by Vet Test 8008 (IDEXX, USA). Mice were then sacrificed by cervical dislocation, and brain, heart, liver, lungs, spleen and kidneys were excised and weighed. The organ weight coefficient was calculated by normalizing the organ weight to the body weight of each mouse. For histopathological examination of off-target major organs, organs were fixed in 10% neutral buffered formalin, embedded in paraffin blocks and frozen at 4 °C before 3 µm sections were cut and stained with H&E. An Olympus optical microscope (Tokyo, Japan) was used to observe the stained slices.

2.19 Statistical analysis

Results are represented as mean $\pm$ standard deviation from a minimum of three independent experiments. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by a post hoc test (Dunnett's test) to compare experimental groups. For *in vivo* data, survival curves were compared using a log-rank (Mantel–Cox) test. Differences were considered significant at $*p<0.05$, $**p<0.01$, or $***p<0.001$. Statistical analysis was performed with the software GraphPad Prism 9 (GraphPad Software, USA).

3. RESULTS AND DISCUSSION

3.1. Chemical synthesis of nanoparticle targeting polymeric conjugates

PLGA-PEG polymers were functionalized with His or Angiopep-2 to trigger tumor accumulation and BBB permeation, respectively. The MW of PEG within each targeting polymeric conjugate was specifically chosen to provide two levels of NP surface decoration, thus avoiding inter-steric hindrance - a lower MW 2K PEG embedded within a PLGA-PEG(2K) polymer was used for His attachment, whereas a higher MW 5K PEG embedded within a PLGA-PEG(5K) polymer was used for Angiopep-2 attachment. For the Angiopep-2 functionalization, two different conjugates were synthesized, presenting either acid-cleavable or non-cleavable properties.

3.1.1. *L*-Histidine functionalized targeting polymeric conjugate

Firstly, a PLGA-PEG(2K)-NH₂ polymer was chemically functionalized with COOH-Histidine-BOC in the L isomeric form (COOH-His-BOC) by a carbodiimide strategy followed by a selective carbamate hydrolysis (**Figure 2A**). The presence of a -BOC group protecting the -NH₂ end-group of COOH-His-BOC assured a site-oriented functionalization, avoiding amino acid cross-reaction. PLGA-PEG(2K)-His-BOC FTIR demonstrated a similar fingerprint region to the PLGA-PEG(2K)-NH₂, comprising C-O stretching, C-N stretching and C-H bending vibrations (**Figure 2B**) [41]. Moreover, it was possible to observe a new band at 1661 cm⁻¹ characteristic of C=O stretching in amides (amide I) in the spectrum of the PLGA-PEG(2K)-His-BOC, confirming the successful

reaction between PLGA-PEG(2K)-NH₂ and COOH-His-BOC. PLGA-PEG(2K)-His-BOC ¹H NMR exhibited low-intensity characteristic peaks of COOH-His-BOC that were absent in the mock reaction control and shifted in comparison to COOH-His-BOC (δ = 7.58 versus 8.98 ppm; δ = 6.98 versus 8.34 ppm; δ = 6.80 versus 8.23 ppm; **Figure S2**). The COOH-His-BOC ¹H NMR peaks at δ = 7.58 ppm and δ = 6.80 ppm are attributed to the imidazole ring protons of His, whereas the peak at δ = 6.98 ppm is attributed to the carbamate proton of the -BOC protecting group [42]. The ¹H NMR peak at δ = 1.35 ppm, characteristic of the -BOC methyl protons, was used to estimate the 90% COOH-His-BOC conjugation efficiency. In order to make the polymeric conjugate fully functional, the -BOC protecting group of PLGA-PEG(2K)-His-BOC was removed with success by a selective carbamate hydrolysis reaction, as revealed by ¹H NMR (**Figure S3**). The MALDI-TOF MS spectrum of PLGA-PEG(2K)-His displayed an ionization profile clearly distinct not only from the PLGA-PEG(2K)-NH₂, but also from the physical mixture of PLGA-PEG(2K)-NH₂ and COOH-His-BOC (**Figure 2C**). Compared with PLGA-PEG(2K)-NH₂, it was observed a shift to higher *m/z* values, and a broader MW distribution, which corroborated a successful coupling of COOH-His-BOC with PLGA-PEG(2K)-NH₂. WCA confirmed an intermediate hydrophilicity degree for PLGA-PEG(2K)-His, compared with COOH-His-BOC and PLGA-PEG(2K)-NH₂ (**Figure S4A** and **S4B**).

3.1.2. *Angiopep-2 functionalized targeting polymeric conjugates – cleavable versus non-cleavable*

Secondly, Thiol-Michael addition was selected to functionalize both PLGA-acetal-PEG(5K)-Mal and PLGA-PEG(5K)-Mal polymers with Angiopep-2 (giving PLGA-acetal-PEG(5K)-ANG2 and PLGA-PEG(5K)-ANG2, respectively), presenting acid-cleavable properties due the presence of the acid-cleavable acetal chemical group and non-cleavable properties, respectively. The Angiopep-2 was modified in advance with a N-terminal cysteine to assure a site-oriented functionalization, endowing the peptide with a -SH reaction site (**Figure 2D**). This modification is not expected to interfere with the Angiopep-2 biological activity since it was inspired by the strategy of Angiochem Inc. to develop an investigational chemotherapeutic drug under Phase III clinical trials, ANG1005, which consisted of Angiopep-2 modified with molecules of paclitaxel, namely at the N-terminus of the peptide [43, 44]. 87% Angiopep-2 conjugation efficiency for PLGA-PEG(5K)-ANG2, and 86% for PLGA-acetal-PEG(5K)-ANG2, were estimated by Bradford assay in reaction supernatants. After media acidification (pH 5.5) and consecutive washings of the polymer, PLGA-acetal-PEG(5K)-ANG2 ¹H NMR revealed a

significant decrease in the intensity of the PEG-related peak, which confirmed the acid-sensitive properties of the acetal group and consequent separation of the PEG(5K)-ANG2 moiety from the PLGA core polymer (**Figure 2E**). For both PLGA-acetal-PEG(5K)-ANG2 and PLGA-PEG(5K)-ANG2, ^1H NMR presented specific low-intensity Angiopep-2 characteristic peaks that were absent in the mock reaction controls (c.a. δ = 6.64, 6.66, 7.02, 7.04, 7.16 and 7.24 ppm), thus confirming the presence of the peptide in the structure of the polymeric conjugates (**Figure 2F**). Such peaks are characteristic of the aromatic ring protons from the phenylalanine and tyrosine amino acids of Angiopep-2 [45, 46].

3.2. Nanoparticle manufacture and characterization

NP formulations were manufactured by a nanoprecipitation-on-chip using a continuous-flow microfluidic technique [32-34]. Lab-scale conventional methods to manufacture drug-loaded polymeric NPs lack full control over the process and are usually associated with low- and slow-throughput, limited loading of the cargo and batch-to-batch variability of NP critical quality attributes [47, 48]. Thus, the continuous-flow technique herein applied takes advantage of a microfluidic chip that enables an automated and time-saving NP manufacturing process, while offering a reproducible in-house production of the nanomedicines, which is expected to facilitate future translation into clinics [49]. The TPC of each formulation was tailored to manufacture distinct NP types, whose composition and designation are schematized in **Figure 1B** and summarized in **Table 1**.

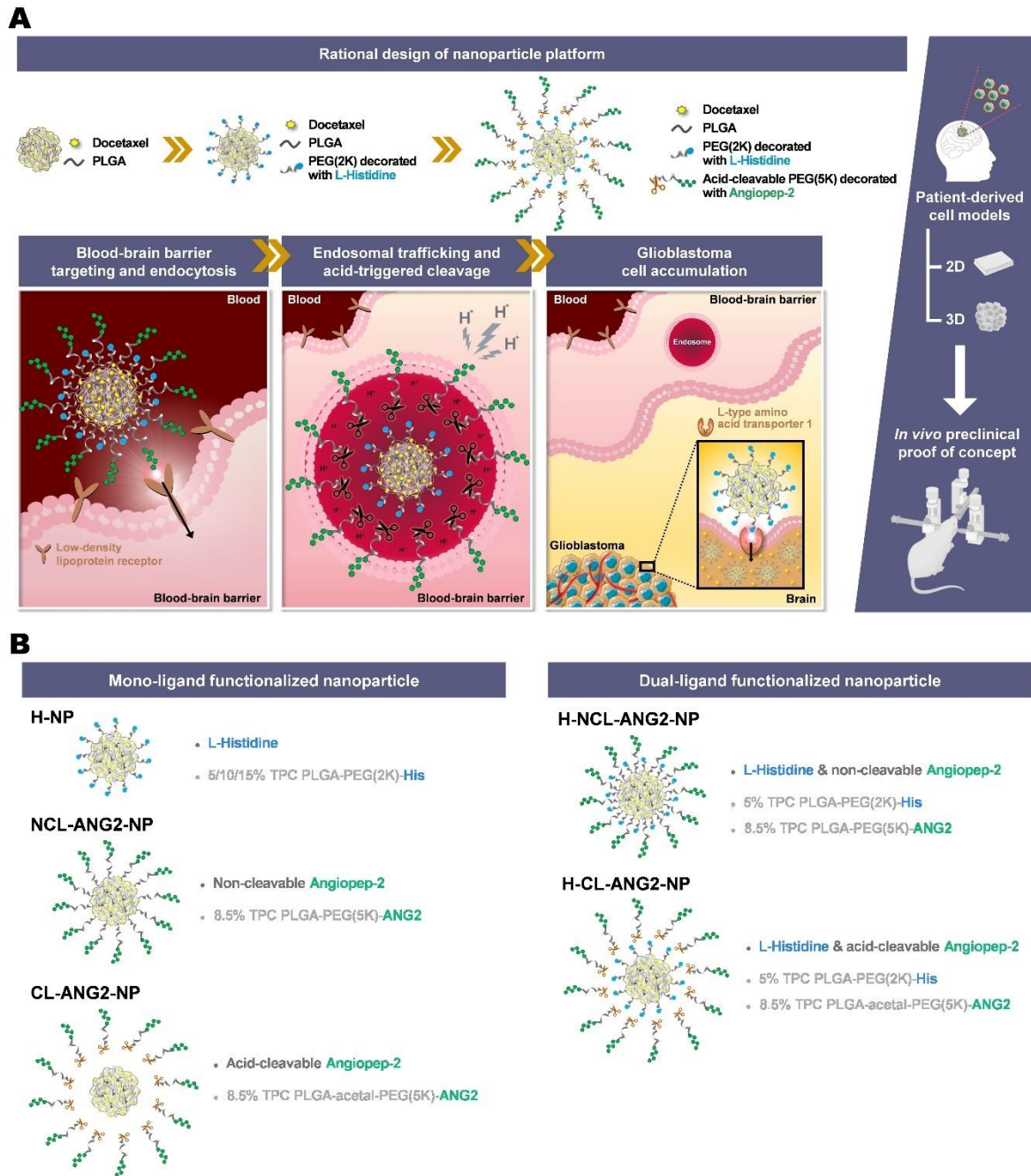


Figure 1. (A) Schematic illustration of the proposed stimuli-responsive multifunctional NP platform design and hypothesized biological mechanism of action. (B) Mono-ligand or dual-ligand surface functionalized NP formulations herein manufactured, and respective composition and designation.

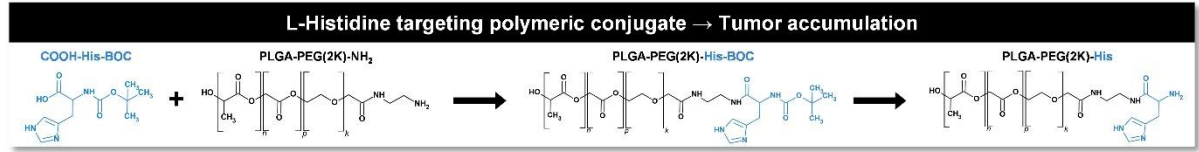
All NP formulations presented, approximately, an average size range of 300 to 400 nm, 0.3 PDI, and -35 to -25 mV zeta-potential (**Figure 2G**). Although the NP average size is not the standard for blood-to-brain crossing, considered to be around 50-100 nm [50], a shift in paradigm has been observed in the field. Recently, BBB crossing has been

reported as highly dependent on the equilibrium between NP size, stiffness, shape, and surface chemistry [51]. 500 nm NPs have been reported to transit across the BBB as efficiently as 100 nm NPs in terms of NP number, when undergoing receptor-mediated endocytosis and if presenting elastic moduli in the kPa–MPa range, as described for PLGA-PEG NPs [51-54]. Depending on their surface chemistry, larger NPs might even deliver cargos more selectively to the targeted cells compared to their smaller counterparts [55]. Taken together, the average NP size range of 300 to 400 nm herein achieved was not considered an exclusion criterion *per se*, since the particles are expected to undergo LDLR-mediated endocytosis across the BBB via their Angiopep-2 moiety. The achieved size range, alongside with a PDI of around 0.3, are attributed to the high polymer concentration and consequent high viscosity of the organic phase used to manufacture the NPs [56]. This resulted from the attempt to provide a better entrapment phase to DTX and increase the loading degree, which usually does not exceed 1% for NPs with a PLGA-PEG matrix [57-59].

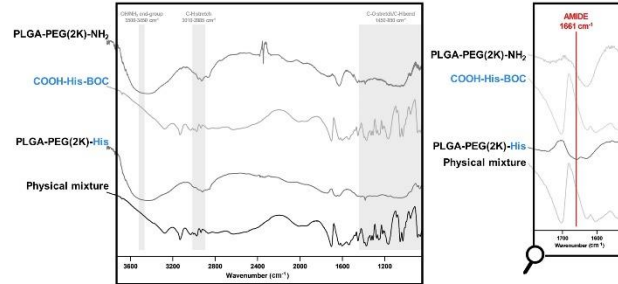
Table 1 – Polymeric conjugate percentage of TPC for each NP formulation, and respective designation. PEGylated NP controls were also manufactured using mPEG(2K)-PLGA or mPEG(5K)-PLGA, and termed mPEG(2K) NPs and mPEG(5K) NPs, respectively. NA: non-applicable.

	NP designation and polymeric conjugate composition (organic phase)				
	H-NP	CL-ANG2- NP	NCL-ANG2- NP	H-CL-ANG2- NP	H-NCL-ANG2- NP
PLGA-PEG(2K)-His	5%/				
	10%/	NA	NA	5%	5%
	20%				
PLGA-acetal-PEG(5K)-ANG2 (acid-cleavable)	NA	8.5%	NA	8.5%	NA
PLGA-PEG(5K)-ANG2 (non-cleavable)	NA	NA	8.5%	NA	8.5%

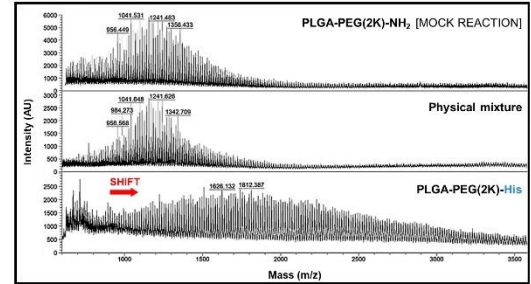
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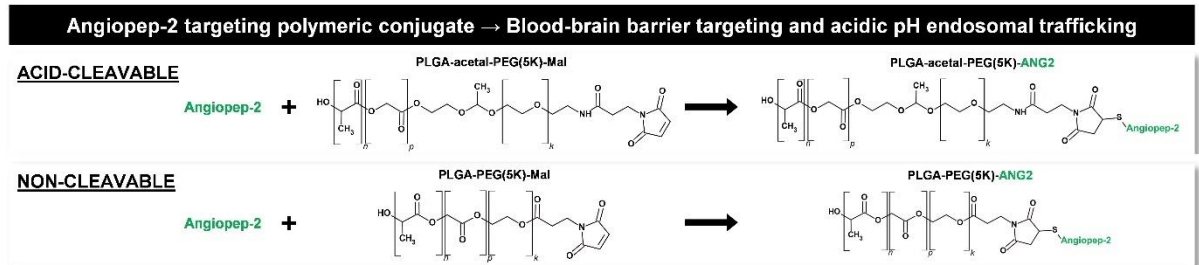
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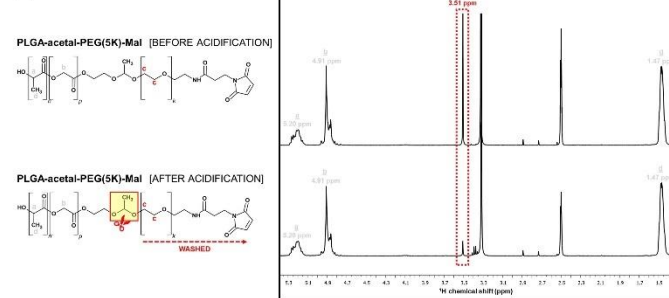
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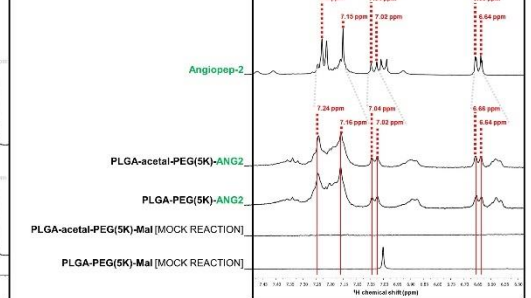
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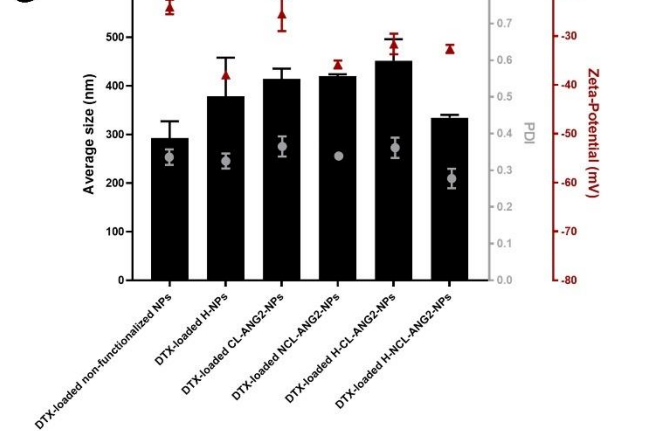
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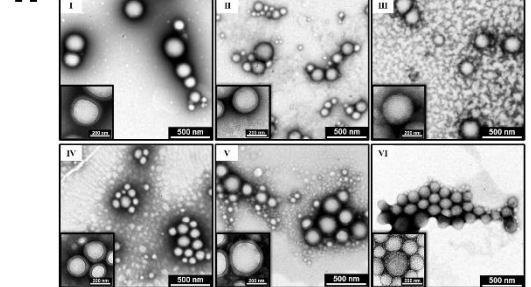


Figure 2. Polymeric conjugate chemical synthesis and NP assembly. PLGA-PEG(2K)-His (A) chemical synthesis, (B) FTIR characterization, and (C) MALDI-TOF MS

characterization. (D) Scheme of the chemical synthesis of the Angiopep-2 functionalized targeting polymeric conjugates. (E) ^1H NMR analysis of the acid-cleavable properties of the PLGA-acetal-PEG(5K)-Mal polymer used to further produce the PLGA-acetal-PEG(5K)-ANG2 targeting polymeric conjugate. (F) ^1H NMR characterization of PLGA-acetal-PEG(5K)-ANG2 and PLGA-PEG(5K)-ANG2. (G) Characterization of average size, PDI and zeta-potential of the DTX-loaded NP formulations assembled from the chemically synthesized targeting polymeric conjugates. (H) TEM characterization of the DTX-loaded NP formulations assembled from the chemically synthesized targeting polymeric conjugates, where I, II, III, IV, V and VI correspond to non-functionalized NPs, H-NPs, CL-ANG2-NPs, NCL-ANG2-NPs, H-CL-ANG2-NPs, and H-NCL-ANG2-NPs, respectively.

Supporting Information presents detailed AE and LD values for all formulations (**Table S2** and **Table S3**), as well as average size, PDI and zeta-potential complementary data (**Figure S5**). TEM analysis revealed spherical shaped particles with relatively smooth surfaces, and a size distribution identical for all NP formulations (**Figure 2H**). The *in vitro* release profile of DTX was evaluated for 24 h under medium mimicking systemic physiological conditions. As depicted in **Figure S6**, NP formulations could retain part of DTX within their hydrophobic PLGA-based core, as about 60-80% of the drug was released in a sustained manner over the testing time window.

3.3. *In vitro* targetability in glioblastoma conventional cell lines and invasive margin patient cells – L-Histidine functionalized nanoparticles

A preliminary screening of the capacity of H-NPs to ameliorate the accumulation of the NPs into GBM cells was performed in GBM conventional cell lines, namely U-87 MG, U-251 MG and U-373 MG. NPs were manufactured with different percentages of PLGA-PEG(2K)-His to understand the impact of the polymeric conjugate on cell-NP interaction. A protocol involving the use of trypsin for cell detachment prior to flow cytometry analysis was developed on purpose to remove the majority of membrane-bound NPs and enrich the signal in cell-internalized NPs [60]. Moreover, a preliminary cytotoxicity experiment was performed to find a non-cytotoxic NP dose (**Figure S7**). As shown in **Figure 3A**, after 4 h treatment, H-NPs presented a significantly higher cell internalization compared to non-functionalized NPs, for all GBM cell lines. This was reflected by an increase of up to 3-times in both MFI and percentage of positive cells (**Figure 3A** and **S8**, respectively).

We hypothesize that the increase in cell internalization is due to the capacity of the His moiety of NPs to recognize and bind LAT1, vastly reported to be overexpressed in human brain gliomas such as GBM, and directly associated with their excessive proliferation and malignant progression [14-16]. Previous RT-PCR studies reported that the LAT1 mRNA expression in the above-mentioned cell lines follows the order U-373 MG > U-251 MG > U-87 MG, which is in agreement with the trend of percentage of positive cells herein observed for H-NP cell internalization (U-373 MG > U-251 MG > U-87 MG, **Figure S8**) [61]. The L-isomeric form of His is extensively reported as one of the LAT1 substrates with highest affinity and stereoselectivity, presenting a kinetics K_m parameter of only 12.7 μ M, which is significantly lower compared to the K_m of other L-amino acid counterparts (e.g. 28.3, 47.2 and 14.2 μ M for L-Tyrosine, L-Valine and L-Phenylalanine, respectively) [62, 63]. Nevertheless, His or His-mimicking imidazole derivatives have been mostly exploited for their endosomal escape properties and pH-triggered conformational changes [64-67], and not for the purpose of drug/NP accumulation into tumor cells such as of GBM. To our best knowledge, only one literature study associated the higher NP internalization of dendrimers modified with His with the general overexpression of amino acid transporters in HeLa cervical cancer cells, necessary for the rapid proliferation and increased antioxidant demands of these cells [68]. Other authors exploited LAT1-specific cancer cell delivery through the use of substrates with lower affinity for the transporter, such as glutamate [69, 70] and L-tyrosine [71]. LAT1-specific delivery has also been widely explored in the field of positron emission tomography (PET) imaging for the detection of tumor tissues. PET probes modified with L-phenylalanine [72], L-methionine [73] and L-tyrosine [74] demonstrated precise tumor-specificity via binding of the amino acid constituents to LAT1.

However, the GBM tumor niche is characterized by a high level of heterogeneity and most of the conventional GBM cell lines are thought to have undergone genetic drift over the years, thus partially losing the invasive behavior characteristic of GBM [75]. Moreover, these cell lines are derived from the core of the tumor, which does not realistically reflect the infiltrative behavior of post-surgical residual GBM cells [76, 77]. Taken together, our efforts focused on using a complementary cell model for drug/NP testing, which could represent better the highly invasive and tumorigenic potential of GBM, such as GIN patient cells (**Figure S9**) [29, 30]. GIN cells are isolated from the invasive margin of GBM tumor tissue of adult patients and evidenced by the 5ALA-GliolanTM fluorescent region at surgery beyond the MRI T1 enhancing region [30]. This invasive margin constitutes therapeutic resistant pressures, and is a molecular snapshot of recurrent tumor cells [29, 30]. Three cell types were selected from the available GIN panel, namely GIN-8, GIN-27 and GIN-31. Similarly to what was observed for the GBM

conventional cell lines, as shown in **Figure 3B**, after 4h treatment, H-NPs presented a significantly higher cell uptake compared to non-functionalized NPs, for all GIN cell types. This was reflected by an increase of up to 3- and 12-fold in MFI and percentage of positive cells, respectively (**Figure 3B** and **S10**). Besides numerous studies on LAT1 overexpression in U-87 MG, U-251 MG and U-373 MG cells, also the GBM invasive margins have been widely described as areas of LAT1 overexpression [78, 79]. In fact, the specific expression of LAT1 in human clinical samples was reported to be even higher in infiltrating glioma cells of the GBM invasive margin compared to glioma cells located at the tumor core [78]. This explains the higher levels of cell internalization herein observed for H-NPs in GIN cells *versus* GBM conventional cell lines (U-87 MG, U-251 MG, U-373 MG), highlighted by the increase of up to 3- *versus* 12-times in the percentage of positive cells, respectively, compared to the non-functionalized NP control (**Figures S8** and **S10**). H-NPs composed by 20% PLGA-PEG(2K)-His were pre-selected for further studies based on the highest levels of cell uptake found in previous flow cytometry studies. H-NP cell uptake was then confirmed by confocal microscopy, where H-NPs presented a considerably higher cell co-localization and internalization compared to the non-functionalized NP control in all GIN cell types (**Figure 3C**).

Lastly, as a proof of concept of cancer cell specificity, the cell internalization of H-NPs was assessed by flow cytometry in a non-tumor astrocyte control cell line. No biologically relevant differences were found between the cell internalization of H-NPs and non-functionalized NPs, as showed by the MFI values (**Figure 3B**). In what concerns the percentage of positive cells, H-NPs composed by 20% PLGA-PEG(2K)-His presented an increase of less than 2-times compared to the non-functionalized control, but only to a maximum of around 15% positive cells, which was attributed to non-specific binding (**Figure S10**).

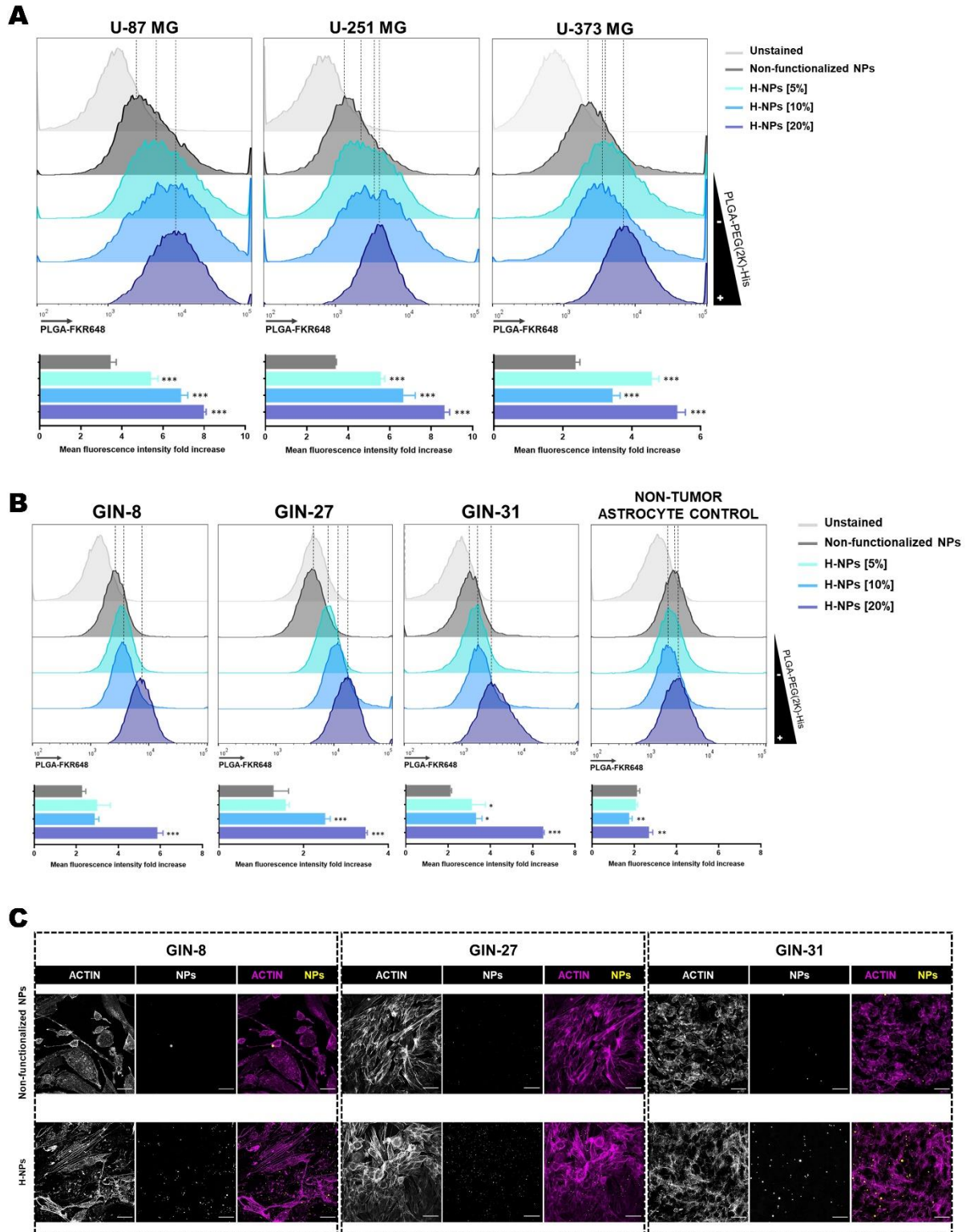


Figure 3. Flow cytometry analysis of cell internalization of non-functionalized NPs and H-NPs assembled from 5%, 10% or 20% PLGA-PEG(2K)-His into (A) GBM conventional cell lines (U-87 MG, U-251 MG, U-373 MG), and (B) GIN patient cells (GIN-8, GIN-27, GIN-31) and non-tumor astrocytes. MFI values were normalized to the unstained control.

Statistical comparison of H-NP groups with non-functionalized NPs. $*p<0.05$, $**p<0.01$, or $***p<0.001$. (C) Confocal microscopy analysis of cell internalization of non-functionalized NPs and H-NPs assembled from 20% PLGA-PEG(2K)-His into GIN patient cells. Cell actin was stained to evidence co-localization with PLGA-FKR648 labeled NPs. Scale bars represent 62 μm .

3.4. 2D and 3D *in vitro* cytotoxicity in glioblastoma invasive margin patient cells – L-Histidine functionalized nanoparticles

The *in vitro* pharmacological activity of the developed NP formulations was assessed in regard to their capacity to compromise the metabolic activity of GIN cells. Due to the highly invasive profile and key role in tumor recurrence [80], GIN cells are expected to mimic better the treatment resistance faced by therapeutics *in vivo*.

GIN-8, GIN-27 and GIN-31 metabolic activity was evaluated after uninterrupted periods of treatment of 48 h and 96 h with DTX doses ranging from 0.45 nM to 4.5 μM . For all GIN cell types, the metabolic activity decreased in a dose-dependent manner, and lower levels were reached at 96 h (day 6) compared to 48 h (day 4). DTX-loaded H-NPs caused a similar impact on metabolic activity compared to DTX-loaded non-functionalized NPs and DTX-loaded mPEG(2K) NPs (**Figure 4B, left panel**). Moreover, DTX-loaded H-NPs demonstrated a similar or slightly lower decrease on cell metabolic activity compared to the non-nanoparticulate drug control, free DTX. This was expected since the assay conditions are static, thus, free drug molecules are readily available to interact with 2D cell monolayers in order to exert an anti-proliferative effect. Whereas, for the nanoparticulate samples, including DTX-loaded H-NPs, drug molecules become only available upon DTX diffusion across the porous PLGA matrix and, in a later stage, PLGA matrix erosion [8].

After confirming the ability of DTX-loaded H-NPs to significantly decrease GIN cell metabolic activity, we hypothesized that 4 h dynamic-like boost treatments could give us more information about the adjuvant effect of the targeting strategy [81]. For the lowest concentration, 4.5 μM DTX, none of the formulations was able to decrease the metabolic activity to values lower than 70% for all GIN cell types, including free DTX (**Figure 4B, right panel**). Thus, a higher concentration was tested, 45 μM DTX, for which a significantly lower metabolic activity was observed for all GIN cell types treated with DTX-loaded H-NPs, in opposition to free DTX and the other NP controls, namely non-functionalized and PEGylated but non-targeted mPEG(2K) NPs (**Figure 4B, right panel**). This was attributed to the enhanced capacity of H-NPs of being internalized by

GIN cells during a contact period of only 4 h, as demonstrated by previous flow cytometry studies, compared to non-functionalized NPs. Therefore, this led to a closer drug-cell interaction and, consequently, a more pronounced effect on the decrease of cell metabolic activity. The 45 μ M DTX boost treatment also implies a boost in the total number of NPs per area, which is hypothesized to benefit NP tumor cell uptake [82] compared to free DTX molecules, which did not present any biologically relevant advantage with respect to cell metabolic activity interference compared to the nanoparticulate samples.

On the other hand, drug diffusivity and therapeutic effect within 3D cell cultures is more predictive of the potential *in vivo* response than simple 2D cultures. 2D cell cultures are considerably more sensitive to anti-cancer medicines compared to 3D counterparts, which behave similarly to human tumors presenting solid and bulky features, such as GBM [75, 83]. Hence, we re-evaluated the previous 2D metabolic activity studies by selecting one of the GIN cell types, namely GIN-31, to assemble a 3D culture. GIN-31 tumor spheroids were generated by high-throughput microwell array technology using non-adhesive agarose hydrogels that promote cell aggregation into 3D spheroid structures with excellent size and shape reproducibility (**Figure 4C**) [84]. After 48 h and 96 uninterrupted periods of treatment, GIN-31 spheroids followed the same trend observed for GIN-31 2D cultures, with the metabolic activity decreasing in a dose-dependent manner and lower levels reached at 96 h (day 6, **Figure 4D, left panel**). In general, the 3D metabolic activity values were higher compared to the 2D cell monolayer setting, which is potentially associated with the higher therapeutic resistance extensively reported for 3D cultures [83, 85]. Similarly to 2D cultures, these uninterrupted periods of treatment did not evidence any biologically relevant differences between free DTX and the NP samples, although all of them resulted in a decrease of metabolic activity up to levels of approximately 35%. Next, we proceeded with the 4 h dynamic-like boost treatment and, herein, in opposition to 2D cultures, not even DTX-loaded H-NPs were able to lower cell metabolic activity (**Figure 4D, right panel**). This 2D *versus* 3D discrepancy is attributed to the insufficient drug penetration into the core of the GIN-31 spheroids over time due to their high interstitial pressure, reduced convective flow and the presence of dense regions of extracellular matrix [86, 87]. Thus, we challenged the spheroids with a broader time window of drug exposure, namely 12 h. In this case, it was possible to recover the significantly higher impact of DTX-loaded H-NPs on the decrease of cell metabolic activity of up to 2-times, as observed for 2D cultures, and became again clear the inability of free DTX or the other NP samples to provide the same effect (**Figure 4D, right panel**). H-NPs improved DTX transport across the spheroid structures most likely by promoting cell internalization via LAT1 binding, and an increased time exposure

allowed DTX to reach deep regions of the spheroid tissue and recover the anti-proliferative effect on GIN-31 cells.

Additional data of the impact of the unloaded formulations on 2D and 3D cell metabolic activity can be found in **Figure S11** and **S12**, respectively.

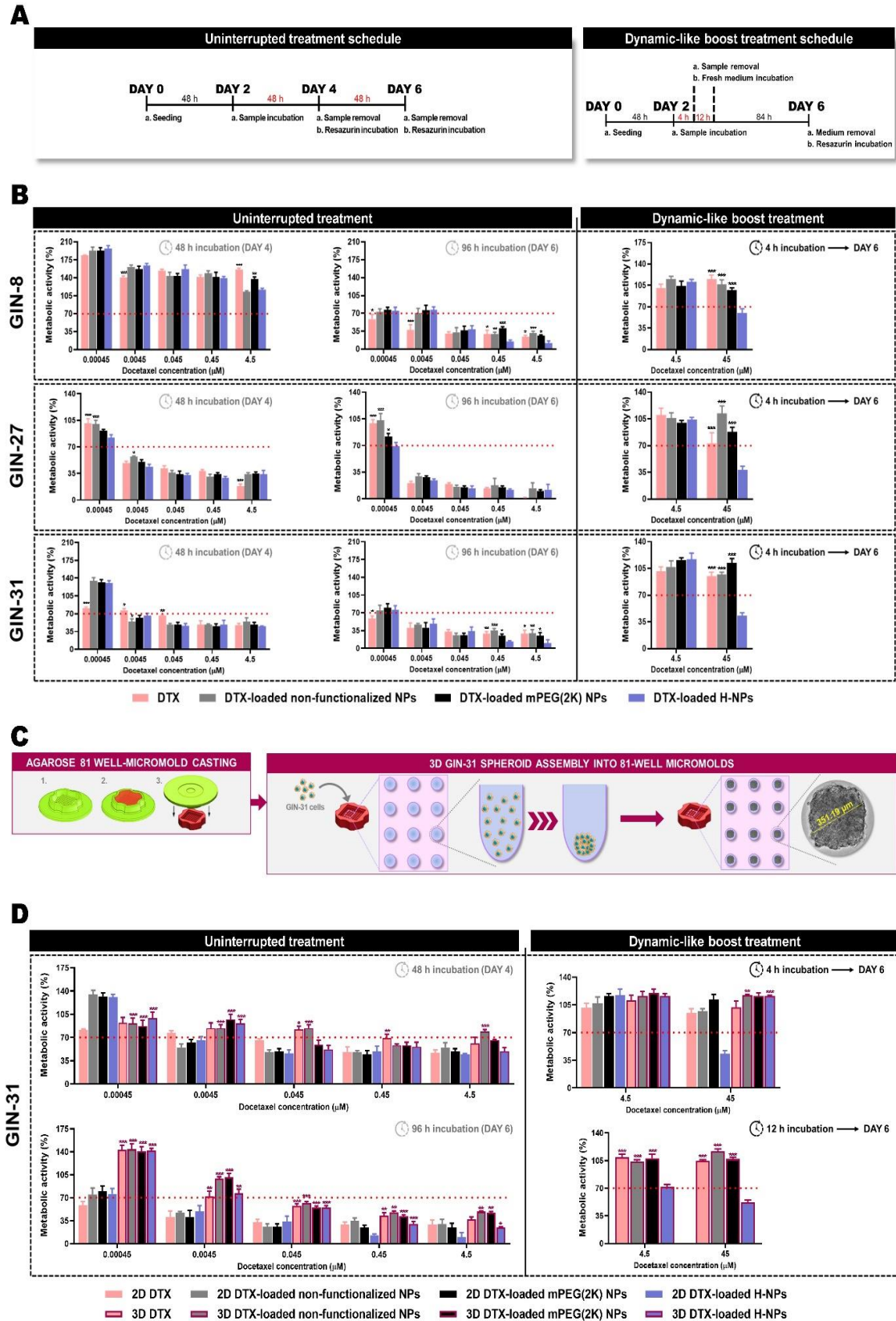


Figure 4. Evaluation of GIN cell metabolic activity after treatment with free DTX, DTX-loaded non-functionalized NPs, DTX-loaded mPEG(2K) NPs, and H-NPs composed by 20% PLGA-PEG(2K)-His. (A) Treatment schedule. (B) 2D metabolic activity of GIN

patient cells (GIN-8, GIN-27, GIN-31). The left panel presents metabolic activity levels after uninterrupted periods of treatment of 48 h and 96 h. The right panel presents a dynamic-like boost treatment of 4 h, integrated in an assessment period of incubation of 96 h. (C) Assembly of GIN-31 tumor spheroids into high-throughput 81-well agarose micro-molds – (1) molds (2) are filled with agarose, and (3) hydrogel micro-molds obtained. The size of spheroids at the day of metabolic activity experiments averaged 350 μm . (D) 3D metabolic activity of GIN-31 tumor spheroids. The left panel presents 2D *versus* 3D metabolic activity after uninterrupted periods of treatment of 48 h and 96 h. The right panel presents a 2D *versus* 3D dynamic-like boost treatment of 4 h or 12 h, integrated in an assessment period of incubation of 96 h. Statistical comparison of DTX-loaded H-NPs with the other drug-loaded groups, within each dose range. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3.5. *In vivo* therapeutic efficacy after intratumoral administration - L-Histidine functionalized nanoparticles

The *in vivo* anti-tumor local effect of DTX-loaded H-NPs was validated in a GBM orthotopic mice model following an intratumoral treatment of 2 μg DTX per brain (**Figure 5**). After MRI assessment of tumor volume (**Figure 5B**) and group randomization at day 8, the formulations were administered intratumorally at day 10, and survival curves determined. Body weight was kept constant throughout 15 days post-administration, for all groups (**Figure 5C**). As presented in **Figure 5D** and **Table 2**, the median survival time was considerably improved by DTX-loaded H-NPs (46.5 days) compared to the untreated (30 days, ** $p < 0.01$), DTX-loaded non-functionalized NPs (33.5 days, ** $p < 0.01$), and free DTX (38 days) groups. Moreover, the DTX-loaded H-NP treatment resulted in a higher number of long-term survivors at 60 days (37.5% *versus* 0% for non-functionalized NPs) and 120 days (12.5% *versus* 0% for both non-functionalized NPs and free DTX). Thus, the DTX-loaded H-NP treatment significantly outperformed the DTX-loaded non-functionalized NP counterpart, which is consistent with *in vitro* studies. This was attributed to the ability of the His moiety to enhance GBM cell uptake, hence resulting in higher DTX accumulation at the tumor site and, consequently, a more potent therapeutic effect. It is of important note that overestimation of the therapeutic effect of free DTX is envisaged since the Taxotere[®] vehicle used for DTX solubilization contains ethanol, which is used *per se* in the treatment of certain types of cancer via percutaneous injection (e.g., liver cancer, liver metastasis) [88]. Overall, these findings offered robust evidence that, once inside the brain parenchyma, H-NPs are able to boost the

chemotherapeutic effect of DTX and provide an *in vivo* therapeutic benefit.

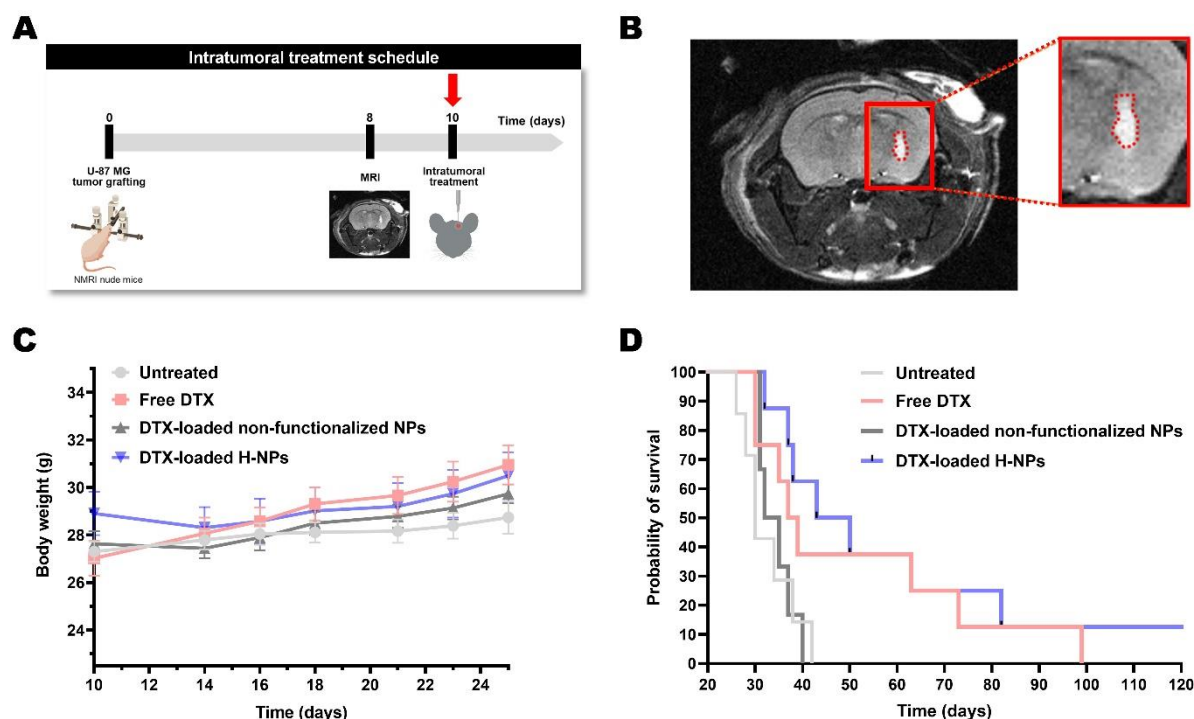


Figure 5. *In vivo* therapeutic efficacy of H-NPs after intratumoral administration. NMRI nude mice were orthotopically grafted with U-87 MG GBM cells at day 0, randomized upon MRI tumor evaluation at day 8, and treated with a dose of 2 μ g DTX per brain at day 10. (A) Treatment schedule. (B) Representative mouse brain MRI of the U-87 MG GBM orthotopic model. (C) Body weight follow-up until day 25. (D) Mice survival curves until day 120.

3.6. *In vitro* permeability across the blood-brain barrier - L-Histidine plus acid-cleavable Angiopep-2 functionalized nanoparticles

A preliminary screening of BBB permeability was conducted in a standard hCMEC/D3 *in vitro* model to understand the ability of Angiopep-2 to ameliorate the accumulation of the NPs into the brain, the additional effect of His, as well as possible differences between acid-cleavable *versus* non-cleavable Angiopep-2 moieties. The optimal Angiopep-2 density for BBB permeability reported in literature data in regard to the TPC of the formulation is highly variable, ranging from around 3% to 20% [89-92]. Thus, we set it up as 8.5% with the ultimate goal of assessing whether this percentage would be already

sufficient to potentiate BBB permeability, before testing other percentages.

As displayed in **Figure 6A**, and surprisingly, the permeability of NCL-ANG2-NPs presented minimum advantage over non-functionalized NPs and PEGylated but non-targeted mPEG(5K) NPs. This can relate to the findings of Tian and colleagues, who demonstrated that Angiopep-2 density on NP surface should be carefully designed in order to avoid ligand-LDLR high avidity and consequent BBB endothelial cell internalization leading to fast degradation but, instead, favor mid avidity to increase the formation of syndapin-2 tubular structures leading to fast shuttling across the BBB [17]. Moreover, H-NPs presented a slightly 1.6-times higher BBB permeability compared to NCL-ANG2-NPs (**Figure 6A**), which can be explained by the fact that the His transporter, LAT1, is highly expressed in the BBB endothelium, apart from tumor tissues [93]. Yet, LAT1 is not the ideal target for brain delivery since the transporter easily achieves saturation by plasma amino acids [94], while LDLR is widely known for its rapidly recycling vesicles and one of the highest BBB trafficking capacity [95]. Interestingly, CL-ANG2-NPs provided a slightly higher BBB permeability compared to H-NPs but, more important, significantly outperformed NCL-ANG2-NPs (**Figure 6A**). A strong basis for this finding is the study of Clark *et al.*, who played around ligand-receptor avidity and receptor-mediated BBB trafficking, by designing a tailored BBB endosomal pH acid-sensitive nanosystem [28]. The authors demonstrated that an acid-cleavable transferrin moiety provided considerably higher brain parenchyma levels of NPs upon systemic administration in mice, while the non-cleavable control remained mostly retained in the BBB endothelium. High-avidity is thus of uppermost importance to promote receptor binding on the luminal side of the BBB, but should be shifted to mild to low avidity during BBB trafficking to allow proper detachment from the receptor and complete the full transport from blood to brain [[28, 96].

The ability of the dual-ligand functionalized NPs to improve BBB permeation was then compared to the simpler system, H-NPs. H-CL-ANG2-NPs increased the permeability levels by approximately 2-times (**Figure 6A**). This could result from a cumulative effect of His and the acid-cleavable Angiopep-2 moiety, and suggested that steric hindrance is not interfering with the activity of the nanosystems presenting dual-ligand functionalization. Steric hindrance was possibly avoided by the careful design of two different PEG length levels on the NP surface, namely of 2K (His attachment) and 5K (Angiopep-2 attachment). On the other hand, H-NCL-ANG2 NPs did not present biological advantage over H-NPs regarding BBB permeability (**Figure 6A**). BBB *in vitro* model transendothelial electrical resistance (TEER) was monitored over maintenance and throughout the permeability experiment (**Figure S13**).

NP formulations were also assessed for their BBB endothelial cell uptake in hCMEC/D3

cells, where acid-cleavable CL-ANG2-NPs and H-CL-ANG2-NPs demonstrated the lowest cell internalization among the other NP samples in both mono-ligand and dual-ligand functionalized groups, respectively (MFI values and percentage of positive cells depicted in **Figure 6B** and **S14**, respectively). This is accordance with literature data and highlighted, once more, that a higher BBB retention does not necessarily correlate with a higher BBB permeability, and contrary trends can be actually found [28, 51, 96].

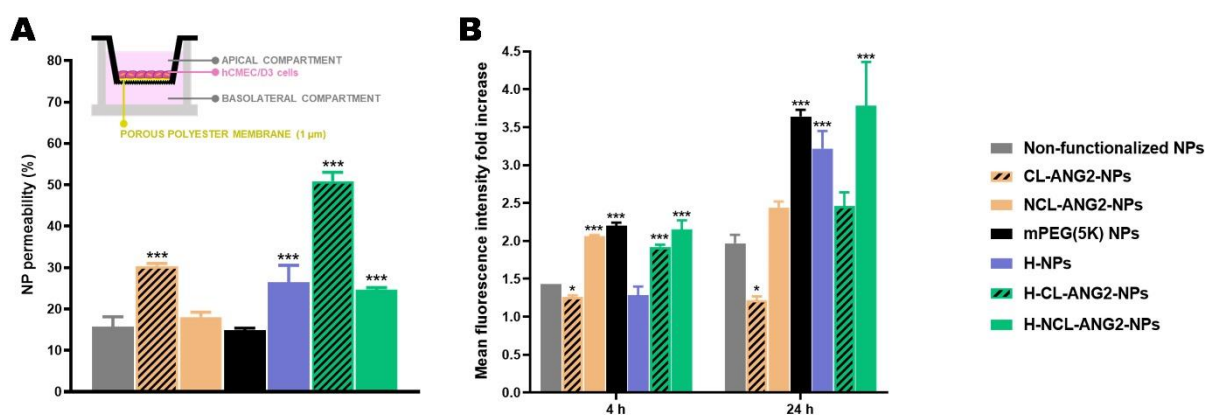


Figure 6. (A) Evaluation of NP permeability across a BBB *in vitro* model, indicating the percentage of NP basolateral permeability across a hCMEC/D3 endothelial cell monolayer, normalized to the initial mass of NPs in the apical compartment. (B) Flow cytometry analysis of cell internalization levels of NP formulations into BBB hCMEC/D3 endothelial cells. Statistical comparison of each group with non-functionalized NPs. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3.7. *In vivo* biodistribution, therapeutic efficacy and toxicity after intravenous administration - L-Histidine plus acid-cleavable Angiopep-2 functionalized nanoparticles

An *in vivo* biodistribution screening trial was performed in a healthy mice model, since there is strong and recent evidence that GBM possesses a gross region beyond the contrast-enhancing tumor bulk with an intact BBB, especially at the invasive margins [97, 98]. Thus, it is generally admitted that pharmacological approaches that succeed in transposing the healthy BBB have better chances of improving the treatment response in further GBM disease models [7, 99]. In order to reduce the number of animals in light of the 3Rs policy, and considering the previous *in vitro* findings, it was possible to eliminate the non-functionalized NP control from this biodistribution trial – non-

functionalized NPs were unable to provide an improved GBM cell internalization (**Figure 3**) and cytotoxicity (**Figure 4B** and **4D**) compared to H-NPs; moreover, non-functionalized NPs were unable to potentiate BBB crossing compared to H-NCL-ANG2-NPs and H-CL-ANG2-NPs (**Figure 6A**). IVIS imaging to track Cy7.5 labeled NPs was performed at 2 h, 6 h and 24 h post-administration. Brain accumulation of the developed formulations followed the trend H-CL-ANG2-NPs > H-NCL-ANG2-NPs > H-NPs, with H-CL-ANG2-NPs outperforming the other formulation counterparts (**Figure 7A**). Actually, differences in the maximum average radiant efficiency observed for H-CL-ANG2-NPs achieved approximately 4- and 3-times higher values than H-NPs and H-NCL-ANG2-NPs, respectively. The trend of brain accumulation was similar to the predictions of *in vitro* studies, highlighting the ability of dual-ligand functionalization and acid-responsiveness to enhance NP permeability across the BBB. All formulations presented a reduced brain signal from 2 h to 24 h, which was expected and can be justified by the physiological clearance of NPs over time. Regarding whole-body biodistribution, NPs were tracked for their accumulation in major organs by excision at 24 h (**Figure S15**). No relevant accumulation was found in the heart, liver and spleen accumulation were expected due to the role of these organs in the reticulo-endothelial system [100], and a certain degree of accumulation was found in kidneys due to renal excretion [101]. Nevertheless, the liver accumulation of free DTX and non-targeted PLGA-based NPs is reported to be 70- and 12.5 times-higher compared with the brain [102, 103], respectively, which is significantly higher than the values obtained for H-NCL-ANG2-NPs (3 times-higher). Interestingly, all NP formulations presented a certain level of accumulation in the lungs, but this was clearly more prominent for the formulations presenting an Angiopep-2 moiety. Since LDLR also plays a vital role in the pulmonary tissue, including regulation of antigen uptake, immune responses and fibrogenesis, the expression of the receptor might induce local sequestration of the NPs [104].

Lastly, the *in vivo* anti-tumor effect of H-CL-ANG2-NPs following intravenous treatment was assessed in a GBM orthotopic mice model. Five independent injections starting at day 10 were administered every 3 days at a 2 mg kg⁻¹ DTX dose, and survival curves determined. Body weight was kept stable throughout the treatment course and up to 8 days after the last injection, for all treatment groups (**Figure 7C**). As presented in **Figure 7D** and **Table 2**, the median survival time of the DTX-loaded H-CL-ANG2-NP treatment was 54 days, which presented a significant improvement compared to the untreated (37 days, **p*<0.05), free DTX (43 days), DTX-loaded non-functionalized NP (38 days), DTX-loaded H-NP (40.5 days) and DTX-loaded H-NCL-ANG2-NP (41 days) groups. Moreover, an increase of more than 50% in the number of survivors at 60 days was observed for DTX-loaded H-CL-ANG2-NPs compared to free DTX, DTX-loaded H-NPs

and DTX-loaded H-NCL-ANG2-NPs, whereas there were no 60 day-survivors among the untreated and DTX-loaded non-functionalized NP groups. DTX-loaded H-CL-ANG2-NPs outperformed the other NP counterparts, which is in accordance with the higher brain accumulation observed in previous biodistribution studies (**Figure 7A**) due to the enhanced BBB permeability provided by the acid-cleavable Angiopep-2 moiety of the NPs. Furthermore, once inside the brain parenchyma, adjuvant effect of His is crucial to cause higher DTX accumulation into GBM cells as demonstrated by previous intratumoral therapeutic efficacy *in vivo* studies (**Figure 5D** and **Table 2**). Taken together, more efficient blood-to-brain trafficking and GBM accumulation provided by the acid-cleavable Angiopep-2 and His surface moieties, respectively, endowed H-CL-ANG2-NPs with a potent anti-cancer effect. It is of important note that the DTX dose of each injection, namely 2 mg kg^{-1} , was lower compared to the standard $7.5\text{-}10 \text{ mg kg}^{-1}$ dose found in literature [27, 105, 106], but still able to provide a prominent anti-GBM effect. Although our H-CL-ANG2-NP prototype is the first proposed system for both BBB and GBM targeting through exploitation of a BBB-responsive transport mechanism, we revised literature reports of the past 10 years of multi-ligand functionalized NPs presenting the same dual-targeting (but lacking BBB responsiveness). By pre-selecting these reports based on the use of the same GBM animal model herein assessed, U-87 MG, the maximum values of median survival were 45 days [18], 25 days [19], 35 days [20-23] and 40 days [24-26], significantly lower than the median survival obtained for our H-CL-ANG2-NP prototype (54 days). The BBB and GBM dual-targeted system (lacking BBB responsiveness) developed by Zhu *et al.* reached 53 days median survival, but using a DTX dose 4-times higher than the one herein proposed ($2 \text{ versus } 7.5 \text{ mg kg}^{-1}$) [27]. Specific examples of these literature reported systems include 10 mg kg^{-1} DTX-loaded HRK-19 functionalized poly(lactic acid)-PEG-hyaluronic acid NPs (both hyaluronic acid and HRK-19 for glioma targeting; 40 days median survival; number of long-term survivors not available; C6 GBM bearing rats) [105], 10 mg kg^{-1} DTX-loaded Angiopep-2 functionalized solid lipid NPs (39 days median survival; no long-term survivors at 60 days; GL261 GBM bearing mice) [107], 7.5 mg kg^{-1} DTX-loaded Angiopep-2 and TAT peptide functionalized micelles (TAT for glioma targeting; 53 days median survival; no long-term survivors at 60 days; U87-MG bearing mice) [27], 6 mg kg^{-1} DTX-loaded TGN peptide and AS1411 aptamer functionalized PEG-polycaprolactone NPs (TGN and AS1411 for BBB and glioma targeting, respectively; 25 days median survival; no long-term survivors at 60 days; C6 GBM bearing mice) [108], and 5 mg kg^{-1} DTX-loaded RGV29 peptide functionalized PLGA-PEG NPs (RGV29 for both BBB and glioma targeting; 42 days median survival; no long-term survivors at 60 days; C6 GBM bearing rats) [109]. This literature review highlights the therapeutic

advantage of the H-CL-ANG2-NPs developed under the scope of this work, and the promising potential of this nanotherapy to increase the survival number of GBM patients in the future. Overall, our findings presented strong evidence that both the acid-cleavable properties of the Angiopep-2 moiety and the presence of the His moiety in the stimuli-responsive multifunctional NPs herein proposed are paramount to achieve higher BBB permeation and accumulation at the brain parenchyma GBM cells, respectively, resulting in a better therapeutic effect.

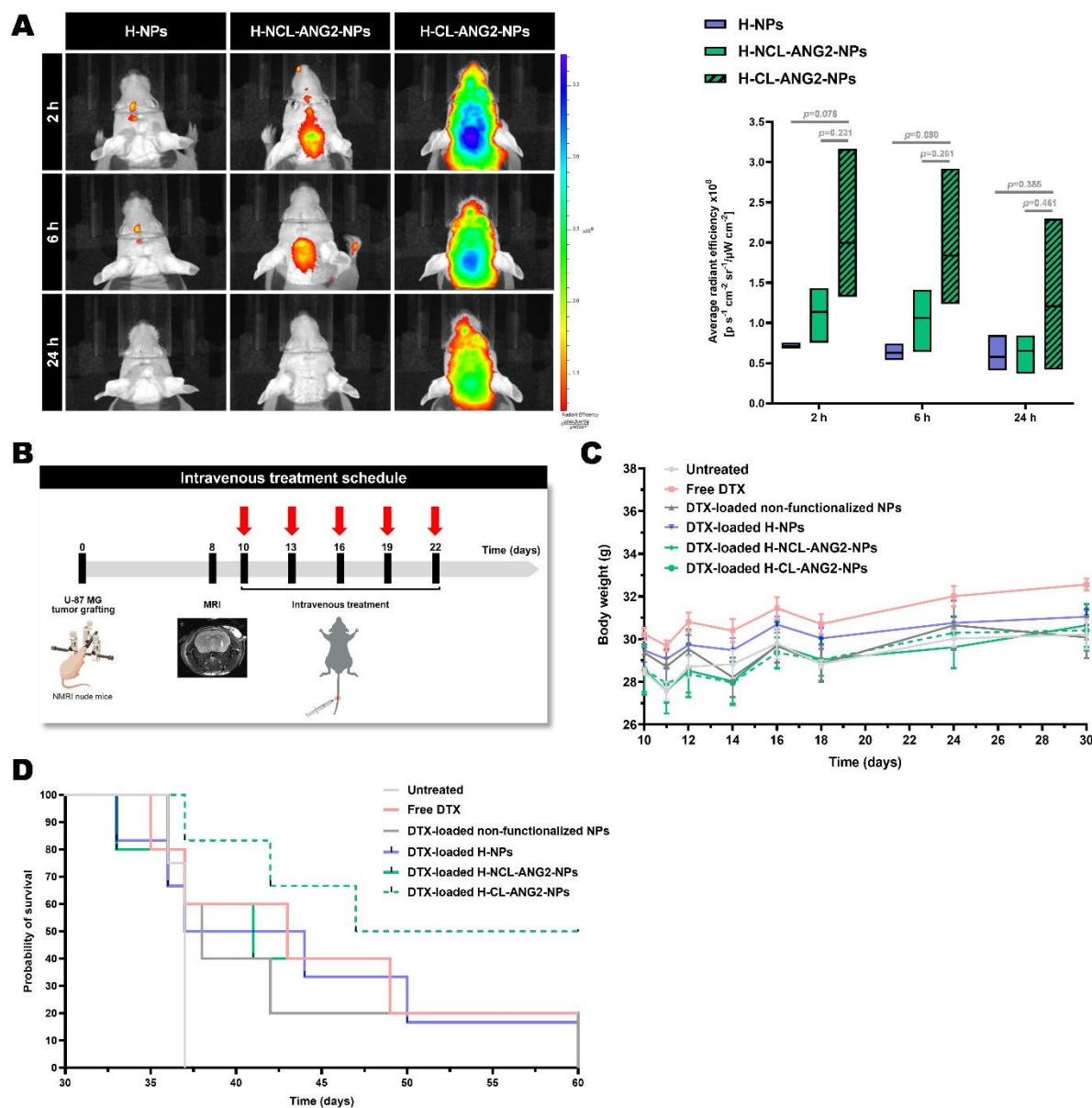


Figure 7. (A) *In vivo* biodistribution of H-CL-ANG2-NPs following intravenous treatment, evidencing brain co-localization of NP formulations at 2 h, 6 h and 24 h post-administration. (B-D) *In vivo* therapeutic efficacy of H-CL-ANG2-NPs following

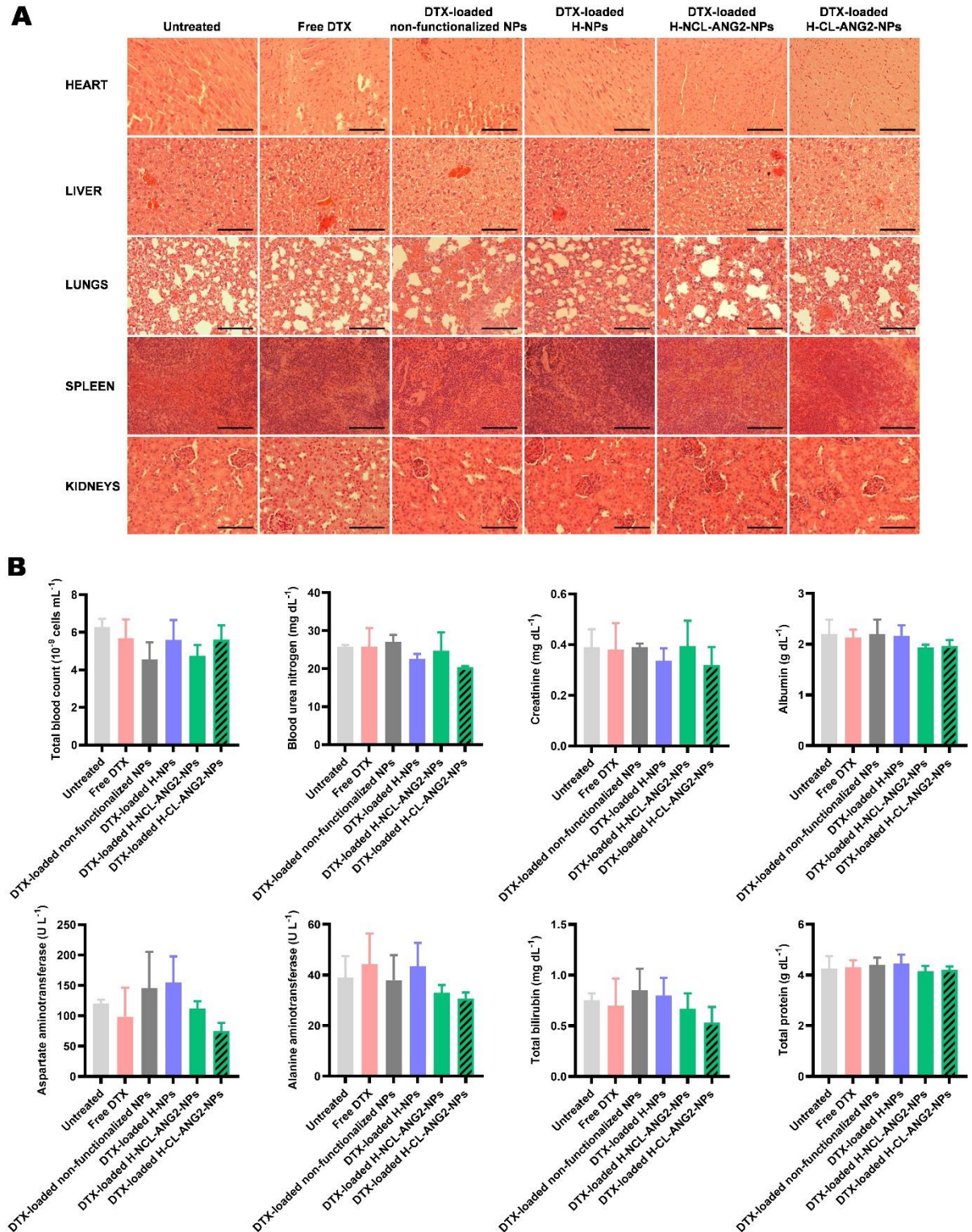
intravenous treatment. NMRI nude mice were orthotopically grafted with U-87 MG GBM cells at day 0, randomized upon MRI tumor evaluation at day 8, and treated with a 2 mg kg⁻¹ DTX dose from day 10, every 3 days, for a total of 5 injections. (B) Treatment schedule. (C) Body weight follow-up until day 30. (D) Mice survival curves until day 60.

Histological analysis of brain sections of treated mice (approximately 80 slides per mouse brain) was also performed (48 h after treatment), namely H&E stainings, and Ki67 and cleaved caspase-3 IHC studies. H&E stainings processed in approximately 20 brain slides presented significant areas of damage, which can be attributed not only to the presence of the tumor, but also to the aggressiveness of the herein used GBM rodent model (tumor inoculation and development processes). For the slide of most significant H&E damage per animal, 2 slides before and 2 slides after (total of 4 slides), were selected for Ki67 and cleaved caspase-3 IHC. However, these brain sections within these slides did not correlate with high intensity signals of Ki67, which is strongly associated with tumor cell proliferation and growth [110]. In line with this, these brain sections did not correlate with high intensity signals of cleaved caspase-3, which is strongly associated with the response to treatment, since DTX triggers apoptosis pathways via cleavage of caspase-3 [110, 111]. Moreover, and of important note, in all brain sections, the presence of the tumor could not be detected macroscopically. Taking this into consideration, it was challenging to select the most appropriate slides to proceed with IHC investigations. It is hypothesized that the time point chosen for mice scarification (48 h post-treatment) was too early, hence making difficult the identification of the tumor bulk, which might still present a dispersive and infiltrative cell behavior. Thus, further *in vivo* studies are required to repeat the experiment and sacrifice the animals at a later stage of tumor development. Finally, possible systemic toxicity effects resulting from the intravenous administration of non-nanoparticulate free DTX and NP formulations were assessed, 48 h after treatment. Anti-cancer therapies often cause severe systemic side effects which directly affect the histological morphology of off-target major organs, organ weight coefficient (organ to body weight normalization), total blood cell count and blood biochemical parameters. In what concerns histopathological analysis of off-target major organs (heart, liver, lungs, spleen, kidneys), no morphological signs of damage were found for the free DTX and DTX-loaded NP formulations (**Figure 8A**), probably due to the low dose of DTX used per administration, namely 2 mg kg⁻¹. Similarly, as shown in **Figure S16**, no significant alterations were found for the brain, heart, liver, lungs, spleen and kidneys weight coefficients compared with the untreated group. Moreover, none of the drug treatments induced significant differences in the total blood cell count of mice (**Figure 8B**). Lastly, blood biochemistry parameters, including blood urea nitrogen,

creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, total bilirubin and total protein levels, were assessed to investigate kidney and liver function, as well as possible signs of infection or inflammation. Compared with the untreated animals, mice treated with free DTX or DTX-loaded NPs did not present significant alterations in any of these blood parameters. Altogether, apart from being relatively safe from negligible loss of weight over the treatment course, the stimuli-responsive multifunctional NP therapy herein proposed presented lack of toxicity regarding alterations in histological, hematologic and blood biochemical indicators, as well as organ weight indices.

Table 2 – Resume of median survival, and percentage of survivors at 60 and 120 days (long-term survivors) for the *in vivo* therapeutic efficacy studies (intratumoral and intravenous treatment). Intratumoral treatment: statistical comparison of DTX-loaded H-NPs with untreated and DTX-loaded non-functionalized NPs groups (** $p < 0.01$). Intravenous treatment: statistical comparison of DTX-loaded H-CL-ANG2-NPs with the untreated group (* $p < 0.05$). NA: non-applicable.

Treatment type	Treatment group	Median survival (days)	Percentage of survivors at 60 days (%)	Percentage of survivors at 120 days (%)
Intratumoral	Untreated	30	0	0
	Free DTX	38	37.5	0
	DTX-loaded non-functionalized NPs	33.5	0	0
	DTX-loaded H-NPs	46.5**	37.5	12.5
Intravenous	Untreated	37	0	NA
	Free DTX	43	20	NA
	DTX-loaded non-functionalized NPs	38	0	NA
	DTX-loaded H-NPs	40.5	20	NA
	DTX-loaded H-NCL-ANG2-NPs	41	20	NA
	DTX-loaded H-CL-ANG2-NPs	54*	50	NA



MRI tumor evaluation at day 8, and treated with a 2 mg kg⁻¹ DTX dose from day 10.

4. CONCLUSION

In this study, stimuli-responsive multifunctional NPs for the delivery of DTX to GBM were developed envisaging BBB targeting and transport to brain, structural rearrangement within the acidic pH of BBB endosomal vesicles (acid-cleavable Angiopep-2 grafting), and enhancement of GBM cell accumulation post-BBB trafficking (His grafting). These multifunctional NPs increased tumor cell uptake, through the use of the His moiety, by up to 4-times in GBM conventional cell lines. Due to the relevance of tumor invasive margins in the recurrence and therapeutic resistance of GBM, GIN cells isolated from patient samples were further used. Herein, multifunctional NPs provided up to 12-times higher GBM cell internalization, and up to 3-times higher cytotoxicity in 2D and 3D cell models. After an *in vivo* intratumoral treatment, NPs presenting the His moiety significantly improved the median survival of mice bearing orthotopically implanted U-87 MG tumors compared to non-functionalized NPs and free DTX. By adding an acid-cleavable Angiopep-2 moiety, with adjuvant effect of the His moiety, the multifunctional NPs presented an increased blood-to-brain transport, by more than 3-fold, in a BBB *in vitro* model. *In vivo* studies in a mice model revealed a clearly higher brain accumulation for this multifunctional NPs, compared to the other NP controls. Lastly, following intravenous treatment at a low DTX dose, the multifunctional NPs increased the median survival of mice by around 20 days and long-term survivors by more than 50%. Moreover, no significant systemic toxicity-related alterations were observed in off-target organs histopathology and weight indices, as well as in blood total cell counts and biochemical indicators. Taken together, the hypothesis proven by our study revealed the key role of both acid-sensitive bioresponsive properties, and BBB and GBM dual-ligand targeting, to achieve an effective multistage anti-GBM therapy. It is of important note to highlight the versatility of the stimuli-responsive multifunctional NPs herein proposed – if in one hand they were proved effective for GBM treatment, they might be also useful in future treatments for brain metastasis and lower grade gliomas; on the other hand, the PLGA core of the NP system has been proved extremely versatile to allow co-encapsulation of therapeutic molecules, which might be useful to include alternative chemotherapies or even immunotherapeutics. In light of this, an exploratory model study on co-encapsulation and co-delivery of protein drugs by the use of continuously manufactured PLGA-based NPs was conducted (**Appendix II**).

5. Supporting information

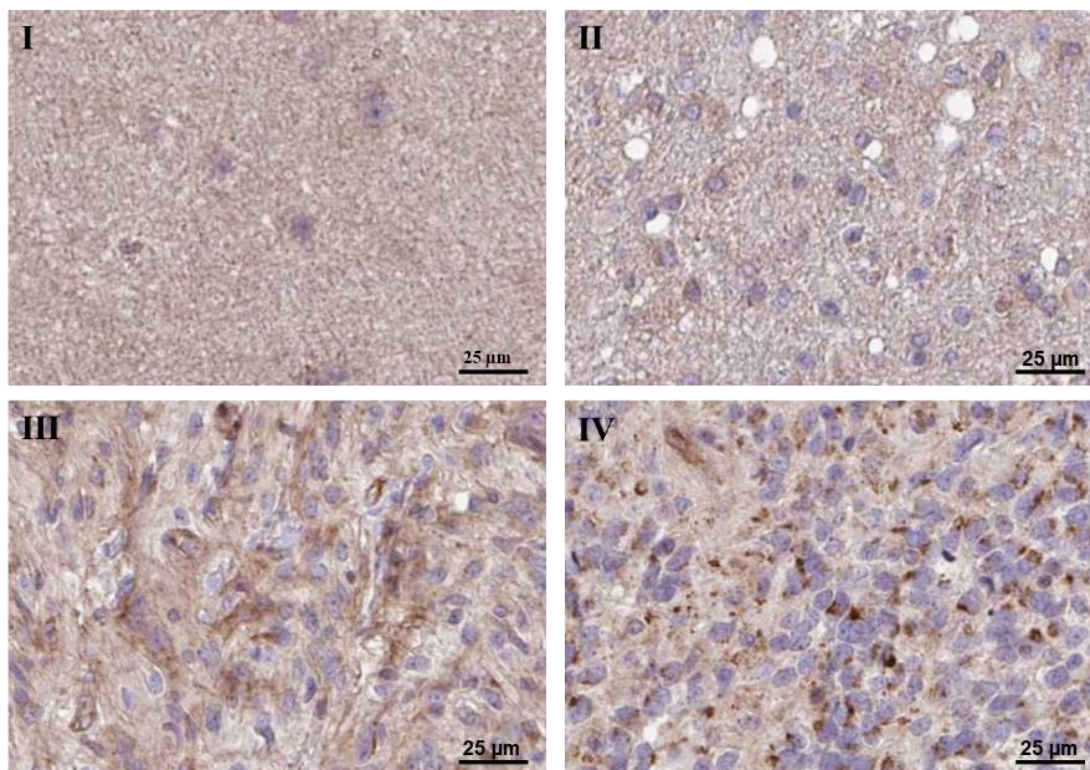


Figure S1. Representative images of high expression of LAT1 in glioma clinical samples (SLC7A5 immunohistochemistry, HPA052673). (I) Normal cerebral cortex sample presenting negative glial staining (Patient ID #4626). (II) Low-grade glioma sample presenting moderate intensity staining (Patient ID #93). (III) High-grade glioma sample presenting moderate intensity staining (Patient ID #2867). (IV) High-grade glioma sample presenting strong intensity staining (Patient ID #2862). Images were retrieved from the Human Protein Atlas (www.proteinatlas.org).

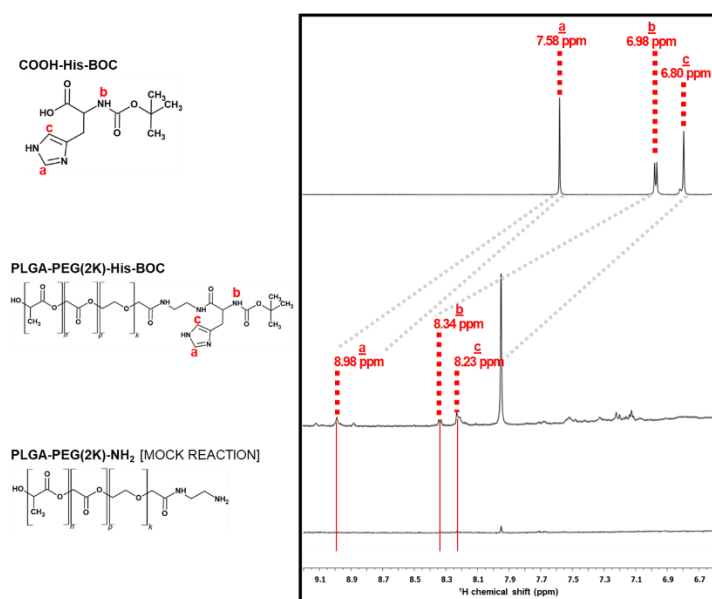


Figure S2. NMR analysis of COOH-His-BOC, PLGA-PEG(2K)-His-BOC and PLGA-PEG(2K)-NH₂.

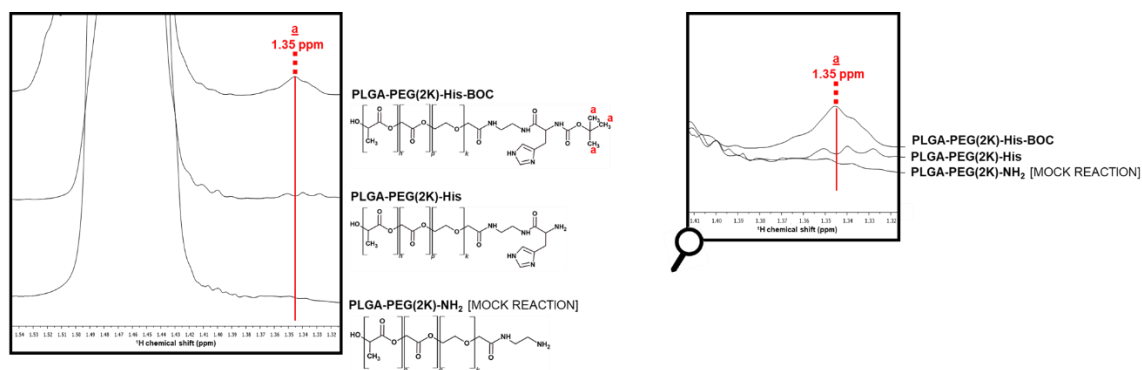


Figure S3. NMR demonstration of removal of the -BOC protecting group of PLGA-PEG(2K)-His-BOC by a selective carbamate hydrolysis reaction.

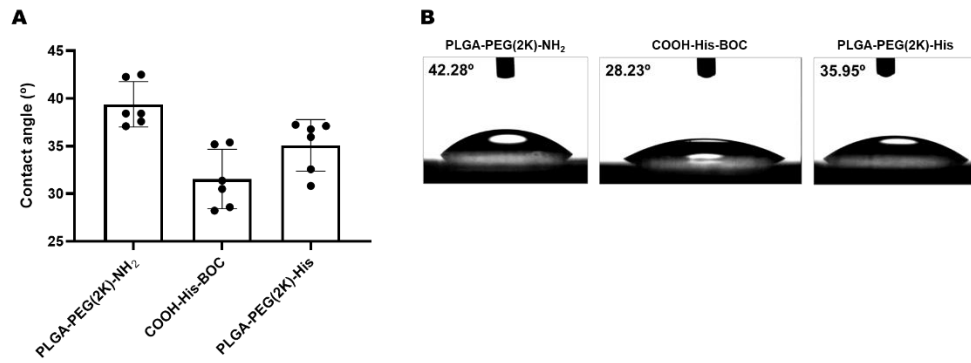


Figure S4. WCA for PLGA-PEG(2K)-His, compared with COOH-His-BOC and PLGA-PEG(2K)-NH₂. (A) Quantitative analysis. (B) Representative images.

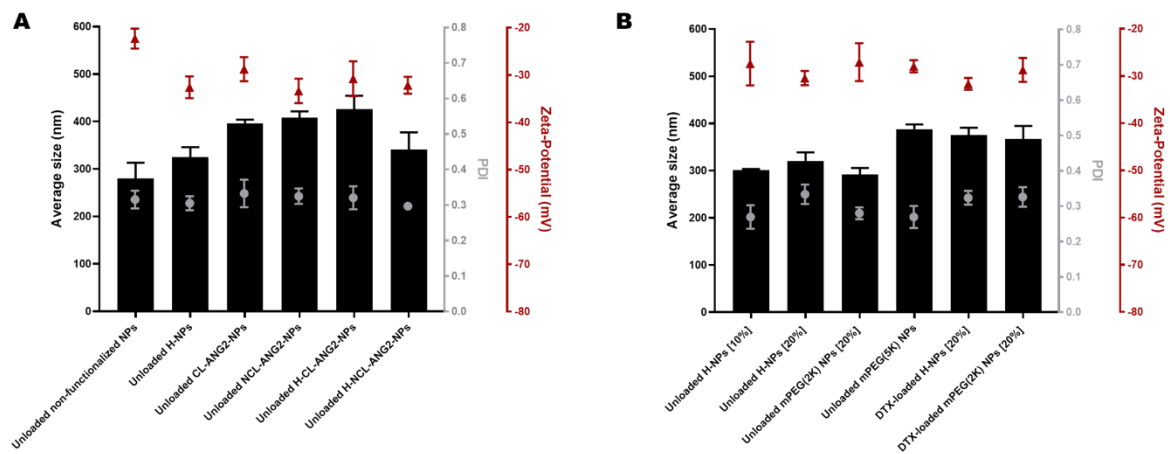


Figure S5. Average size, PDI and zeta-potential complementary data for unloaded NP formulations, and DTX-loaded PEGylated but non-targeted NP controls.

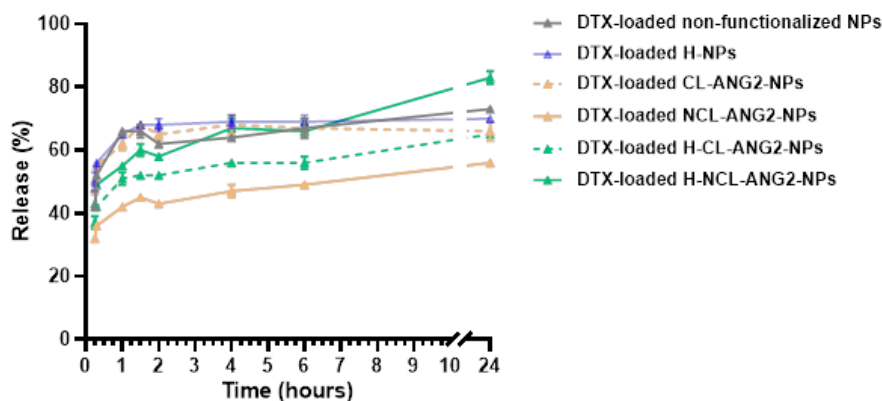


Figure S6. *In vitro* DTX release from NP formulations over a time period of 24 h in medium mimicking systemic physiological conditions (PBS, pH 7.4).

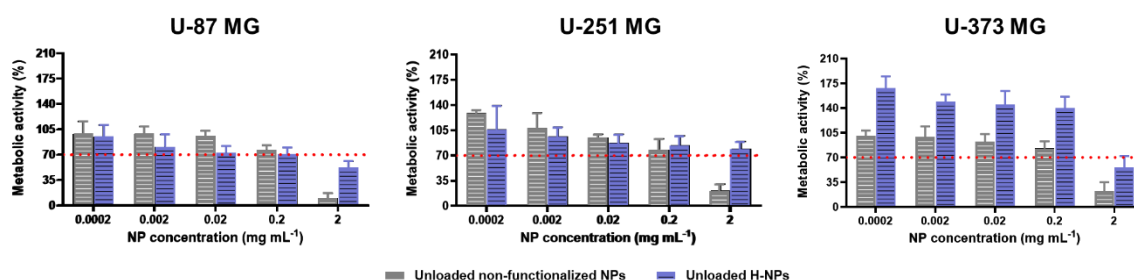


Figure S7. Preliminary metabolic activity assessment of U-87 MG, U-251 MG and U-373 MG cells after exposure to different concentrations of PLGA-FKR648 labeled H-NPs composed by 20% PLGA-PEG(2K)-His over a 4 h period.

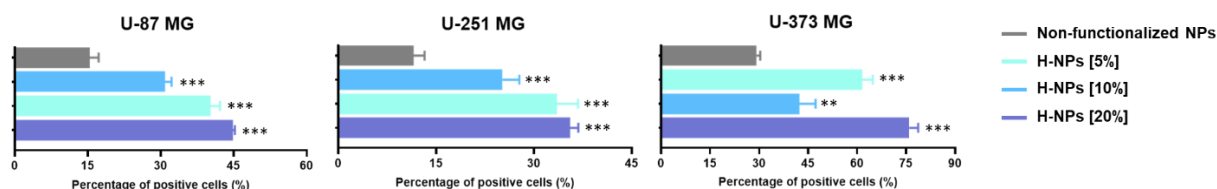


Figure S8. Flow cytometry analysis of cell internalization of non-functionalized NPs and H-NPs assembled from 5%, 10% or 20% PLGA-PEG(2K)-His into GBM conventional cell lines (U-87 MG, U-251 MG, U-373 MG). Statistical comparison of H-NPs with non-functionalized NPs. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

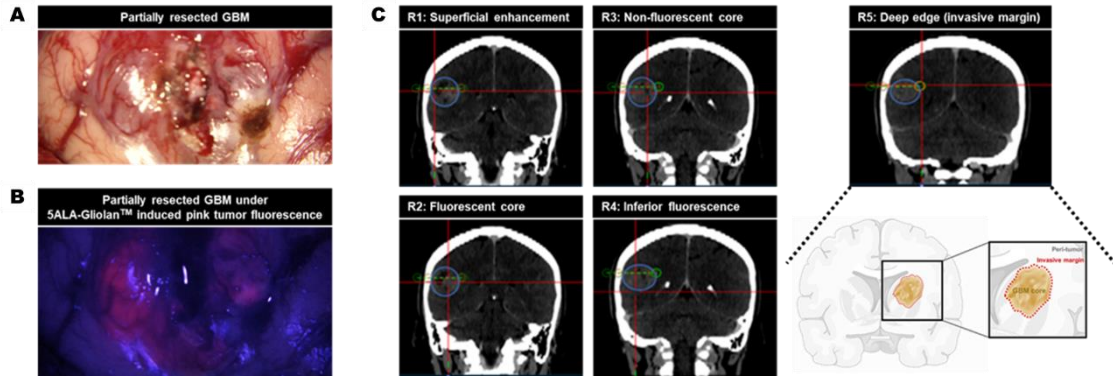


Figure S9. Scheme of the GIN cell isolation procedure. (A) White light conventional view through the operating microscope of a patient temporal lobe with partially resected GBM. (B) Same area of (A) under blue light, evidencing areas of 5ALA-Gliolan™ induced pink tumor fluorescence. (C) Representative MRI of multiregion surgical sampling from a GBM patient indicating the typical regional (R) location of samples, highlighting the deep edge/invasive margin, from which GIN cells are isolated.

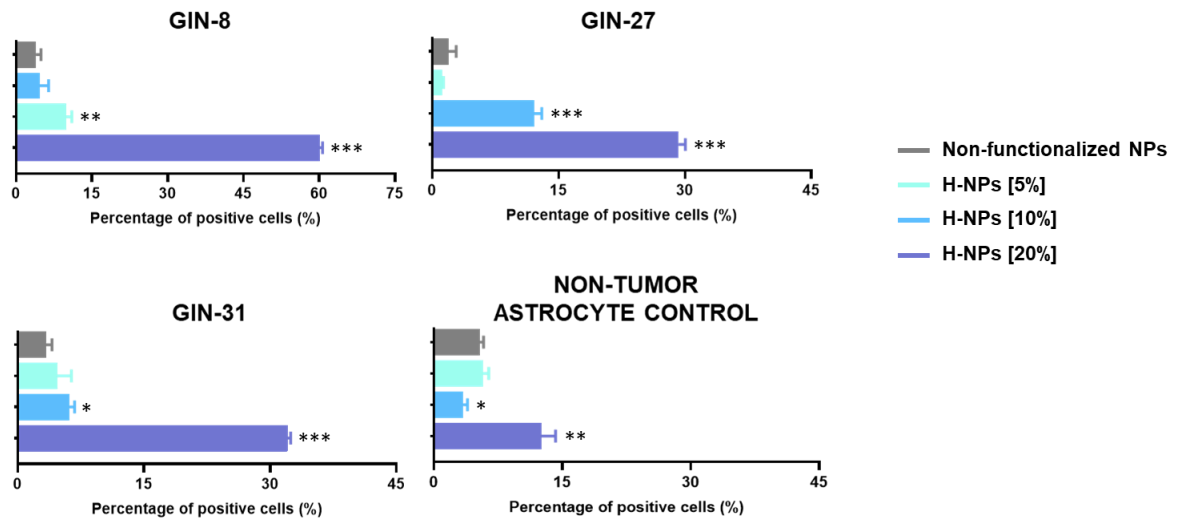


Figure S10. Flow cytometry analysis of cell internalization of non-functionalized NPs and H-NPs assembled from 5%, 10% or 20% PLGA-PEG(2K)-His into GIN patient cells (GIN-8, GIN-27, GIN-31) and a non-tumor astrocyte control cell line. Statistical comparison of H-NPs with non-functionalized NPs. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

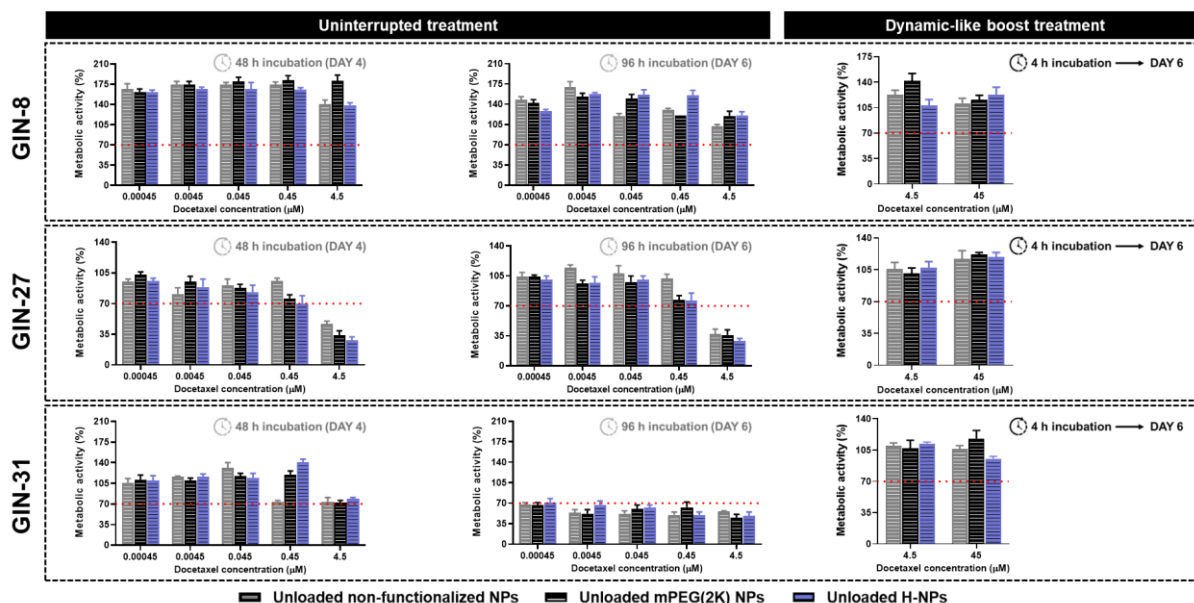


Figure S11. 2D evaluation of the metabolic activity of GIN patient cells after exposure to different concentrations of unloaded NP formulations. The left panel presents metabolic activity levels after uninterrupted periods of treatment of 48 h and 96 h. The right panel presents a dynamic-like boost treatment of 4 h, integrated in an assessment period of incubation of 96 h. H-NPs were assembled from 20% PLGA-PEG(2K)-His.

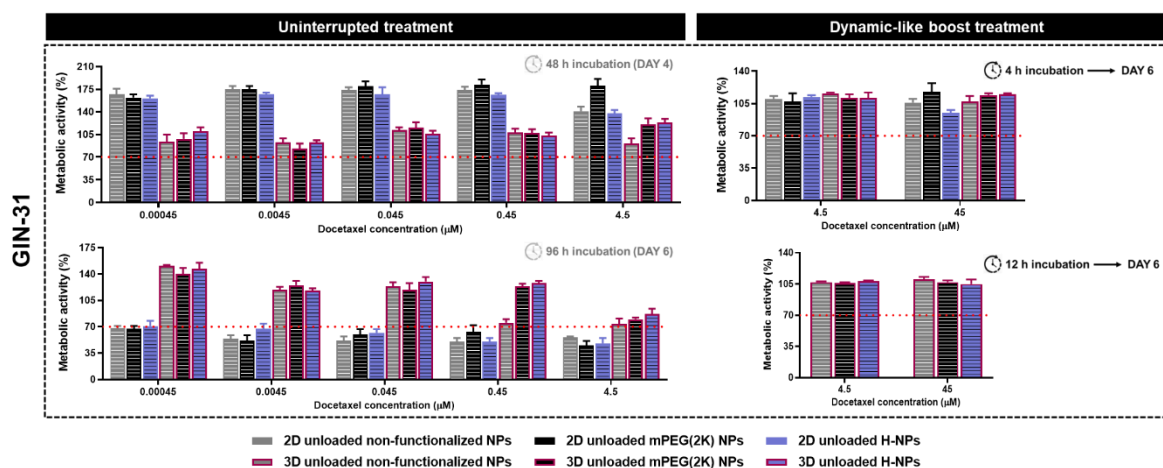


Figure S12. 3D evaluation of the metabolic activity of GIN cells after exposure to different concentrations of unloaded NP formulations. The left panel presents 2D *versus* 3D metabolic activity levels after uninterrupted periods of treatment of 48 h and 96 h. The right panel presents a 2D *versus* 3D dynamic-like boost treatments of 4 h or 12 h, integrated in an assessment period of incubation of 96 h. H-NPs were assembled from

20% PLGA-PEG(2K)-His.

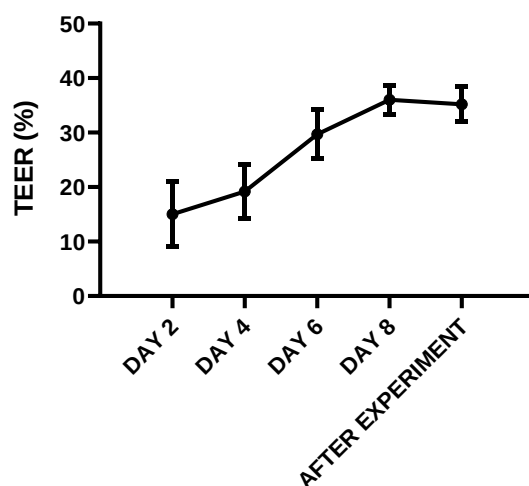


Figure S13. BBB *in vitro* model TEER values over maintenance period and at the day of the permeability experiment.

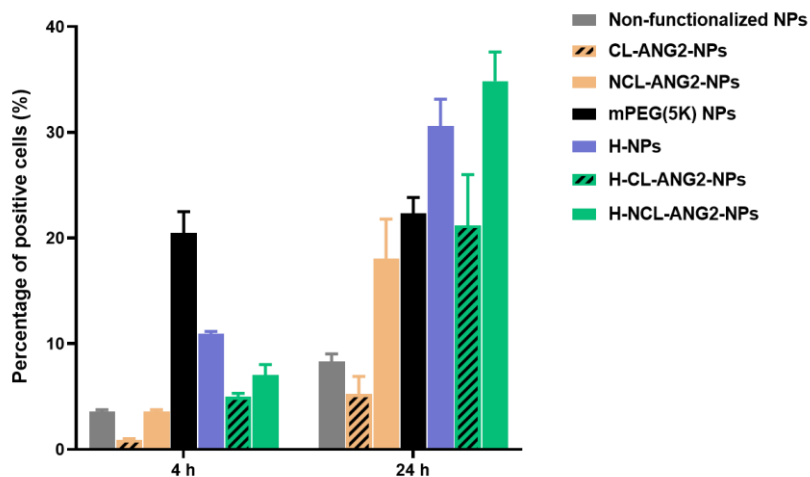


Figure S14. Flow cytometry analysis of cell internalization levels of the different NP formulations. The evaluation was performed in BBB hCMEC/D3 endothelial cells. Statistical comparison of each group with non-functionalized NPs. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

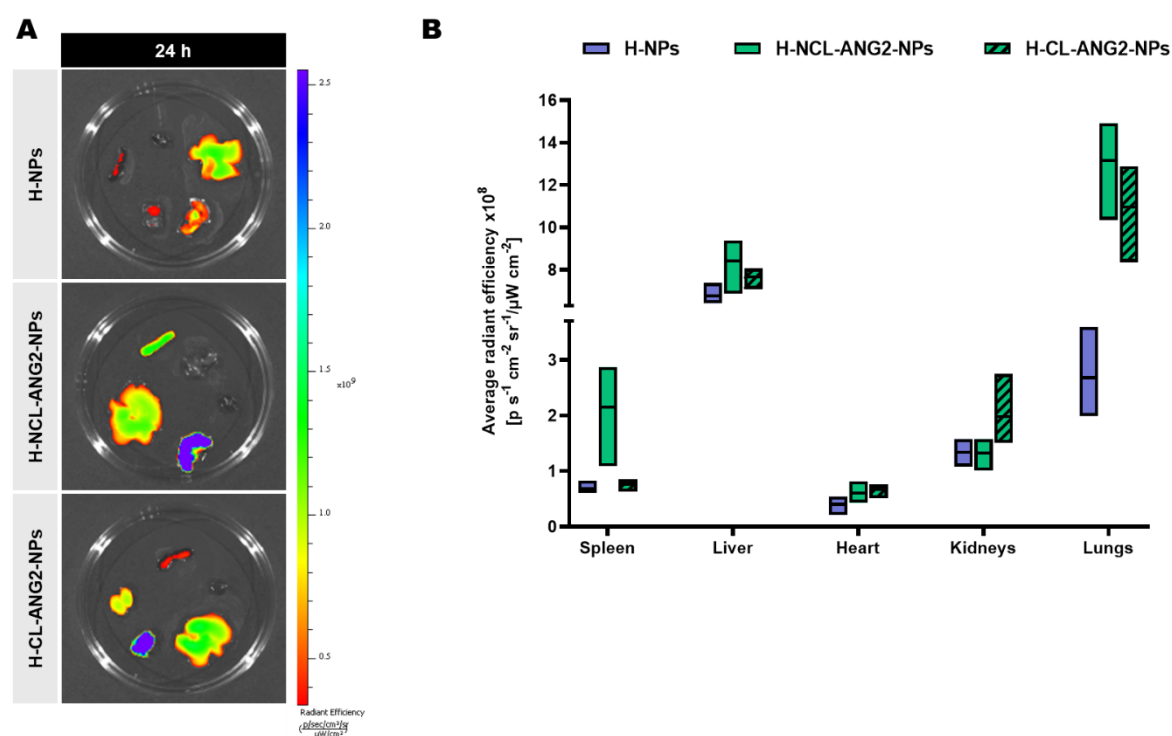


Figure S15. *In vivo* biodistribution trial of different NP formulations to track their accumulation in major organs by excision at 24 h analysis. (A) Representative images of organ accumulation. (B) Quantification of signal intensities at different organs for each NP formulation.

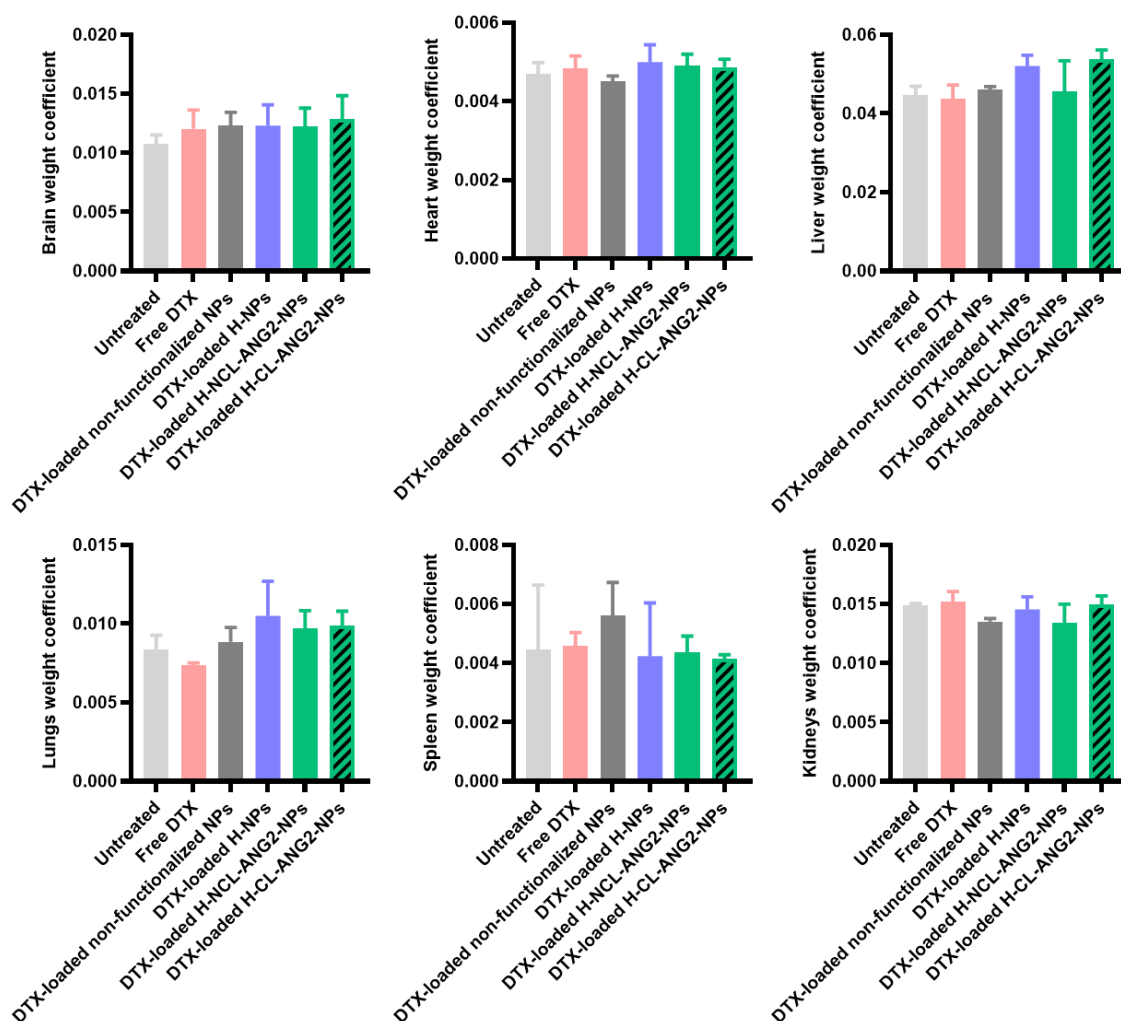


Figure S16. *In vivo* systemic toxicity of free DTX and NP formulations after intravenous treatment evaluated by organ weight coefficient. NMRI nude mice were orthotopically grafted with U-87 MG GBM cells at day 0, randomized upon MRI tumor evaluation at day 8, and treated with a 2 mg kg⁻¹ DTX dose at day 10.

Table S1. Representative results of single tandem repeat (STR) genotyping of GIN cells. The GIN-8 line compared across two passages (p11 and p33), showed identical STR genotypes, which matched the STR genotype from primary invasive margin GBM tissue (T8.3) from which the cells were derived. The GIN-27 line compared across p7 and p14, showed identical STR genotypes, which fully matched the STR genotype from their primary invasive margin GBM tissue (T27.5). GIN-31 p11 cells fully retain the genotype from the primary invasive margin GBM tissue (T31.5); GIN-31 p33 retains a matched STR genotype in 13/21 loci, with amplifications acquired in culture likely a result of the 22 passages between this sample and p11. These results confirm that the primary lines derived from the GBM invasive margin broadly retain the genotype of the tumor. Adapted with permission[29], 2020, Oxford University Press, the Society for Neuro-Oncology and the European Association of Neuro-Oncology.

DNA system	GIN-8		GIN-27				GIN-31		
	p11	p33	T8.3	p7	p14	T27.5	p11	p33	T31.5
AM	X,X	X,X	X,X	X,X	X,X	X,X	X,X	X,X	X,X
D3S1358	15,16	15,16	15,16	15,16	15,16	15,16	16,18	16,18	16,18
D1S1656	12,16	12,16	12,16	15,16	15,16	15,16	12,15	12,15	12,15
D6S1043	14,18	14,18	14,18	11,12	11,12	11,12	12,18	12,18	12,18
D13S317	12,12	12,12	12,12	8,12	8,12	8,12	11,13	11,13	13,13
Penta E	6,9	6,9	6,9	15,17	15,17	15,17	7,12	7,12	7,12
D16S539	12,12	12,12	12,12	10,13	10,13	10,13	10,11	10,11	10,11
D18S51	16,17	16,17	16,17	12,16	12,16	12,16	17,18	16,17,18	17,18
D2S1338	17,19	17,19	17,19	20,23	20,23	20,23	19,25	11,19,25	19,25
CSF1PO	11,11	11,11	11,11	11,12	11,12	11,12	11,12	11,12	11,12
Penta D	12,13	12,13	12,13	9,11	9,11	9,11	11,12	11,12	11,12
TH01	9,9	9,9	9,9	7,9	7,9	7,9	6,9	6,9	6,9
D21S11	30,30	30,30	30,30	29,31	29,31	29,31	29,29	28,29	29,29
D7S820	9,10	9,10	9,10	10,12	10,12	10,12	7,10	7,10	7,10
D5S818	12,13	12,13	12,13	10,13	10,13	10,13	11,12	11,1,12	11,12
TPOX	8,11	8,11	8,11	8,9	8,9	8,9	9,9	9,9	9,9
D8S1179	10,13	10,13	10,13	12,14	12,14	12,14	11,13	11,13,14.1	11,13
D12S391	18,18	18,18	18,18	15,22	15,22	15,22	17,23	16,17,22.3,23	23,23
FGA	21,22	21,22	21,22	21,24	21,24	21,24	22,26	19.3,22,26	22,26

Table S2. AE and LD values for the developed DTX-loaded NP formulations.

DTX-loaded NP formulation	AE (%)	LD (%)
Non-functionalized NPs	30.42 ± 5.11	1.52 ± 0.26
H-NPs	30.98 ± 7.26	1.55 ± 0.36
CL-ANG2 NPs	30.98 ± 4.46	1.55 ± 0.22
NCL-ANG2 NPs	36.43 ± 0.20	1.82 ± 0.01
H-CL-ANG2 NPs	31.37 ± 1.39	1.57 ± 0.07
H-NCL-ANG2 NPs	26.10 ± 0.80	1.30 ± 0.04

Table S3. LD values for the developed Cy7.5 labeled NP formulations.

Cy7.5-loaded NP formulation	LD (%)
H-NPs	0.13 ± 0.02
H-CL-ANG2 NPs	0.14 ± 0.00
H-NCL-ANG2 NPs	0.12 ± 0.02

REFERENCES

- [1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J Clin*, 71 (2021) 209-249.
- [2] M.W. Yu, D.F. Quail, Immunotherapy for glioblastoma: Current progress and challenges, *Front Immunol*, 12 (2021) 676301.
- [3] W. Yang, J. Soares, P. Greninger, E.J. Edelman, H. Lightfoot, S. Forbes, N. Bindal, D. Beare, J.A. Smith, I.R. Thompson, S. Ramaswamy, P.A. Futreal, D.A. Haber, M.R. Stratton, C. Benes, U. McDermott, M.J. Garnett, Genomics of Drug Sensitivity in Cancer (GDSC): A resource for therapeutic biomarker discovery in cancer cells, *Nucleic Acids Res* 41 (2012) D955-D961.
- [4] S. Ostermann, C. Csajka, T. Buclin, S. Leyvraz, F. Lejeune, L.A. Decosterd, R. Stupp, Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients, *Clin Cancer Res*, 10 (2004) 3728-3736.
- [5] A.J. ten Tije, W.J. Loos, M. Zhao, S.D. Baker, R.H. Enting, H. van der Meulen, J. Verweij, A. Sparreboom, Limited cerebrospinal fluid penetration of docetaxel, *Anticancer Drugs*, 15 (2004) 715-718.
- [6] S. Wilhelm, A.J. Tavares, Q. Dai, S. Ohta, J. Audet, H.F. Dvorak, W.C.W. Chan, Analysis of nanoparticle delivery to tumours, *Nat Rev Mater*, 1 (2016) 16014.
- [7] S. Quader, K. Kataoka, H. Cabral, Nanomedicine for brain cancer, *Adv Drug Deliv Rev*, 182 (2022) 114115.
- [8] C. Martins, F. Sousa, F. Araújo, B. Sarmiento, Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications, *Adv Healthc Mater*, 7 (2018) 1701035.
- [9] M. Souiri, M. Soltani, F. Moradi Kashkooli, M. Kiani Shahvandi, M. Chiani, F.S. Shariati, M.R. Mehrabi, L.L. Munn, Towards principled design of cancer nanomedicine to accelerate clinical translation, *Mater Today Bio*, 13 (2022) 100208.
- [10] A.M. Wagner, D.S. Spencer, N.A. Peppas, Advanced architectures in the design of responsive polymers for cancer nanomedicine, *J Appl Polym Sci*, 135 (2018).
- [11] X. Zhang, S. Malhotra, M. Molina, R. Haag, Micro- and nanogels with labile crosslinks – from synthesis to biomedical applications, *Chem Soc Rev*, 44 (2015) 1948-1973.
- [12] W. Zhang, A. Mehta, Z. Tong, L. Esser, N.H. Voelcker, Development of polymeric nanoparticles for blood–brain barrier transfer—strategies and challenges, *Adv Sci*, 8 (2021) 2003937.

- [13] A. Duro-Castano, D. Moreira Leite, J. Forth, Y. Deng, D. Matias, C. Noble Jesus, G. Battaglia, Designing peptide nanoparticles for efficient brain delivery, *Adv Drug Deliv Rev*, 160 (2020) 52-77.
- [14] Z. Haining, N. Kawai, K. Miyake, M. Okada, S. Okubo, X. Zhang, Z. Fei, T. Tamiya, Relation of LAT1/4F2hc expression with pathological grade, proliferation and angiogenesis in human gliomas, *BMC Clin Pathol*, 12 (2012) 4.
- [15] H. Nawashiro, N. Otani, N. Shinomiya, S. Fukui, H. Ooigawa, K. Shima, H. Matsuo, Y. Kanai, H. Endou, L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors, *Int J Cancer*, 119 (2006) 484-492.
- [16] P. Häfliger, R.-P. Charles, The L-Type amino acid transporter LAT1 - an emerging target in cancer, *Int J Mol Sci*, 20 (2019) 2428.
- [17] X. Tian, D.M. Leite, E. Scarpa, S. Nyberg, G. Fullstone, J. Forth, D. Matias, A. Apriceno, A. Poma, A. Duro-Castano, M. Vuyyuru, L. Harker-Kirschneck, A. Šarić, Z. Zhang, P. Xiang, B. Fang, Y. Tian, L. Luo, L. Rizzello, G. Battaglia, On the shuttling across the blood-brain barrier via tubule formation: Mechanism and cargo avidity bias, *Sci Adv*, 6 (2020) eabc4397.
- [18] Z. Jin, L. Piao, G. Sun, C. Lv, Y. Jing, R. Jin, Dual functional nanoparticles efficiently across the blood–brain barrier to combat glioblastoma via simultaneously inhibit the PI3K pathway and NKG2A axis, *J Drug Target*, 29 (2021) 323-335.
- [19] X. Wang, Y. Zhao, S. Dong, R.J. Lee, D. Yang, H. Zhang, L. Teng, Cell-penetrating peptide and transferrin co-modified liposomes for targeted therapy of glioma, *Molecules*, 24 (2019) 3540.
- [20] S. Lakkadwala, B. dos Santos Rodrigues, C. Sun, J. Singh, Dual functionalized liposomes for efficient co-delivery of anti-cancer chemotherapeutics for the treatment of glioblastoma, *J Control Release*, 307 (2019) 247-260.
- [21] C. Liu, Z. Zhao, H. Gao, I. Rostami, Q. You, X. Jia, C. Wang, L. Zhu, Y. Yang, Enhanced blood-brain-barrier penetrability and tumor-targeting efficiency by peptide-functionalized poly(amidoamine) dendrimer for the therapy of gliomas, *Nanotheranostics*, 3 (2019) 311-330.
- [22] Z. Belhadj, C. Zhan, M. Ying, X. Wei, C. Xie, Z. Yan, W. Lu, Multifunctional targeted liposomal drug delivery for efficient glioblastoma treatment, *Oncotarget*, 8 (2017) 66889-66900.
- [23] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, Glioma targeting and blood–brain barrier penetration by dual-targeting doxorubicin liposomes, *Biomaterials*, 34 (2013) 5628-5639.

- [24] X. Zhu, H. Zhou, Y. Liu, Y. Wen, C. Wei, Q. Yu, J. Liu, Transferrin/aptamer conjugated mesoporous ruthenium nanosystem for redox-controlled and targeted chemo-photodynamic therapy of glioma, *Acta Biomater*, 82 (2018) 143-157.
- [25] C. Chen, Z. Duan, Y. Yuan, R. Li, L. Pang, J. Liang, X. Xu, J. Wang, Peptide-22 and cyclic RGD functionalized liposomes for glioma targeting drug delivery overcoming BBB and BBTB, *ACS Appl Mater Interfaces*, 9 (2017) 5864-5873.
- [26] P. Zhang, L. Hu, Q. Yin, L. Feng, Y. Li, Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy, *Mol Pharm*, 9 (2012) 1590-1598.
- [27] Y. Zhu, Y. Jiang, F. Meng, C. Deng, R. Cheng, J. Zhang, J. Feijen, Z. Zhong, Highly efficacious and specific anti-glioma chemotherapy by tandem nanomicelles co-functionalized with brain tumor-targeting and cell-penetrating peptides, *J Control Release*, 278 (2018) 1-8.
- [28] A.J. Clark, M.E. Davis, Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core, *Proc Natl Acad Sci USA*, 112 (2015) 12486-12491.
- [29] S.J. Smith, J. Rowlinson, M. Estevez-Cebrero, D. Onion, A. Ritchie, P. Clarke, K. Wood, M. Diksin, A. Lourdasamy, R.G. Grundy, R. Rahman, Metabolism-based isolation of invasive glioblastoma cells with specific gene signatures and tumorigenic potential, *Neurooncol Adv*, 2 (2020) vdaa087.
- [30] S.J. Smith, M. Diksin, S. Chhaya, S. Sairam, M.A. Estevez-Cebrero, R. Rahman, The invasive region of glioblastoma defined by 5ALA guided surgery has an altered cancer stem cell marker profile compared to central tumour, *Int J Mol Sci*, 18 (2017).
- [31] S.J. Smith, B.M. Tyler, T. Gould, G.J. Veal, N. Gorelick, J. Rowlinson, R. Serra, A. Ritchie, P. Berry, A. Otto, J. Choi, N. Skuli, M. Estevez-Cebrero, K.M. Shakesheff, H. Brem, R.G. Grundy, R. Rahman, Overall survival in malignant glioma is significantly prolonged by neurosurgical delivery of etoposide and temozolomide from a thermo-responsive biodegradable paste, *Clin Cancer Res*, 25 (2019) 5094-5106.
- [32] C. Martins, F. Araújo, M.J. Gomes, C. Fernandes, R. Nunes, W. Li, H.A. Santos, F. Borges, B. Sarmiento, Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy, *Eur J Pharm Biopharm*, 138 (2018) 111-124.
- [33] C. Martins, V.M. Chauhan, A.A. Selo, M. Al-Natour, J.W. Aylott, B. Sarmiento, Modelling protein therapeutic co-formulation and co-delivery with PLGA nanoparticles continuously manufactured by microfluidics, *React Chem Eng*, 5 (2020) 308-319.
- [34] C. Martins, B. Sarmiento, Microfluidic manufacturing of multitargeted PLGA/PEG nanoparticles for delivery of taxane chemotherapeutics, in: K.K. Jain (Ed.) *Drug Delivery Systems*, Springer New York, New York, NY, 2020, pp. 213-224.

- [35] M.J. Gomes, C. Fernandes, S. Martins, F. Borges, B. Sarmento, Tailoring lipid and polymeric nanoparticles as siRNA carriers towards the blood-brain barrier—from targeting to safe administration, *J Neuroimmune Pharmacol*, 12 (2016) 107-119.
- [36] A. ISO 10993-5: 2009 Biological Evaluation of Medical Devices-Part 5: Tests for in Vitro Cytotoxicity; ISO: Phoenix, USA, 2007 (2009) 1-11.
- [37] B. Mendes, C. Marques, I. Carvalho, P. Costa, S. Martins, D. Ferreira, B. Sarmento, Influence of glioma cells on a new co-culture in vitro blood–brain barrier model for characterization and validation of permeability, *Int J Pharm*, 490 (2015) 94-101.
- [38] M.J. Gomes, P.J. Kennedy, S. Martins, B. Sarmento, Delivery of siRNA silencing P-gp in peptide-functionalized nanoparticles causes efflux modulation at the blood–brain barrier, *Nanomedicine*, 12 (2017) 1385-1399.
- [39] F. Danhier, K. Messaoudi, L. Lemaire, J.P. Benoit, F. Lagarce, Combined anti-Galectin-1 and anti-EGFR siRNA-loaded chitosan-lipid nanocapsules decrease temozolomide resistance in glioblastoma: In vivo evaluation, *Int J Pharm*, 481 (2015) 154-161.
- [40] J. Bianco, C. Bastiancich, N. Joudiou, B. Gallez, A. des Rieux, F. Danhier, Novel model of orthotopic U-87 MG glioblastoma resection in athymic nude mice, *J Neurosci Methods*, 284 (2017) 96-102.
- [41] C. Cannavà, R. Stancanelli, M.R. Marabeti, V. Venuti, C. Cascio, P. Guarneri, C. Bongiorno, G. Sortino, D. Majolino, A. Mazzaglia, S. Tommasini, C.A. Ventura, Nanospheres based on PLGA/amphiphilic cyclodextrin assemblies as potential enhancers of Methylene Blue neuroprotective effect, *RSC Adv*, 6 (2016) 16720-16729.
- [42] R. Cheng, A.C. Weitz, J. Paris, Y. Tang, J. Zhang, H. Song, N. Naowarojna, K. Li, L. Qiao, J. Lopez, M.W. Grinstaff, L. Zhang, Y. Guo, S. Elliott, P. Liu, OvoAMtht from *Methyloversatilis thermotolerans* ovothiol biosynthesis is a bifunction enzyme: Thiol oxygenase and sulfoxide synthase activities, *Chem Sci*, 13 (2022) 3589-3598.
- [43] F.C. Thomas, K. Taskar, V. Rudraraju, S. Goda, H.R. Thorsheim, J.A. Gaasch, R.K. Mittapalli, D. Palmieri, P.S. Steeg, P.R. Lockman, Q.R. Smith, Uptake of ANG1005, a novel paclitaxel derivative, through the blood-brain barrier into brain and experimental brain metastases of breast cancer, *Pharm Res*, 26 (2009) 2486-2494.
- [44] F. Marcucci, A. Corti, A.J.M. Ferreri, Breaching the blood-brain tumor barrier for tumor therapy, *Cancers (Basel)*, 13 (2021).
- [45] L. Costantino, F. Gandolfi, G. Tosi, F. Rivasi, M.A. Vandelli, F. Forni, Peptide-derivatized biodegradable nanoparticles able to cross the blood–brain barrier, *J Control Release*, 108 (2005) 84-96.
- [46] G.P. Hoyos-Ceballos, B. Ruozzi, I. Ottonelli, F. Da Ros, M.A. Vandelli, F. Forni, E. Daini, A. Vilella, M. Zoli, G. Tosi, J.T. Duskey, B.L. López-Osorio, PLGA-PEG-ANG-2

nanoparticles for blood-brain barrier crossing: Proof-of-concept study, *Pharmaceutics*, 12 (2020).

[47] R. Juliano, Nanomedicine: Is the wave cresting?, *Nat Rev Drug Discov*, 12 (2013) 171-172.

[48] J. Ma, S.M.-Y. Lee, C. Yi, C.-W. Li, Controllable synthesis of functional nanoparticles by microfluidic platforms for biomedical applications – a review, *Lab Chip*, 17 (2017) 209-226.

[49] A. Gdowski, K. Johnson, S. Shah, I. Gryczynski, J. Vishwanatha, A. Ranjan, Optimization and scale up of microfluidic nanolipomer production method for preclinical and potential clinical trials, *J Nanobiotechnology*, 16 (2018) 12-12.

[50] D. Furtado, M. Björnmalm, S. Ayton, A.I. Bush, K. Kempe, F. Caruso, Overcoming the blood–brain barrier: The role of nanomaterials in treating neurological diseases, *Adv Mater*, 30 (2018) 1801362.

[51] T.D. Brown, N. Habibi, D. Wu, J. Lahann, S. Mitragotri, Effect of nanoparticle composition, size, shape, and stiffness on penetration across the blood–brain barrier, *ACS Biomater Sci Eng*, 6 (2020) 4916-4928.

[52] J. Key, A.L. Palange, F. Gentile, S. Aryal, C. Stigliano, D. Di Mascolo, E. De Rosa, M. Cho, Y. Lee, J. Singh, P. Decuzzi, Soft discoidal polymeric nanoconstructs resist macrophage uptake and enhance vascular targeting in tumors, *ACS Nano*, 9 (2015) 11628-11641.

[53] Z. Wang, J. Ding, X. Ma, S. Luo, Selective ultrasound contrast enhancement in the tumor by nanocapsules with perfluorooctylbromide: Effect of PLGA–PEG proportion, *RSC Adv*, 8 (2018) 17958-17966.

[54] L. Ribovski, E. de Jong, O. Mergel, G. Zu, D. Keskin, P. van Rijn, I.S. Zuhorn, Low nanogel stiffness favors nanogel transcytosis across an in vitro blood–brain barrier, *Nanomedicine*, 34 (2021) 102377.

[55] T. Tsuji, H. Yoshitomi, J. Usukura, Endocytic mechanism of transferrin-conjugated nanoparticles and the effects of their size and ligand number on the efficiency of drug delivery, *Microscopy*, 62 (2013) 341-352.

[56] P.P. Di Mauro, S. Borrós, Development of high drug loaded and customizing novel nanoparticles for modulated and controlled release of paclitaxel, *Pharm Res*, 31 (2014) 3461-3477.

[57] J. Cheng, B.A. Teply, I. Sherifi, J. Sung, G. Luther, F.X. Gu, E. Levy-Nissenbaum, A.F. Radovic-Moreno, R. Langer, O.C. Farokhzad, Formulation of functionalized PLGA–PEG nanoparticles for in vivo targeted drug delivery, *Biomaterials*, 28 (2007) 869-876.

[58] S. Maiolino, A. Russo, V. Pagliara, C. Conte, F. Ungaro, G. Russo, F. Quaglia, Biodegradable nanoparticles sequentially decorated with polyethyleneimine and

hyaluronan for the targeted delivery of docetaxel to airway cancer cells, *J Nanobiotechnology*, 13 (2015) 29.

[59] P. Rafiei, A. Haddadi, Docetaxel-loaded PLGA and PLGA-PEG nanoparticles for intravenous application: Pharmacokinetics and biodistribution profile, *Int J Nanomed*, 12 (2017) 935-947.

[60] Z. Xiao, E. Levy-Nissenbaum, F. Alexis, A. Lupták, B.A. Teply, J.M. Chan, J. Shi, E. Digga, J. Cheng, R. Langer, O.C. Farokhzad, Engineering of targeted nanoparticles for cancer therapy using internalizing aptamers isolated by cell-uptake selection, *ACS Nano*, 6 (2012) 696-704.

[61] K. Kobayashi, A. Ohnishi, J. Promsuk, S. Shimizu, Y. Kanai, Y. Shiokawa, M. Nagane, Enhanced tumor growth elicited by L-type amino acid transporter 1 in human malignant glioma cells, *Neurosurgery*, 62 (2008) 493-503.

[62] O. Yanagida, Y. Kanai, A. Chairoungdua, D.K. Kim, H. Segawa, T. Nii, S.H. Cha, H. Matsuo, J. Fukushima, Y. Fukasawa, Y. Tani, Y. Taketani, H. Uchino, J.Y. Kim, J. Inatomi, I. Okayasu, K. Miyamoto, E. Takeda, T. Goya, H. Endou, Human L-type amino acid transporter 1 (LAT1): Characterization of function and expression in tumor cell lines, *Biochim Biophys Acta*, 1514 (2001) 291-302.

[63] H.-C. Chien, C. Colas, K. Finke, S. Springer, L. Stoner, A.A. Zur, B. Venteicher, J. Campbell, C. Hall, A. Flint, E. Augustyn, C. Hernandez, N. Heeren, L. Hansen, A. Anthony, J. Bauer, D. Fotiadis, A. Schlessinger, K.M. Giacomini, A.A. Thomas, Reevaluating the substrate specificity of the L-type amino acid transporter (LAT1), *J Med Chem*, 61 (2018) 7358-7373.

[64] D. Brevet, O. Hocine, A. Delalande, L. Raehm, C. Charnay, P. Midoux, J.O. Durand, C. Pichon, Improved gene transfer with histidine-functionalized mesoporous silica nanoparticles, *Int J Pharm*, 471 (2014) 197-205.

[65] V. Jahed, E. Vasheghani-Farahani, F. Bagheri, A. Zarrabi, T. Fink, K. Lambertsen Larsen, Enhanced cellular uptake of phenamil through inclusion complex with histidine functionalized β -cyclodextrin as penetrative osteoinductive agent, *Int J Nanomedicine*, 14 (2019) 8221-8234.

[66] G. Tan, J. Li, D. Liu, H. Pan, R. Zhu, Y. Yang, W. Pan, Amino acids functionalized dendrimers with nucleus accumulation for efficient gene delivery, *Int J Pharm*, 602 (2021) 120641.

[67] E. Gallon, T. Matini, L. Sasso, G. Mantovani, A. Armiñan de Benito, J. Sanchis, P. Caliceti, C. Alexander, M.J. Vicent, S. Salmaso, Triblock copolymer nanovesicles for pH-responsive targeted delivery and controlled release of siRNA to cancer cells, *Biomacromolecules*, 16 (2015) 1924-1937.

- [68] S.-Y. Wu, H.-Y. Chou, H.-C. Tsai, R. Anbazhagan, C.-H. Yuh, J.M. Yang, Y.-H. Chang, Amino acid-modified PAMAM dendritic nanocarriers as effective chemotherapeutic drug vehicles in cancer treatment: A study using zebrafish as a cancer model, *RSC Adv*, 10 (2020) 20682-20690.
- [69] L. Li, X. Di, M. Wu, Z. Sun, L. Zhong, Y. Wang, Q. Fu, Q. Kan, J. Sun, Z. He, Targeting tumor highly-expressed LAT1 transporter with amino acid-modified nanoparticles: Toward a novel active targeting strategy in breast cancer therapy, *Nanomedicine*, 13 (2017) 987-998.
- [70] L. Li, X. Di, S. Zhang, Q. Kan, H. Liu, T. Lu, Y. Wang, Q. Fu, J. Sun, Z. He, Large amino acid transporter 1 mediated glutamate modified docetaxel-loaded liposomes for glioma targeting, *Colloids Surf B*, 141 (2016) 260-267.
- [71] Z. Wang, D. Chi, X. Wu, Y. Wang, X. Lin, Z. Xu, H. Liu, J. Sun, Z. He, Y. Wang, Tyrosine modified irinotecan-loaded liposomes capable of simultaneously targeting LAT1 and ATB(0,+) for efficient tumor therapy, *J Control Release*, 316 (2019) 22-33.
- [72] S. Nozaki, Y. Nakatani, A. Mawatari, N. Shibata, W.E. Hume, E. Hayashinaka, Y. Wada, H. Doi, Y. Watanabe, 18F-FIMP: A LAT1-specific PET probe for discrimination between tumor tissue and inflammation, *Sci Rep*, 9 (2019) 15718.
- [73] S. Okubo, H.-N. Zhen, N. Kawai, Y. Nishiyama, R. Haba, T. Tamiya, Correlation of L-methyl-11C-methionine (MET) uptake with L-type amino acid transporter 1 in human gliomas, *J Neurooncol*, 99 (2010) 217-225.
- [74] A. Habermeier, J. Graf, B.F. Sandhöfer, J.P. Boissel, F. Roesch, E.I. Closs, System L amino acid transporter LAT1 accumulates O-(2-fluoroethyl)-L-tyrosine (FET), *Amino Acids*, 47 (2015) 335-344.
- [75] A. Hahn, J. Bode, T. Krüwel, G. Solecki, S. Heiland, M. Bendszus, B. Tews, F. Winkler, M.O. Breckwoldt, F.T. Kurz, Glioblastoma multiforme restructures the topological connectivity of cerebrovascular networks, *Sci Rep*, 9 (2019) 11757.
- [76] S.L. Perrin, M.S. Samuel, B. Koszyca, M.P. Brown, L.M. Ebert, M. Oksdath, G.A. Gomez, Glioblastoma heterogeneity and the tumour microenvironment: Implications for preclinical research and development of new treatments, *Biochem Soc Trans*, 47 (2019) 625-638.
- [77] C.E. Vasey, R.J. Cavanagh, V. Taresco, C. Moloney, S. Smith, R. Rahman, C. Alexander, Polymer pro-drug nanoparticles for sustained release of cytotoxic drugs evaluated in patient-derived glioblastoma cell lines and in situ gelling formulations, *Pharmaceutics*, 13 (2021).
- [78] H. Nawashiro, N. Otani, Y. Uozumi, H. Ooigawa, T. Toyooka, T. Suzuki, H. Katoh, N. Tsuzuki, A. Ohnuki, K. Shima, N. Shinomiya, H. Matsuo, Y. Kanai, High expression

of L-type amino acid transporter 1 in infiltrating glioma cells, *Brain Tumor Pathol*, 22 (2005) 89-91.

[79] D.Y. Lewis, R. Mair, A. Wright, K. Allinson, S.K. Lyons, T. Booth, J. Jones, R. Bielik, D. Soloviev, K.M. Brindle, [18F]fluoroethyltyrosine-induced Cerenkov luminescence improves image-guided surgical resection of glioma, *Theranostics*, 8 (2018) 3991-4002.

[80] K. Petrecca, M.-C. Guiot, V. Panet-Raymond, L. Souhami, Failure pattern following complete resection plus radiotherapy and temozolomide is at the resection margin in patients with glioblastoma, *J Neuro-Oncol*, 111 (2013) 19-23.

[81] M.A. Islam, Y. Xu, W. Tao, J.M. Ubellacker, M. Lim, D. Aum, G.Y. Lee, K. Zhou, H. Zope, M. Yu, W. Cao, J.T. Oswald, M. Dinarvand, M. Mahmoudi, R. Langer, P.W. Kantoff, O.C. Farokhzad, B.R. Zetter, J. Shi, Restoration of tumour-growth suppression in vivo via systemic nanoparticle-mediated delivery of PTEN mRNA, *Nat Biomed Eng*, 2 (2018) 850-864.

[82] B. Ouyang, W. Poon, Y.-N. Zhang, Z.P. Lin, B.R. Kingston, A.J. Tavares, Y. Zhang, J. Chen, M.S. Valic, A.M. Syed, P. MacMillan, J. Couture-Sen cal, G. Zheng, W.C.W. Chan, The dose threshold for nanoparticle tumour delivery, *Nat Mater*, 19 (2020) 1362-1371.

[83] A.S. Nunes, A.S. Barros, E.C. Costa, A.F. Moreira, I.J. Correia, 3D tumor spheroids as in vitro models to mimic in vivo human solid tumors resistance to therapeutic drugs, *Biotechnol Bioeng*, 116 (2019) 206-226.

[84] T. Bauleth-Ramos, T. Feij o, A. Gon alves, M.-A. Shahbazi, Z. Liu, C. Barrias, M.J. Oliveira, P. Granja, H.A. Santos, B. Sarmiento, Colorectal cancer triple co-culture spheroid model to assess the biocompatibility and anticancer properties of polymeric nanoparticles, *J Control Release*, 323 (2020) 398-411.

[85] C. Dubois, R. Dufour, P. Daumar, C. Aubel, C. Szczepaniak, C. Blavignac, E. Mounetou, F. Penault-Llorca, M. Bamdad, Development and cytotoxic response of two proliferative MDA-MB-231 and non-proliferative SUM1315 three-dimensional cell culture models of triple-negative basal-like breast cancer cell lines, *Oncotarget*, 8 (2017) 95316-95331.

[86] M.A.G. Barbosa, C.P.R. Xavier, R.F. Pereira, V. Petrikait , M.H. Vasconcelos, 3D cell culture models as recapitulators of the tumor microenvironment for the screening of anti-cancer drugs, *Cancers (Basel)*, 14 (2021).

[87] M.E. Ackerman, D. Pawlowski, K.D. Wittrup, Effect of antigen turnover rate and expression level on antibody penetration into tumor spheroids, *Mol Cancer Ther*, 7 (2008) 2233-2240.

[88] S.J. Yu, J.H. Yoon, J.M. Lee, J.Y. Lee, S.H. Kim, Y.Y. Cho, J.J. Yoo, M. Lee, D.H. Lee, Y. Cho, E.J. Cho, J.H. Lee, Y.J. Kim, C.Y. Kim, Percutaneous ethanol injection

therapy is comparable to radiofrequency ablation in hepatocellular carcinoma smaller than 1.5cm: A matched case-control comparative analysis, *Medicine (Baltimore)*, 95 (2016) e4551.

[89] Y. Hao, B. Zhang, C. Zheng, R. Ji, X. Ren, F. Guo, S. Sun, J. Shi, H. Zhang, Z. Zhang, L. Wang, Y. Zhang, The tumor-targeting core-shell structured DTX-loaded PLGA@Au nanoparticles for chemo-photothermal therapy and X-ray imaging, *J Control Release*, 220 (2015) 545-555.

[90] H. Xin, X. Sha, X. Jiang, L. Chen, K. Law, J. Gu, Y. Chen, X. Wang, X. Fang, The brain targeting mechanism of Angiopep-conjugated poly(ethylene glycol)-co-poly(ϵ -caprolactone) nanoparticles, *Biomaterials*, 33 (2012) 1673-1681.

[91] M. Liu, A. Apriceno, M. Sipin, E. Scarpa, L. Rodriguez-Arco, A. Poma, G. Marchello, G. Battaglia, S. Angioletti-Uberti, Combinatorial entropy behaviour leads to range selective binding in ligand-receptor interactions, *Nat Commun*, 11 (2020) 4836.

[92] Y. Jiang, W. Yang, J. Zhang, F. Meng, Z. Zhong, Protein toxin chaperoned by LRP-1-targeted virus-mimicking vesicles induces high-efficiency glioblastoma therapy in vivo, *Adv Mater*, 30 (2018) e1800316.

[93] R.J. Boado, J.Y. Li, M. Nagaya, C. Zhang, W.M. Pardridge, Selective expression of the large neutral amino acid transporter at the blood-brain barrier, *Proc Natl Acad Sci USA*, 96 (1999) 12079-12084.

[94] E.M. del Amo, A. Urtti, M. Yliperttula, Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2, *Eur J Pharm Sci*, 35 (2008) 161-174.

[95] Y. Bertrand, J.C. Currie, J. Poirier, M. Demeule, A. Abulrob, D. Fatehi, D. Stanimirovic, H. Sartelet, J.P. Castaigne, R. Béliveau, Influence of glioma tumour microenvironment on the transport of ANG1005 via low-density lipoprotein receptor-related protein 1, *Br J Cancer*, 105 (2011) 1697-1707.

[96] D.T. Wiley, P. Webster, A. Gale, M.E. Davis, Transcytosis and brain uptake of transferrin-containing nanoparticles by tuning avidity to transferrin receptor, *Proc Natl Acad Sci U S A*, 110 (2013) 8662-8667.

[97] J.N. Sarkaria, L.S. Hu, I.F. Parney, D.H. Pafundi, D.H. Brinkmann, N.N. Laack, C. Giannini, T.C. Burns, S.H. Kizilbash, J.K. Laramy, K.R. Swanson, T.J. Kaufmann, P.D. Brown, N.Y.R. Agar, E. Galanis, J.C. Buckner, W.F. Elmquist, Is the blood-brain barrier really disrupted in all glioblastomas? A critical assessment of existing clinical data, *Neuro-Oncology*, 20 (2017) 184-191.

[98] A.T. Kessler, A.A. Bhatt, Brain tumour post-treatment imaging and treatment-related complications, *Insights Imaging*, 9 (2018) 1057-1075.

[99] M. Osswald, J. Blaes, Y. Liao, G. Solecki, M. Gömmel, A.S. Berghoff, L. Salphati, J.J. Wallin, H.S. Phillips, W. Wick, F. Winkler, Impact of blood-brain barrier integrity on

tumor growth and therapy response in brain metastases, *Clin Cancer Res*, 22 (2016) 6078-6087.

[100] K.M. Tsoi, S.A. MacParland, X.-Z. Ma, V.N. Spetzler, J. Echeverri, B. Ouyang, S.M. Fadel, E.A. Sykes, N. Goldaracena, J.M. Kathis, J.B. Conneely, B.A. Alman, M. Selzner, M.A. Ostrowski, O.A. Adeyi, A. Zilman, I.D. McGilvray, W.C.W. Chan, Mechanism of hard-nanomaterial clearance by the liver, *Nat Mater*, 15 (2016) 1212-1221.

[101] C. Ghimire, H. Wang, H. Li, M. Vieweger, C. Xu, P. Guo, RNA nanoparticles as rubber for compelling vessel extravasation to enhance tumor targeting and for fast renal excretion to reduce toxicity, *ACS Nano*, 14 (2020) 13180-13191.

[102] A.K. Mohammad, J.J. Reineke, Quantitative detection of PLGA nanoparticle degradation in tissues following intravenous administration, *Mol Pharm*, 10 (2013) 2183-2189.

[103] A.A. van der Veldt, N.H. Hendrikse, E.F. Smit, M.P. Mooijer, A.Y. Rijnders, W.R. Gerritsen, J.J. van der Hoeven, A.D. Windhorst, A.A. Lammertsma, M. Lubberink, Biodistribution and radiation dosimetry of ¹¹C-labelled docetaxel in cancer patients, *Eur J Nucl Med Mol Imaging*, 37 (2010) 1950-1958.

[104] X. Yao, S.J. Levine, The long and winding road from GWAS to obstructive lung disease: Is there a role for LRP1?, *Am J Respir Cell Mol Biol*, 64 (2021) 279-280.

[105] R. Fan, D. Chuan, H. Hou, H. Chen, B. Han, X. Zhang, L. Zhou, A. Tong, J. Xu, G. Guo, Development of a hybrid nanocarrier-recognizing tumor vasculature and penetrating the BBB for glioblastoma multi-targeting therapy, *Nanoscale*, 11 (2019) 11285-11304.

[106] H. Gao, Z. Yang, S. Zhang, S. Cao, Z. Pang, X. Yang, X. Jiang, Glioma-homing peptide with a cell-penetrating effect for targeting delivery with enhanced glioma localization, penetration and suppression of glioma growth, *J Control Release*, 172 (2013) 921-928.

[107] A. Kadari, D. Pooja, R.H. Gora, S. Gudem, V.R.M. Kolapalli, H. Kulhari, R. Sistla, Design of multifunctional peptide collaborated and docetaxel loaded lipid nanoparticles for antiglioma therapy, *Eur J Pharm Biopharm*, 132 (2018) 168-179.

[108] H. Gao, J. Qian, S. Cao, Z. Yang, Z. Pang, S. Pan, L. Fan, Z. Xi, X. Jiang, Q. Zhang, Precise glioma targeting of and penetration by aptamer and peptide dual-functioned nanoparticles, *Biomaterials*, 33 (2012) 5115-5123.

[109] H. Hua, X. Zhang, H. Mu, Q. Meng, Y. Jiang, Y. Wang, X. Lu, A. Wang, S. Liu, Y. Zhang, Z. Wan, K. Sun, RVG29-modified docetaxel-loaded nanoparticles for brain-targeted glioma therapy, *Int J Pharm*, 543 (2018) 179-189.

[110] F.C. Lam, S.W. Morton, J. Wyckoff, T.-L. Vu Han, M.K. Hwang, A. Maffa, E. Balkanska-Sinclair, M.B. Yaffe, S.R. Floyd, P.T. Hammond, Enhanced efficacy of

combined temozolomide and bromodomain inhibitor therapy for gliomas using targeted nanoparticles, *Nat Commun*, 9 (2018) 1991.

[111] R. Cristofani, M. Montagnani Marelli, M.E. Cicardi, F. Fontana, M. Marzagalli, P. Limonta, A. Poletti, R.M. Moretti, Dual role of autophagy on docetaxel-sensitivity in prostate cancer cells, *Cell Death Dis*, 9 (2018) 889.

CHAPTER IV. Glioblastoma multicellular microtissue as a 3D *in vitro* evaluation tool of anti-cancer nano-therapeutics

This chapter was based on the following publication:

- **C Martins**, C Pacheco, C Moreira-Barbosa, A Marques-Magalhães, S Dias, M Araújo, MJ Oliveira, B Sarmiento. “Glioblastoma immuno-endothelial multicellular microtissue as a 3D in vitro evaluation tool of anti-cancer nano-therapeutics”. *Journal of Controlled Release*, 353, 77-95 (2023)

ABSTRACT

Despite being the most prevalent and lethal type of adult brain cancer, glioblastoma (GBM) remains intractable. Promising anti-GBM nanoparticle (NP) systems have been developed to improve the anti-cancer performance of difficult-to-deliver therapeutics, with particular emphasis on tumor targeting strategies. However, current disease modeling toolboxes lack close-to-native *in vitro* models that emulate GBM microenvironment and bioarchitecture, thus partially hindering translation due to poorly predicted clinical responses. Herein, human GBM heterotypic multicellular tumor microtissues (MCTMs) are generated through high-throughput 3D modeling of U-251 MG tumor cells, tissue differentiated macrophages isolated from peripheral monocytes, and brain microvascular primary endothelial cells. GBM MCTMs mimicked tumor spatial organization, extracellular matrix production and necrosis areas. The bioactivity of a model drug, docetaxel (DTX), and of tumor-targeted DTX-loaded polymeric NPs with a surface L-Histidine moiety (H-NPs), were assessed in the MCTMs. MCTMs cell uptake and anti-proliferative effect was 8- and 3-times higher for H-NPs, respectively, compared to the non-targeted NPs and to free DTX. H-NPs provided a decrease of MCTMs anti-inflammatory M2-macrophages, while increasing their pro-inflammatory M1 counterparts. Moreover, H-NPs showed a particular biomolecular signature through reduced secretion of an array of medium cytokines (IFN- γ , IL-1 β , IL-1Ra, IL-6, IL-8, TGF- β). Overall, MCTMs provide an *in vitro* biomimetic model to recapitulate key cellular and structural features of GBM and improve *in vivo* drug response predictability, fostering future clinical translation of anti-GBM nano-therapeutic strategies.

KEYWORDS

3D *in vitro* models; Glioblastoma; Multicellular tumor microtissues; Nanomedicine; Preclinical nanomedicine screening; Tumor microenvironment.

1. INTRODUCTION

Brain cancers account for approximately 300,000 new cases and 250,000 deaths per year worldwide, according to the Global Cancer Observatory 2020 [1]. Among brain cancers, glioblastoma (GBM) is the most common and deadly primary malignant tumor in adults, with an average median survival of less than 15 months [2]. Focusing on the tumor microenvironment (TME) of GBM, tumor-associated macrophages (TAMs) constitute the major non-neoplastic cell population, composed by brain-intrinsic microglia and monocyte (MON)-derived macrophages originated from peripheral blood [3]. In human primary GBMs, it is known that TAMs may constitute over 30-50% of the cell bulk [3-5] and are mostly derived from peripheral MONs (CCR2⁺/CX3CR1⁻) [6]. Despite macrophages can be polarized into either a M1 (pro-inflammatory; anti-tumor) or M2 (anti-inflammatory; pro-tumor) phenotype, or even present a continuum of phenotypes, TAMs in the GBM TME are mainly M2-like macrophages and key to support tumor proliferation, stemness, angiogenesis, immune suppression, among others [7]. Endothelial cells (ECs) constitute another crucial GBM TME non-neoplastic cell population, which typically represents 5-30% of the tumor cell bulk in GBM patient biopsies [8]. ECs are responsible for tumor angiogenesis, which is especially critical at the GBM perivascular niche, due to its essential role in tumor maintenance and growth, modulation of the immune pro-tumor response and therapeutic resistance [9].

The current GBM multimodal standard of care consists of surgical resection, followed by radiotherapy and temozolomide (TMZ) chemotherapy. Other chemotherapeutics such as docetaxel [10], paclitaxel [11], doxorubicin [12] and cisplatin [13] have also been exploited. However, due to the heterogeneity of the TME, tumor infiltration ability and highly inaccessible brain location of GBM, the majority of patients experiences minimal progression-free survival and an eventual tumor relapse [14]. Over recent years, GBM nanomedicines based on nanoparticle (NP) systems have been developed to improve the anti-cancer performance of difficult-to-deliver therapeutics by providing drug-protecting vehicles that (i) enhance drugs' suboptimal aqueous solubility, (ii) improve brain bioavailability through a more efficient blood-brain barrier (BBB) translocation, and (iii) provide a navigation function for tumor cell targeting or accumulation [15-17]. This ultimately leads to the increase of drug therapeutic efficacy against GBM, and fewer side effects [15].

Despite the promising advances made in the field of anti-GBM nanomedicines, unresolved hurdles have hampered their clinical translation, including the lack of appropriate GBM *in vitro* models that recapitulate the *in vivo* complexity of the disease

and accurately predict the therapeutic response in a clinical setting [18, 19]. Conventional 2D cell cultures fail to reassemble the tumor heterogeneous cell populations, intrinsic production of extracellular matrix (ECM), and dynamic cell-cell and cell-ECM interactions [19]. As an alternative, heterotypic multicellular tumor spheroids, giving rise to multicellular tumor microtissues (MCTMs), arose as 3D cell culture models that mimic better these physiological characteristics of solid tumors and offer a more realistic representation of the dynamic interplay between the TME components [20]. MCTMs recreate the intercellular and cell-ECM communication, tumor growth kinetics and metabolic rates, as well as gradients of pH, oxygen and nutrients [21, 22]. Importantly, they also recreate the higher therapeutic resistance of 3D tissues, compared to 2D cell models, while avoiding the ethical issues and interspecies discrepancies of *in vivo* studies [23]. Therefore, MCTMs are promising platforms to investigate newly developed anti-GBM drugs, namely those based on nanomedicines, due to their ability to reliably predict *in vivo* therapeutic responses [24].

In this work, a heterotypic MCTM model of GBM comprising U-251 MG tumor cells, macrophages differentiated *in situ* and isolated from healthy blood donors peripheral MONs, and brain microvascular primary ECs, was developed to test the efficacy of non-targeted *versus* tumor-targeted polymeric NPs loaded with a model chemotherapeutic, namely docetaxel (DTX). Although DTX is not part of the GBM current standard of care, it holds great potential as an alternative for the conventional temozolomide (TMZ) chemotherapy, due to a 150,000-times lower IC₅₀ (0.008 μ M for DTX *versus* 1250 μ M for TMZ, U-251 MG GBM cell line) [25] and avoidance of the TMZ-related tumor self-repair mechanisms [26]. Moreover, the pharmaceutical constrains of DTX, which have hampered its use in clinics, can be easily overcome by NP solutions: (i) depot systems that enhance the poor aqueous solubility of DTX [27-29], (ii) BBB shuttling systems to circumvent the lack of BBB permeation ability of DTX (blood plasma to cerebrospinal fluid ratio of 0.001-0.09 for DTX *versus* 0.2-0.3 for TMZ) [30, 31], and (iii) tumor targeting systems that surpass the typical low percentage of only 1% tumor uptake of the total administered dose [16]. Well-established polymeric NPs of a biocompatible and biodegradable poly(lactic-co-glycolic acid) (PLGA) matrix [32], modified with polyethylene glycol (PEG) for surface functionalization and brain-penetrating properties [33, 34], were chosen for DTX encapsulation. These tumor-targeted PLGA-PEG NPs were obtained by a straightforward functionalization of their polymeric matrix with L-Histidine (His), given the capacity of this L isomeric amino acid to recognize and bind the L-type amino acid transporter 1 (LAT1). LAT1 is extensively reported to be overexpressed by human GBMs, specifically by U-251 MG cells, and directly correlated with malignant progression [35-38]. The 3D *in vitro* model herein proposed is the first

one combining GBM cells with both primary macrophages and ECs of human and brain origin, respectively, instead of only GBM cells and macrophages (as cell line) or ECs [39-42], thus increasing its fidelity for preclinical testing. Besides U-251 MG cells, the model was designed to comprise the TAMs content of clinical GBMs (30-50%) [3-5], and a well-spread surface distribution of ECs, typical of highly angiogenic GBM tissues, and actively contributing to vasculature-related signaling alongside with the tumor cells' vasculogenic mimicry [43]. The GBM MCTM was optimized, followed by assessment of NP or free DTX uptake, anti-proliferative effect, macrophage polarization and biomolecular signature (cytokine array).

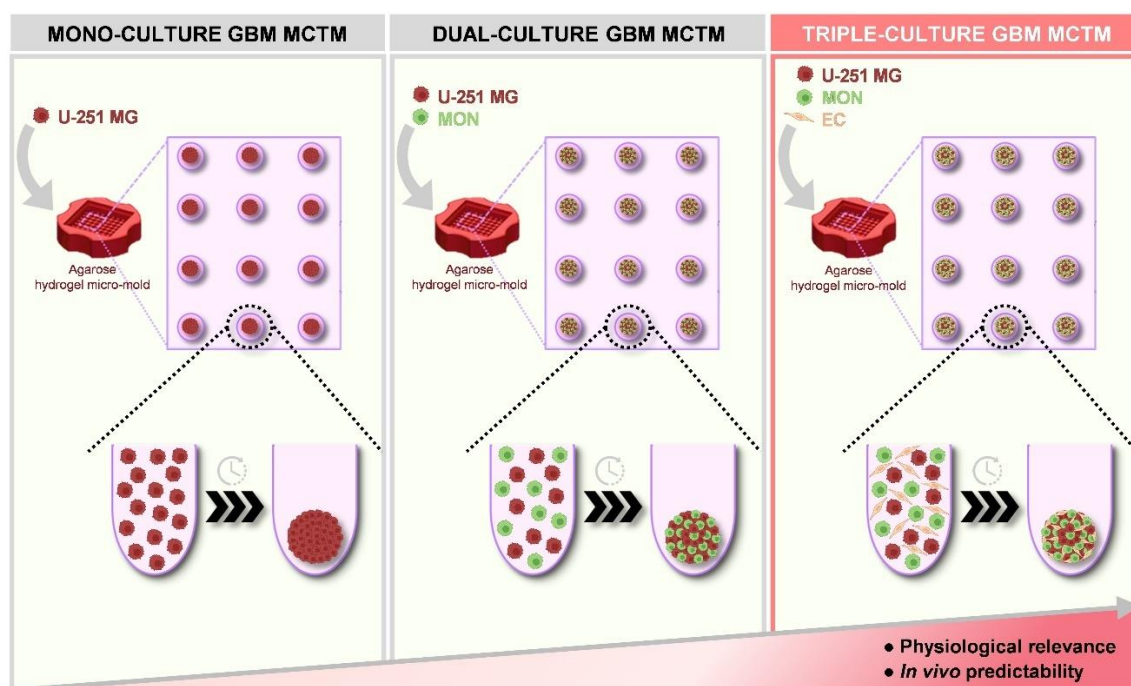


Figure 1. Schematic illustration of the proposed U-251 MG tumor cells, macrophages differentiated *in situ* from blood peripheral MONs and ECs triple-culture GBM MCTM. Created with BioRender.com.

2. MATERIALS AND METHODS

2.1. Materials

PLGA-COOH was kindly provided by Corbion (Netherlands). Resazurin, dimethylformamide, N-hydroxysuccinimide, N,N-dicyclohexylcarbodiimide, N,N-diisopropylethylamine, uranyl acetate, Poloxamer 407, diethyl ether, methanol,

dichloromethane, potassium bromide, paraformaldehyde, neutral buffered formalin, trypsin, xylene, graded alcohol series, hematoxylin and eosin (H&E) staining solution, Tris, EDTA-Na, sodium citrate, DAPI, L-Histidine (COOH-Histidine-BOC, termed COOH-His-BOC or simply His) and anti-fibronectin were acquired from Sigma-Aldrich (USA); DTX from LC Laboratories (USA); endothelial basal medium-2, high glucose Dulbecco's Modified Eagle medium and SeaKem® LE agarose from Lonza (Switzerland); acetonitrile, Versene and Accutase® from Thermo Fisher Scientific (USA); Triton X-100 from Spi-Chem (USA); RosetteSep-Human Monocyte Enrichment Cocktail from StemCell Technologies (France); RPMI1640 medium, Alexa Fluor 488-anti rabbit and Alexa Fluor 594-anti mouse were purchased from Invitrogen (Belgium); fetal bovine serum and penicillin-streptomycin from Gibco (USA); PLGA-PEG(2K)-NH₂ and mPEG(2K)-PLGA were acquired from Ruixibio (China). PLGA-FKR648 was acquired from Akina (USA). The total protein content analysis kit was acquired from Bio-Rad (Dc Protein kit, USA). Anti-Vimentin (V9) was acquired from Santa Cruz Biotechnology Inc (USA). Anti-CD68 and anti-CD31 were purchased from Agilent (USA). Anti-CD14/APC and anti-CD86/FITC were obtained from ImmunoTools (Germany). Anti-206/PE was purchased from BioLegend (USA). Anti-CD163/PE was acquired from BD Biosciences (USA). The X-junction microfluidic chip (Quartz Droplet Junction Chip, 190 µm etch depth) was acquired from Dolomite (UK). Amicon Ultra-15 centrifugal filter units (molecular weight cutoff of 100K) were acquired from Merck Millipore (USA). U-251-MG cells were purchased from Biochrom (Germany); human microvascular primary endothelial cells from Innoprot (Spain).

2.2. Ethics statement

MONs were isolated from surplus buffy coats from healthy blood donors, kindly provided by the Immunohemotherapy Department of Centro Hospitalar Universitário São João (CHUSJ), Porto, Portugal. Procedures were approved by the Centro Hospitalar Universitário São João Ethics Committee (protocol 90/19). Blood donors provided informed written consent that the byproducts of their blood collections could be used for research purposes.

2.3. Cells and culturing

U-251 MG GBM cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (FBS, 10% v/v) and penicillin-

streptomycin (1% v/v). Brain microvascular primary ECs were maintained in endothelial basal medium-2 supplemented with FBS (5% v/v) and penicillin-streptomycin (1% v/v). Human peripheral MONs were isolated from buffy coats from healthy blood donors (using a RosetteSep-Human Monocyte Enrichment Cocktail, following the manufacturer's instructions), and maintained in RPMI1640 medium with FBS (10% v/v) and penicillin-streptomycin (1% v/v). Cell cultures were kept in an incubator (CellCulture CO₂ incubator, ESCO GB Ltd., UK) at 37 °C with 5% CO₂ and 95% relative humidity. Cell culture medium was changed every 2–3 days.

2.4 Glioblastoma multicellular tumor microtissue assembly

MCTM spheroids were formed using commercially available molds (3D Petri Dish®, MicroTissues, USA). Agarose was dissolved at 2% (w/v) in 0.9% NaCl (w/v) and casted into 3D Petri Dish® molds to form hydrogel micro-molds with 81 homogenous circular recesses (each micro-mold was filled with 190 µL of medium and placed in a well of a 12-well plate filled with 2 mL of medium). For mono-culture MCTMs, 0.0025x10⁶, 0.005x10⁶ or 0.01x10⁶ U-251 MG cells per spheroid were added to the micro-molds (190 µL) and allowed to settle for 30 min, before adding complete DMEM medium to each well (2 mL). For dual- and triple-culture MCTMs, the U-251 MG cell number was kept, but adding human peripheral MONs (1:1 or 1:2 ratio of U-251 MG:MON, respectively), or human peripheral MONs plus brain microvascular primary ECs (1:2:0.2 ratio of U-251 MG:MON:EC), respectively. MCTM macrophage differentiation from human peripheral MONs occurred spontaneously *in situ* in absence of colony-stimulating factor or other exogenous factors. Medium was replaced every two days.

2.5. Glioblastoma multicellular tumor microtissue average diameter, metabolic activity and circularity characterization

At determined time-points (1, 4 and 7 days), MCTMs images were acquired by brightfield microscopy (ZOE™ Fluorescent Cell Imager, Bio-Rad Laboratories, USA), and MCTMs average diameter (two diameter measurements randomly evaluated in three different MCTMs per micro-mold core) and circularity estimated by the open-source software ImageJ (NIH, USA). For metabolic activity measurements at days 1, 4 and 7, MCTM micro-molds were incubated with DMEM complete medium containing resazurin (20% v/v; 190 µL per micro-mold, and 2 mL per each 12 well-plate well where the micro-mold was placed) and incubated in the dark at 37 °C for 2 h. Following incubation, the

fluorescence of a supernatant aliquot (100 μ L) was measured at the excitation and emission wavelengths of 530 nm and 590 nm, respectively, using a SynergyMx™ MultiMode microplate reader (BioTek™, USA). All samples were analyzed at least in triplicate.

2.6. Glioblastoma multicellular tumor microtissue histological analysis

At days 1, 4 and 7, medium was removed from micro-molds and MCTMs fixed in neutral buffered formalin (10% v/v; 190 μ L per micro-mold, and 2 mL per each 12 well-plate well where the micro-mold was placed) for 24 h at room temperature (RT). Afterwards, the micro-molds were washed with phosphate buffered saline (PBS), and a sealing layer of agarose (2% w/v) dissolved in NaCl (0.9% w/v) was added to the top of each micro-mold. The micro-molds were then embedded in paraffin using an automated embedding system (Thermo Scientific™ STP 120 Spin Tissue Processor, USA). Paraffin embedded samples were sectioned into 3 μ m sections, deparaffinized in xylene and rehydrated in graded alcohol series. Staining was performed with H&E.

2.7. Glioblastoma multicellular tumor microtissue immunofluorescence analysis

At day 7, fixed paraffin MCTMs embedded samples, prepared as described above, were sectioned in 5 μ m sections, followed by deparaffinization in xylene and rehydration in sequentially decreasing ethanol concentrations. Before staining, to retrieve the antigens, sections were treated with Tris-EDTA (10 mM Tris, 1 mM EDTA-Na, pH 9) or sodium citrate (10 mM sodium citrate, pH 6) buffers, at 96 °C for 30 min. Next, samples were washed 3-times by immersing sections in PBS (pH 7.4), under agitation (60 rpm) for 5 min. To permeabilize the samples, sections were immersed in Triton X-100 (0.25% v/v) at 60 rpm for 10 min and then washed 3-times, as described above. Before adding the antibodies, samples were blocked with FBS in PBS (10% v/v) for 1 h at RT. Primary human anti-vimentin (1:50), anti-CD68 (1:100), anti-fibronectin (1:200) or anti-CD31 (1:20) antibodies were added to the samples and incubated overnight, in a wet chamber, at 4 °C. Samples were washed 3-times, and incubated with species specific secondary antibodies (at 1:400 dilution) and DAPI (0.5 μ g mL⁻¹) in a wet chamber, in the dark, for 1 h at RT. Sections were then washed 3-times in PBS, mounted with mounting media and dried overnight at RT in the dark. Sections were imaged with a SP5 confocal microscope

(Leica Microsystems, Germany) and image analysis was performed with the LAS AF Lite software (Leica Microsystems, Germany).

2.8 Chemical synthesis and characterization of PLGA-PEG-His

For the manufacture of tumor-targeted PEGylated NPs (H-NPs), a targeting polymeric conjugate based on His grafted PLGA-PEG polymer (PLGA-PEG-His) was synthesized previously to NP production. This procedure was adapted from a protocol described elsewhere [44, 45]. A solution of COOH-His-BOC (MW ~ 0.255 kDa, 30.6 mg, 120 μ mol), N-hydroxysuccinimide (41.4 mg, 360 μ mol) and N,N-dicyclohexylcarbodiimide (74.3 mg, 360 μ mol) in 9 ml anhydrous dimethylformamide was treated with PLGA-PEG(2K)-NH₂ (Mn ~ 17 kDa, 360 mg, 22 μ mol) and N,N-diisopropylethylamine (37.8 mg, 293 μ mol) in 5 ml anhydrous dimethylformamide. The reaction was stirred for 24 h at room temperature (RT) under inert atmosphere. The resultant mixture was poured into cold diethyl ether (80 mL), washed 3-times with methanol (40 mL) and dried under vacuum overnight. The polymeric conjugate was re-dissolved and filtered in 15 mL anhydrous dichloromethane, and dried under vacuum overnight. The obtained polymeric conjugate was termed PLGA-PEG-His-BOC. A selective carbamate hydrolysis was further employed to remove the -BOC protecting group. Here, trifluoroacetic acid (455 μ L, 5.9 mmol) was added to PLGA-PEG-His-BOC (150 mg, 0.009 mmol) dissolved in anhydrous dichloromethane (16 mL), and the reaction mixture was stirred overnight at RT under inert atmosphere. The reaction mixture was then extracted and neutralized 3-times with a 1 M NaOH aqueous solution (8.5 mL). The organic layer was dried under vacuum overnight, washed with cold diethyl ether (10 mL) and dried again. To characterize PLGA-PEG-His, FTIR spectra were recorded with a FTIR-RAMAN Perkin Elmer 2000 (Perkin Elmer, USA) spectrometer using the potassium bromide (KBr) pellet method. Each pellet was prepared by blending 2 mg of the polymeric conjugate with 200 mg KBr. After a 5-min purge of the sample chamber with N₂, IR spectra were immediately recorded by accumulation of 200 interferograms at a 4 cm⁻¹ spectral resolution over the range from 400 to 8,000 cm⁻¹ with background subtraction.

2.9 Nanoparticle manufacture

The microfluidic protocol for non-targeted PEGylated NP (mPEG NPs) manufacture was adapted from elsewhere [46-48]. Briefly, the organic phase consisted of a polymer solution at 40 mg mL⁻¹. The total polymer concentration was based on 95% PLGA-COOH

backbone (Mn ~ 35 kDa), and 5% mPEG(2K)-PLGA (Mn ~ 17 kDa) or the previously synthesized PLGA-PEG-His, and DTX at 2 mg mL⁻¹ in acetonitrile (for preliminary assays in 2D cultures, 10% and 20% PLGA-PEG-His were also assessed). Whereas, the aqueous phase consisted of a Poloxamer 407 aqueous solution (1% w/v). Both phases were injected into a X-junction microfluidic chip with a three-inlet layout, in which the organic (0.75 mL min⁻¹) and aqueous (2.25 mL min⁻¹) phases were injected through the central and the two outer inlets, respectively. Each experiment was conducted until 20 mg of NPs were produced (equivalent to a 40 s run time). NPs were washed 3-times with water and recovered by ultrafiltration using Amicon Ultra-15 centrifugal filter units. For the obtention of fluorescent labeled NPs, 10% of the backbone polymer content of the organic phase was replaced by PLGA-FKR648 (Mn ~ 20 kDa).

2.10 Nanoparticle characterization

NPs were characterized for their average size and polydispersity index (PDI) by dynamic light scattering (DLS), and zeta-potential through laser Doppler anemometry (LDA), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). For these measurements, samples were diluted (1:100) in an ionic solution of 10 mM sodium chloride. The morphological features of the NPs were analyzed by transmission electron microscopy (TEM) with a JEOL JEM 1400 microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 120 kV. Images were digitally recorded using a Gatan SC 1100 ORIUS CCD camera (Gatan, Warrendale, PA). Samples were prepared by dropping 10 µL of NPs suspension onto a 300-mesh nickel grid, and stained with uranyl acetate. The amount of loaded DTX was directly quantified by high-performance liquid chromatography (HPLC) in order to determine the association efficiency (AE) and loading degree (LD) of the developed formulations by dissolving the NPs in 2 mL dimethylformamide. The calculation of AE and LD followed Equation (1) and (2), respectively.

$$AE (\%) = \frac{\text{Quantified loaded mass of cargo}}{\text{Initial mass of cargo}} \times 100 \quad (1)$$

$$LD (\%) = \frac{\text{Quantified loaded mass of cargo}}{\text{Total mass of NPs}} \times 100 \quad (2)$$

The quantification of DTX was performed by reversed-phase HPLC with ultraviolet detection using a Merck-Hitachi LaChrom® HPLC system (Merck, USA). A Symmetry C18 column (5 µm, 125×4 mm; Milford, USA) with a LiChrospher 100 RP-18 guard

column (Merck, USA) was used as stationary phase. Chromatographic runs were performed using a gradient method at a flow rate of 1 mL/min. Initial stage was 10% acetonitrile and 90% water from 0 to 5 min; from 5 to 14 min, the conditions were defined as 60% acetonitrile and 40% water; final stage returned to 10% acetonitrile and 90% water from 14 to 17 min. The injection volume was 50 μ L per sample, analyzed at a detection wavelength of 227 nm.

2.11 Glioblastoma multicellular tumor microtissue uptake of nanoparticle formulations

For preliminary assays in 2D cultures, 0.25×10^6 U-251 MG (6-well plate) cells were seeded and allowed to attach overnight, washed with PBS, and PLGA-FKR648 labeled NPs in complete medium ($25 \mu\text{g mL}^{-1}$ NPs, corresponding to approximately $0.5 \mu\text{M}$ DTX dose) were added for 4 h at 37°C . Afterwards, cells were washed twice with PBS, detached with trypsin ($250 \mu\text{L}$), and $500 \mu\text{L}$ media were added to each well. Cells were subsequently washed with PBS, fixed with 2% paraformaldehyde (PFA) for 20 min at RT, washed again, and placed into cytometer tubes for further analysis. Cells were analyzed using an Accuri C6 cytometer (BD Biosciences, USA). For MCTM assays, at day 1, each micro-mold was washed with PBS, and PLGA-FKR648 labeled NPs in complete medium ($100 \mu\text{g mL}^{-1}$ NPs, corresponding to approximately $2 \mu\text{M}$ DTX dose; $190 \mu\text{L}$ per micro-mold, and 2 mL per each 12 well-plate well where the micro-mold was placed) were added for 4 h or 12 h at 37°C . After, medium was removed and the spheroids were transferred to a 15 mL plastic tube. Per tube, spheroids were incubated with Versene ($400 \mu\text{L}$) for 15 min, and Accutase[®] ($200 \mu\text{L}$) for 30 min, at 37°C , followed by vigorous pipetting for dissociation into single cells. Isolated cells were afterwards washed twice with PBS, fixed with 2% PFA for 20 min at RT, washed again, and placed into cytometer tubes for further analysis. Cells were analyzed using an Accuri C6 cytometer (BD Biosciences, USA). Cells incubated with only cell culture medium were used as the unstained control, and all cell constituents were grouped in a single gate. Mean fluorescence intensity (MFI) was measured for at least 10,000 viable plus single cell gated events per sample, and all data was processed with FlowJo software (Tree Star, USA). Experiments were performed at least in triplicate.

2.12 Glioblastoma multicellular tumor microtissue anti-proliferative effect of drug and nanoparticle formulations

At day 1, each MCTM micro-mold was washed with PBS, and different concentrations of drug or NP samples (0.0045 to 4.5 μ M, in regard to the drug) in medium (190 μ L per micro-mold, and 2 mL per each 12 well-plate well where the micro-mold was placed) were added and incubated up to 48 h and 96 h, at 37 °C. This setup allowed to mimic the clinical situation, when the treatment is applied while the tumor is evolving. For the dynamic-like boost treatment, the same procedure was followed, but drug or NP samples at a 4.5 μ M concentration were added for 12 h, micro-molds washed 3-times with PBS, and incubated with fresh medium up to 96 h. At each time point, the medium was removed and the spheroids transferred to a 15 mL plastic tube. Per tube, spheroids were incubated with Versene (400 μ L) for 15 min, and Accutase® (200 μ L) for 30 min, at 37 °C, followed by vigorous pipetting to dissociate the spheroids into single cells. The cells were washed 3-times with PBS and resuspended in 200 μ L medium, from which 30 μ L were transferred to 96-well plates and allowed to attach overnight. At the following day, resazurin (200 μ L, 20% v/v) was added to each well and incubated for 4 h at 37 °C in the dark. After, fluorescence was measured at the excitation and emission wavelengths of 530 nm and 590 nm, respectively, using a SynergyMx™ MultiMode microplate reader (BioTek™, USA). Experiments were performed at least in quadruplicate, and all data was normalized in regard to the positive control (medium), which was considered as 100% metabolic activity. Graphical data for cell metabolic activity was referenced to a threshold of 70% cell viability according to the ISO 10993–5 standard [49].

2.13 Glioblastoma multicellular tumor microtissue macrophage polarization after treatment with drug and nanoparticle formulations

At day 1, each MCTM micro-mold was washed with PBS, and different concentrations of drug or NP samples (0.0045 μ M, in regard to the drug) in medium (190 μ L per micro-mold, and 2 mL per each 12 well-plate well where the micro-mold was placed) were added and incubated for 48 h, at 37 °C. Medium was removed and the spheroids transferred to a 15 mL plastic tube. Per tube, spheroids were incubated with Versene (400 μ L) for 15 min, and Accutase® (200 μ L) for 30 min, at 37 °C, followed by vigorous pipetting to dissociate the spheroids into single cells. The cells were washed with PBS,

resuspended in PBS containing 2% FBS (50 μ L) with anti-CD14/APC (1:25), anti-CD86/FITC (1:25), anti-CD163/PE (1:10) or anti-206/PE (1:50) antibodies, and incubated in the dark for 40 min at 4 °C. After incubation, cells were washed twice and resuspended in PBS containing 2% FBS, fixed with 2% PFA for 30 min at RT, washed again, and placed into cytometer tubes for further analysis. Samples were analyzed with a BD FACSCanto™ II flow cytometer (BD Biosciences, USA). MFI was measured for at least 10,000 viable plus single cell gated events per sample, and all data was processed with FlowJo software (Tree Star, USA). Experiments were performed at least in triplicate.

2.14 Glioblastoma multicellular tumor microtissue cytokine secretion levels after treatment with drug and nanoparticle formulations

Each MCTM micro-mold was exposed to a 0.0045 μ M DTX equivalent dose for 48 h at 37 °C and 5% CO₂, at day 1. MCTM supernatants were collected, centrifuged at 300 *g* for 5 min, frozen at -20 °C and further analyzed through ELISA by EVE Technologies (Canada). The supernatant concentration of IFN- γ , IL-1 β , IL-1Ra, IL-6, IL-8, TGF- β , TNF- α and MCP-1 was measured and normalized towards the total protein concentration (total protein content estimated according to manufacturer's instructions).

2.15 Statistical analysis

Results are represented as mean $\pm$ standard deviation from a minimum of three independent experiments. Statistical analysis was performed by 2-tailed paired Student's t-test, or two-way analysis of variance (ANOVA) followed by a post hoc test (Dunnett's test), to compare experimental groups. Differences were considered significant at * p <0.05, ** p <0.01, or *** p <0.001. Statistical analysis was performed with the software GraphPad Prism 9 (GraphPad Software, USA).

3. RESULTS AND DISCUSSION

3.1 Optimization and characterization of the heterotypic glioblastoma multicellular tumor microtissue

In this work, a novel GBM-recapitulating *in vitro* model was developed based on a 3D heterotypic MCTM spheroid. The main goal of this model was to closely mimic the tumor 3D structure and interactions between three of the most representative cellular components of the GBM TME – tumor cells, macrophages derived from peripheral MONs, and ECs – to further allow the testing of efficacy and biocompatibility of nanomedicines with chemotherapeutic, immunotherapeutic and/or anti-angiogenic roles. The GBM MCTMs were assembled using microwell array technology, by performing the cell seeding in high-throughput 81-well agarose hydrogel micro-molds [50]. The agarose hydrogel inhibits cell-surface adhesion, thus inducing cell-cell interaction and aggregation, and ultimately resulting in large-scale production of GBM MCTM spheroids with excellent reproducibility in shape and size [51]. The optimization of the GBM MCTM started with a U-251 MG tumor core mono-culture, followed by step-by-step addition of peripheral MONs and ECs (**Figure 1**).

3.1.1 Mono-culture glioblastoma multicellular tumor microtissue

To assemble the U-251 tumor mono-culture MCTMs, three different cell densities were seeded on agarose micro-molds, namely 2,500, 5,000 and 10,000 cells per MCTM. Brightfield microscopy demonstrated that, at day 1, loose cell aggregates were obtained for all cell densities (**Figure 2A**). Yet, over time and up to day 7, the cell aggregates became spherical-shaped 3D structures with a high level of compactness. For the two higher cell densities, either the MCTM metabolic activity regressed over time (reduction of 10,000 resazurin fluorescence units for 10,000 cells per MCTM; **Figure 2B**) or a decrease in average size was observed (100 μm and 200 μm regression for 5,000 and 10,000 cells per MCTM, respectively; **Figure 2C**) from day 1 to day 7. The decrease in size was also noticeable by brightfield microscopy at day 7 (**Figure 2A**), alongside with the appearance of irregular protuberances on the MCTM surface. This suggested that these high cell densities exhausted the medium nutrients and provoked cell death *per se*, which disturbed the intercellular organization [52]. For the lowest cell density, the metabolic activity was, however, kept relatively constant over the culture period, as well

as the average size (approximately 300-350 nm), which is in accordance with the typical slow proliferation rates of compact cellular tissues [53]. Regarding MCTM circularity, no biologically relevant differences were found among the different initial cell densities at day 1, 4 and 7 (values averaging 0.9; **Figure 2D**). H&E histological studies corroborated the cell looseness and lack of intercellular organization of the MCTMs at day 1, which was gradually replaced by highly organized cell compactness up to day 7 (**Figure 2E**). Focal sites of necrosis were observed for all initial cell densities from day 4, without a well-organized necrotic core, as in accordance with what has been reported for glioma cellular tissues of around 300 μm average size [22, 54]. The focal sites of necrosis are associated with localized deprivation of oxygen and nutrients, leading to the formation of hypoxic areas with a certain degree of cell death, characteristic of tumor tissues [50]. Considering these findings, and to minimize the impact on cell viability, we opted to fix the U-251 initial cell density as 2,500 cells per MCTM. Higher cell densities, with a higher number of cells per micro-mold area, led to a metabolic activity and/or average size regression over time, suggesting spatially-induced cell death and uncontrolled intercellular disorganization. 2,500 cells per MCTM kept a constant metabolic activity and average size over time, while still retaining focal sites of tumor-representative necrosis.

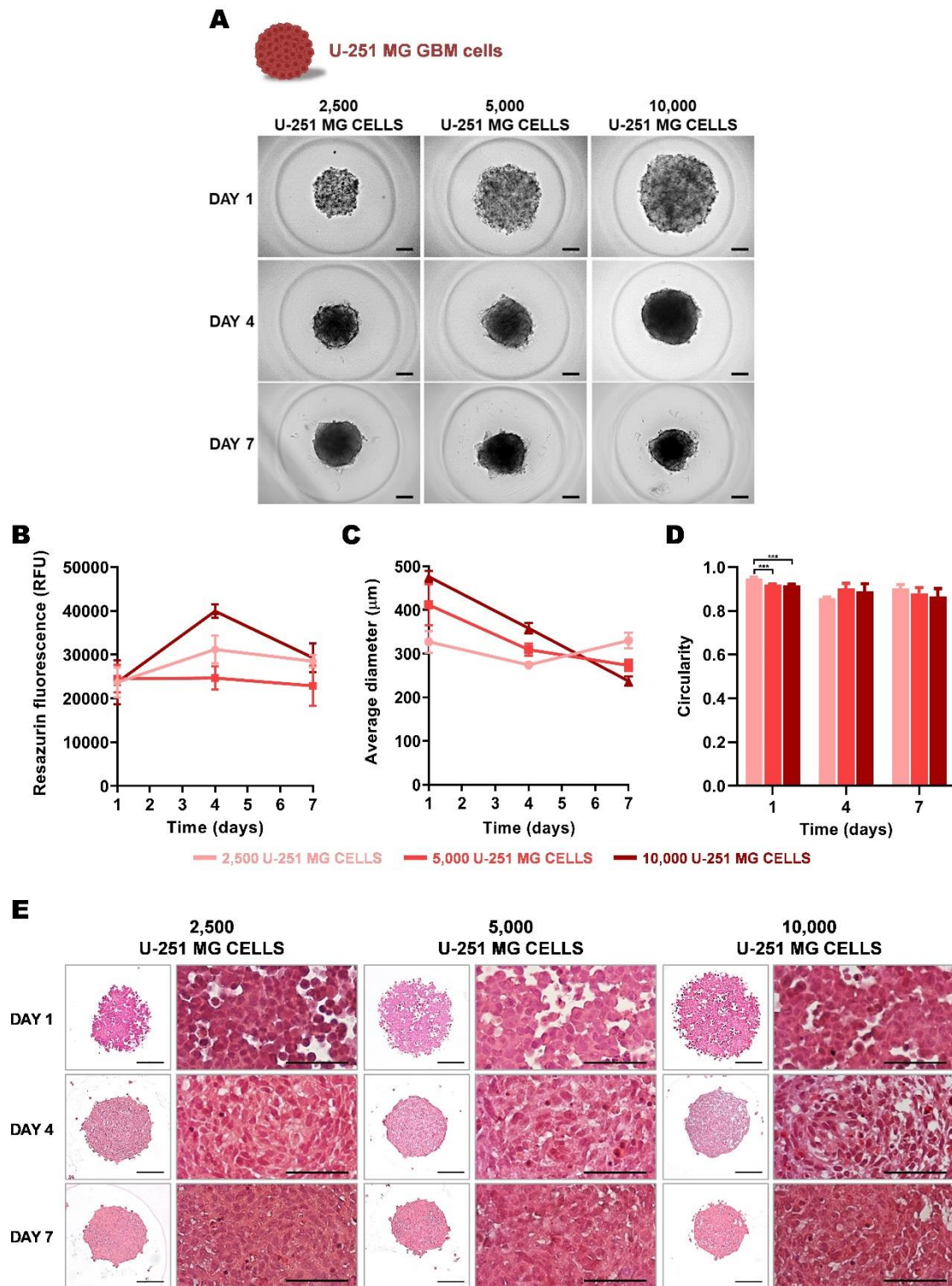


Figure 2. Human U-251 tumor mono-culture GBM MCTMs formation and characterization along 7 days. A) Over time brightfield microscopy images of the morphology of mono-culture GBM MCTMs prepared with three different initial cell densities. Scale bar represents 100 μm. B) MCTMs metabolic activity, C) average diameter, and D) circularity progression over time as function of the initial seeding cell

density. E) H&E staining of the formed MCTMs (20x magnification left panel, 100 μm scale bar; 40x magnification right panel, 50 μm scale bar). Necrotic areas are characterized by a white color. Statistical comparison of the lowest initial seeding cell density with the other conditions within each evaluation day. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3.1.2 Dual-culture glioblastoma multicellular tumor microtissue

To assemble the dual-culture MCTMs, donor-isolated peripheral MON were cultured with U-251 tumor cells in two different ratios, namely 1:1 and 1:2 (U-251:MON).

At day 1, similarly to what was observed by brightfield microscopy for mono-culture MCTMs, dual-culture MCTMs presented loose cell aggregates for both tested ratios (**Figure 3A**). However, over time and up to day 7, a high cell compactness and spherical-shaped 3D organization was developed. For both ratios, the MCTM metabolic activity regressed over time from day 1 to day 7 (reduction of approximately 10,000 resazurin fluorescence units; **Figure 3B**). This might be attributed to the compromised viability of excess MONs that were not able to infiltrate the MCTM and progress in the regular immunological process by differentiating into macrophages *in situ*. Another explanation could be the mechanical pressure imposed by tumor cells on MONs due to their higher proliferation rate or to their ability to secrete factors that might impair MONs differentiation, hence leading to immune cell death [55]. For both cell ratios, the average size of the MCTMs was kept relatively constant over time (approximately 350 μm ; **Figure 3C**), and similar to the mono-culture system. In what concerns circularity, no biologically relevant differences were found between the two ratios and in relation to the mono-culture MCTMs at day 1, 4 and 7 (values averaging 0.8-0.9; **Figure 3D**). H&E histological analysis demonstrated cell looseness, structural disorganization and significant surface protuberances at day 1, which were replaced by a highly organized spherical shaped structure over time, with significant cell compactness, particularly at day 7 (**Figure 3E**). Despite not forming a highly organized necrotic core, the size of focal sites of necrosis increased from day 1 to day 7 for both ratios, which is characteristic of the tumor spheroid growing process [56].

Altogether, we opted to fix the cell ratio of the dual-culture GBM MCTMs as 1:2 (U-251:MON). This ratio allowed to keep their metabolic activity and average size relatively constant over time, while also presenting a certain degree of tumor-representative necrosis and, possibly, a higher number of macrophages per microtissue.

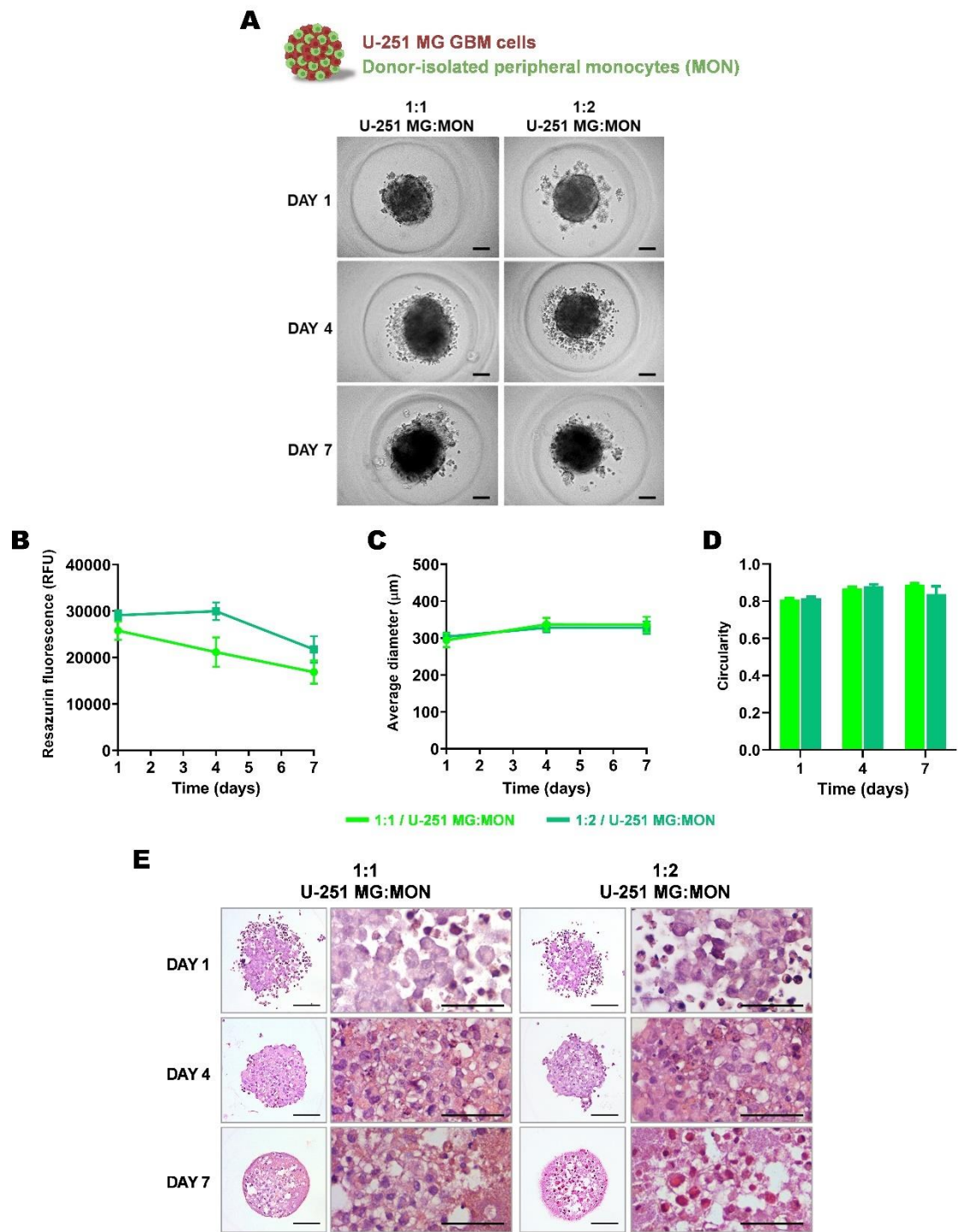


Figure 3. Human U-251 tumor cells and macrophages, differentiated from donor-isolated peripheral MONs, dual-culture GBM MCTMs formation and characterization along 7 days. A) Over time brightfield images of the dual-culture GBM MCTMs morphology, considering two different initial cell ratios (1:1 and 1:2). Scale bar represents 100 μm. B) MCTMs metabolic activity, C) average diameter, and D) circularity progression over time as function of the initial seeding cell ratio. E) H&E staining of the formed MCTMs (20x magnification left panel, 100 μm scale bar; 40x magnification right panel, 50 μm scale

bar). Necrotic areas are characterized by a white color.

3.1.3 Triple-culture glioblastoma multicellular tumor microtissue

In order to assemble the triple-culture MCTMs, brain primary ECs were co-cultured with U-251 tumor cells and donor-isolated peripheral MONs at a 1:2:0.2 ratio (U-251:MON:EC).

As previously observed by brightfield microscopy for mono- and dual-culture MCTMs, triple-culture MCTMs presented highly disorganized and loose cell aggregates at day 1 (**Figure 4A**). Over time and up to day 7, the triple-culture MCTMs acquired a densely organized, compact and tightly packed core, surrounded by a looser surface cell layer. This reassembles the *in vivo* scenario, as GBM often presents a compact lesion core, surrounded by a more diffusive tumor invasive margin [57]. Compared to mono- and dual-culture MCTMs, triple-culture MCTMs presented a higher metabolic activity (50,000-60,000 *versus* 20,000-30,000 resazurin fluorescence units; **Figure 4B**) and average size (approximately 500 μm *versus* 350 μm ; **Figure 4C**), which might be explained by the higher cell proliferation and the presence of the looser surface cell layer, respectively. Regarding circularity, no biologically relevant differences were found between the mono-culture, dual-culture and triple-culture MCTMs (values averaging 0.8-0.9; **Figure 4D**). H&E histological analysis showed a similar pattern to dual-culture MCTMs, with considerable cell looseness and disorganization at day 1, but a highly organized and packed structure at day 7 (**Figure 4E**). Moreover, the size of focal sites of necrosis within the MCTM structure increased over time from day 1 to day 7, which most probably results from the tumor spheroid growing process [56].

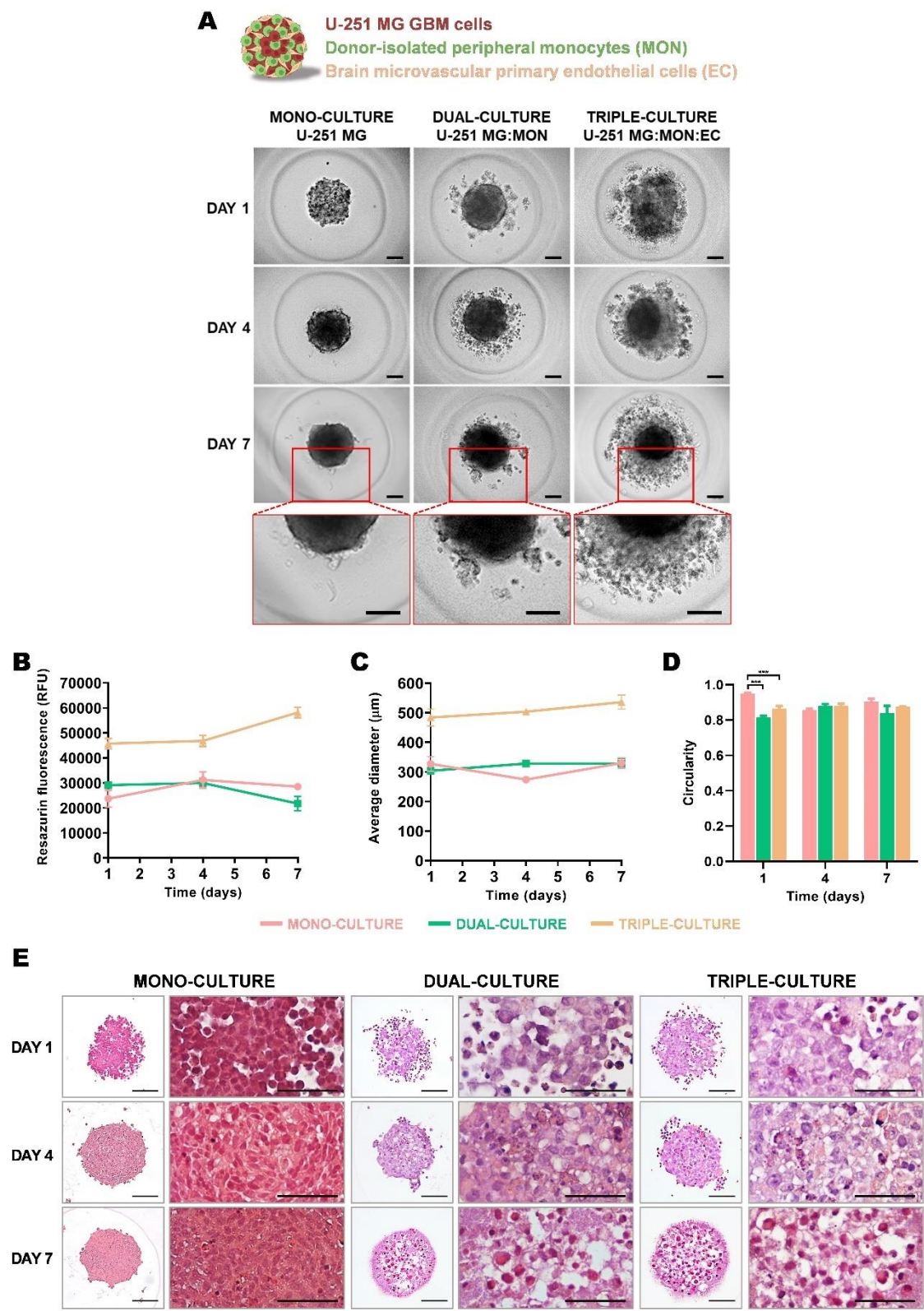


Figure 4. Human U-251 tumor cells, macrophages differentiated from donor-isolated peripheral MONs, and brain microvascular primary ECs triple-culture GBM MCTMs formation and characterization along 7 days. A) Over time brightfield images of the triple-culture GBM MCTMs morphology. Scale bar represents 100 μm. B) MCTMs metabolic

activity, C) average diameter, and D) circularity progression over time as function of the initial seeding cell ratio. E) H&E staining of the formed MCTMs (20x magnification left panel, 100 μm scale bar; 40x magnification right panel, 50 μm scale bar). Necrotic areas are characterized by a white color. Statistical comparison of triple-culture GBM MCTMs with the other MCTM types within each evaluation day. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3.2 Glioblastoma multicellular tumor microtissue cell co-localization and production of extracellular matrix

Immunofluorescence studies were performed to investigate the MCTM location of U-251 MG tumor cells (vimentin), macrophages (CD68) and ECs (CD31). Since abundant production of ECM-representative fibronectin has been found in GBM biopsy samples and correlated with disease progression [58], MCTM location of this ECM constituent was also investigated.

For the mono-culture GBM MCTMs, a homogeneous and spherical distribution of the vimentin tumor marker was observed (**Figure 5**). Moreover, the MCTMs presented massive intrinsic production of fibronectin, specially located at the tissue core. This is consistent with the reported capacity of GBM cells to synthesize and secrete their own ECM with high level of cross-linking, making the tumor tissue more rigid compared to the normal brain [59]. Regarding the dual-culture GBM MCTMs, the CD68 macrophage marker was predominately co-localized with the MCTM surface. This suggested a poor penetration of the macrophages into deeper regions of the MCTM, and a preferential accumulation at the MCTM surface, which has been observed in other tumor cell-macrophage co-culture spheroid models [50]. Whereas, the vimentin tumor marker was well-spread throughout the 3D structure, and the MCTM maintained the capacity to secrete its own ECM fibronectin. For the triple-culture MCTMs, the vimentin tumor marker pattern was similar to the mono- and dual-culture MCTMs, presenting spreading throughout the overall structure of the tissue, and with a well-defined distribution at the MCTM periphery. Similarly, it was found a homogeneous spreading of the CD31 EC marker throughout the MCTMs. Interestingly, compared to the dual-culture MCTMs, the CD68 macrophage marker presented a more spread distribution across the triple-culture MCTM structure, and not only an accumulation at the MCTM surface. Furthermore, the intensity of ECM fibronectin was considerably reduced in the triple-culture MCTM model, compared to mono- and dual-culture MCTMs. Since ECs are reported to be one of the major cell population responsible for ECM-degrading proteolytic activity within the GBM TME [60] and are exclusively present in the triple-culture MCTM model, it is hypothesized

that these cells contributed to the partial elimination of ECM-representative fibronectin, thus allowing macrophages to migrate towards deeper regions of the MCTMs. Further studies will be performed to confirm such hypothesis. This recapitulates the *in vivo* scenario in a more precise manner, compared to macrophage confinement to the MCTM surface, since there is evidence that these cells are located not only at the tumor periphery (invasive margin), but also intratumorally (tumor bulk) [4].

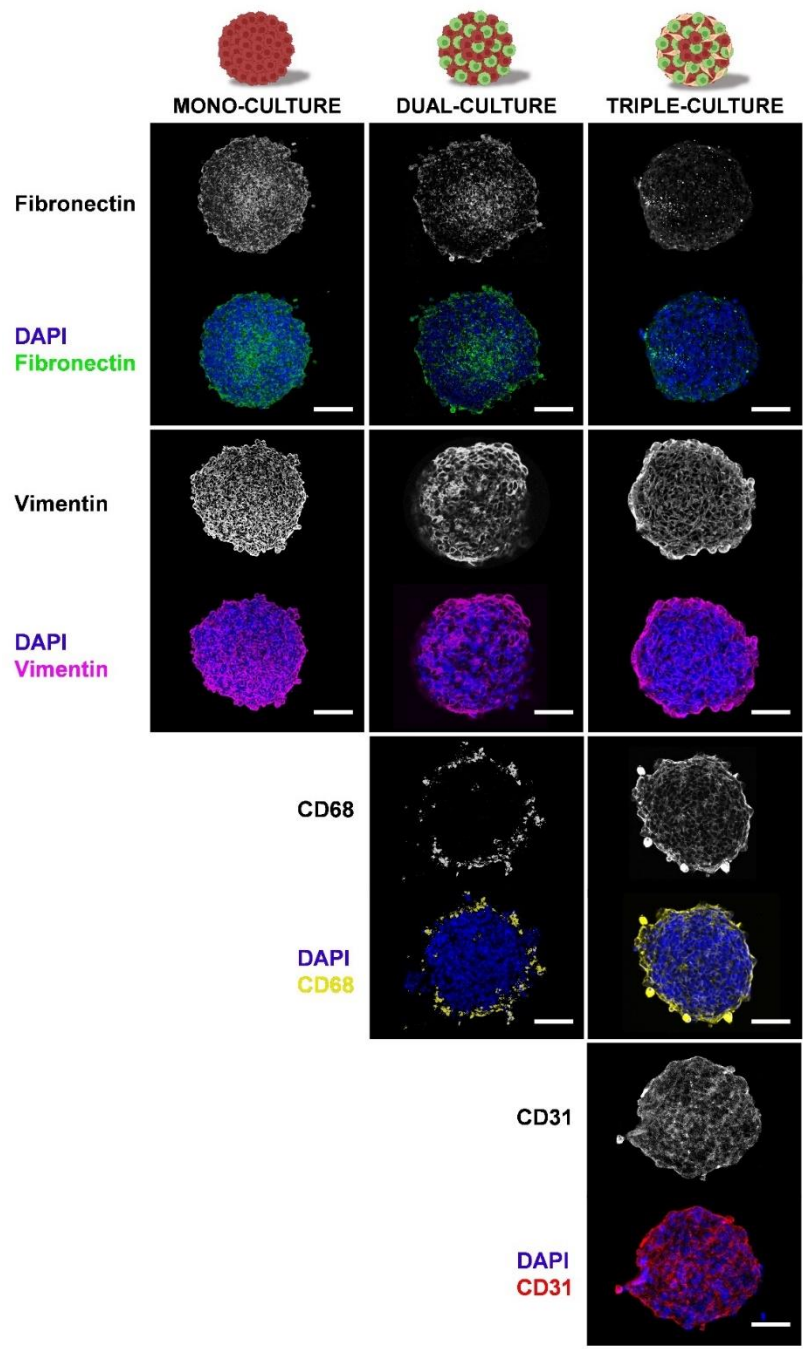


Figure 5. Immunofluorescence microscopy images of day 7 mono-, dual- and triple-culture GBM MCTMs. Representative images of cellular organization within the MCTMs

of: fibronectin (green); U-251 MG-vimentin (pink); macrophages–CD68 (yellow); ECs-CD31 (red); nuclei counterstaining with DAPI (blue). Scale bars represent 80 μm .

3.3 Nanoparticle production and characterization

In this work, non-targeted and tumor-targeted His-decorated NPs (**Figure 6A**) of PLGA and PEG encapsulating DTX were manufactured by a microfluidic technique previously optimized by our group [46-48].

Previously to NP manufacture, a PLGA-PEG polymer was chemically functionalized with His via a carbodiimide strategy to obtain a PLGA-PEG-His polymeric conjugate (around 70% yield and 90% conjugation efficiency, data not shown). The successful functionalization was confirmed by FTIR spectroscopy (**Figure S1**), which demonstrated a similar chemical fingerprint to PLGA-PEG (C-H bending, C-O and C-N stretching, [61]), and a new band at 1668 cm^{-1} characteristic of the C=O stretching in amides (amide I). Both non-targeted and tumor-targeted NP formulations presented a similar average size (approximately 200-250 nm), relatively homogenous size distribution (PDI of approximately 0.2-0.25), and a negative zeta-potential (approximately -30 mV), as schematized in **Figure 6B**. These properties were similar to the unloaded formulation counterparts (**Figure S2**). AE and LD values for the DTX-loaded NP formulations are shown in **Table S1**. TEM analysis evidenced spherical shaped particles with relatively smooth surfaces, with a size distribution identical for both formulations (**Figure 6C**). Considering that MCTM *in vitro* studies were performed over 7 days in FBS supplemented cell culture medium, we further determined the colloidal stability of the NP formulations. Although there is an increasing trend in both average size and PDI over time, due to the well reported adsorption of medium proteins on the NP surface and consequent aggregation of the nanosystems [62], no biologically relevant differences were found between day 0 and day 7 for both non-targeted and tumor-targeted His-decorated NPs (average size < 300 nm and PDI < 0.3 at day 7, **Figure 6D**). Zeta-potential values were kept constant over the 7-day duration of the stability assay (-36.2 ± 1.0 , -33.5 ± 2.4 and -36.2 ± 2.5 mV for H-NPs at day 0, 4 and 7, respectively; -35.1 ± 0.9 , -36.1 ± 3.4 and -35.3 ± 1.9 mV for mPEG NPs at day 0, 4 and 7, respectively).

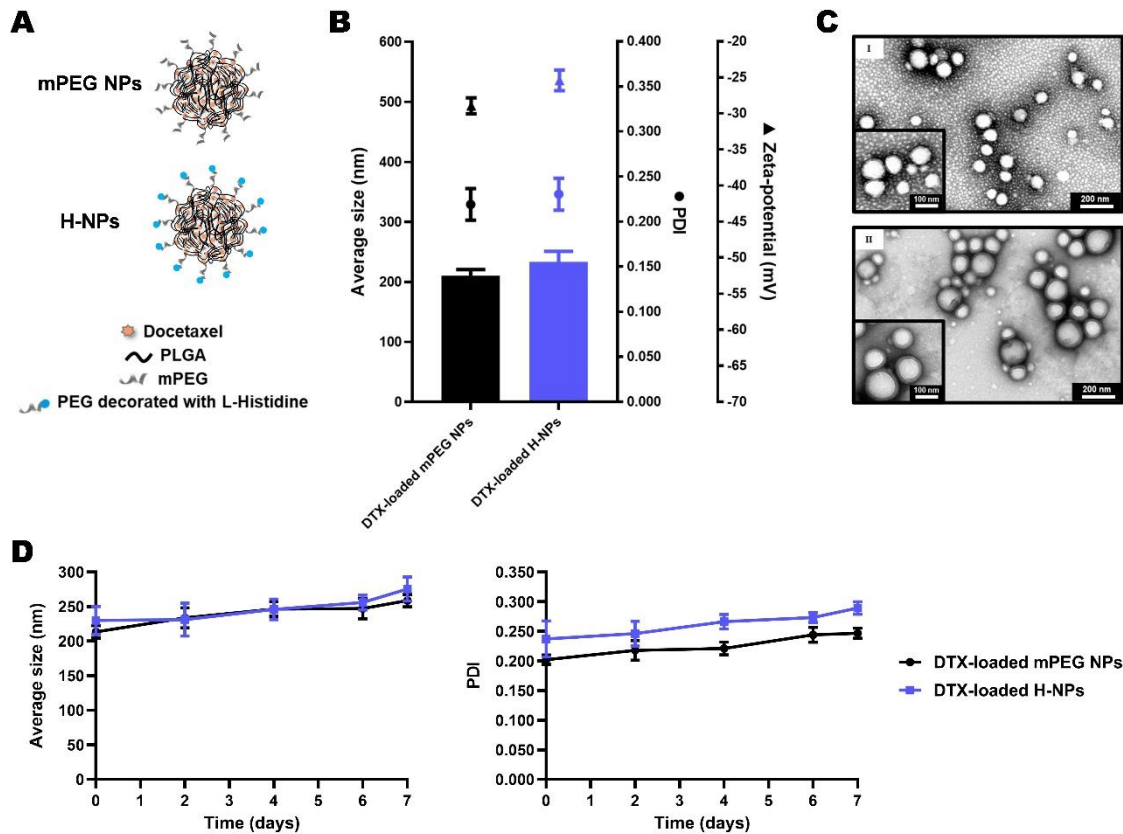


Figure 6. NP manufacture and characterization. A) Schematic illustration of DTX-loaded mPEG NPs and H-NPs. B) Characterization of average size, PDI and zeta-potential of DTX-loaded mPEG NPs and H-NPs. C) TEM characterization of DTX-loaded formulations, where I and II correspond to DTX-loaded mPEG NPs and DTX-loaded H-NPs, respectively. D) NP stability over 7 days in FBS supplemented cell culture medium.

3.4 Cell uptake

MCTMs were assessed for their capacity to differently uptake H-NPs or the respective PEGylated control without surface His, mPEG NPs, by flow cytometry.

As a preliminary assay, H-NPs manufactured using different percentages of the PLGA-PEG-His polymeric conjugate (5%, 10% and 20%) were tested regarding U-251 MG cell uptake after a 4 h treatment (**Figure S3**). In general, an increasing cell uptake was observed for increasing percentages of PLGA-PEG-His polymer. The lowest percentage (5% PLGA-PEG-His) was selected to proceed with further studies, since it already resulted in a 2-times or higher percentage of positive cells and MFI compared to mPEG NPs without surface functionalization with His. Regarding the GBM MCTMs, as shown in **Figure 7**, after 4 h treatment, H-NPs presented a significantly higher cell association

compared to mPEG NPs, which was reflected by a 1.5- and 2-fold increase in percentage of positive cells (**Figure 7A**) and MFI (**Figure 7B**), respectively. While for a 12 h treatment, a 1.5- and 9-fold increase was found for H-NPs regarding percentage of positive cells (**Figure 7A**) and MFI (**Figure 7B**), respectively. His on the H-NPs surface is a well-known ligand of LAT1, which is a transporter vastly described to be overexpressed by human gliomas such as GBM, and directly correlated with their aberrant proliferation and malignant development [35-37]. RT-PCR studies confirmed the overexpression of LAT1 mRNA by U-251 MG cells, being this the tumor cell component of the GBM MCTM [38]. A higher NP internalization of dendrimers modified with His has been correlated with the overexpression of amino acid transporters, such as LAT1 in HeLa cervical cancer cells, required for the rapid proliferation and increased antioxidant demands of these cells [63]. LAT1-mediated cancer cell delivery has also been exploited through the use of alternative LAT1 amino acid substrates, including glutamate [64, 65] and L-tyrosine [66]. Moreover, LAT1 overexpression by tumor tissues has been widely explored in positron emission tomography (PET) imaging through the development of tumor-enhancing PET probes modified with other L-amino acids, such as L-methionine [67], L-phenylalanine [68], or L-tyrosine [69]. Altogether, the increased cell association of H-NPs herein observed was attributed to a higher MCTM tumor cell internalization mediated by the binding of the H-NP surface His to the U-251 MG-overexpressed LAT1 transporter. Although ECs comprise a minor population within the GBM MCTM, we envisage that they are also responsible for the higher MCTM cell internalization of H-NPs compared to mPEG NPs. LAT1 is reported to present overexpression in the glioma microvascular endothelium and to play a key role in the regulation of tumor angiogenesis and proliferation, as well as in pro-angiogenic VEGF-A signaling [67, 70]. Macrophages differentiated from peripheral MONs, in turn, present low or no expression of LAT1, unless stimulated by pro-inflammatory environments [71-73]. Thus, we believe that the macrophagic population within the GBM MCTM is not responsible for the enhanced cell internalization observed for H-NPs. Importantly, these cell uptake experiments were performed in an early phase of MCTMs development, which can correlate with an early phase of GBM development, and the findings should not be extrapolated to a later stage of tumor progression. Moreover, despite the high concentration of competing endogenous His (around 1 nm average diameter) in plasma and cerebrospinal fluid of approximately 90 μ M and 20 μ M, respectively, this is not expected to interfere with the tumor-targeting efficiency of H-NPs (around 200-300 nm average diameter) [74, 75]. Other works demonstrated that ligand-functionalized NP systems increase the affinity of the NPs towards the receptor of interest by several-orders of magnitude compared to the free form of the ligand, mainly because a single

NP can spatially bind to several cell transporters at a time and lead to the so-called “multiligand binding” phenomenon [76, 77]. To corroborate this, when incubating both ligand-functionalized NPs and the respective ligand alone, several-orders of magnitude higher concentration of the ligand alone might be needed to cause 50% displacement of the NPs from the receptor (inhibitory constant) in comparison with the concentration usually needed to cause 50% displacement of the free form of the ligand [76, 78]. This is, moreover, strongly supported by the poor capacity of displacement of multiligand binding molecules or particles offered by monovalent ligands [79, 80].

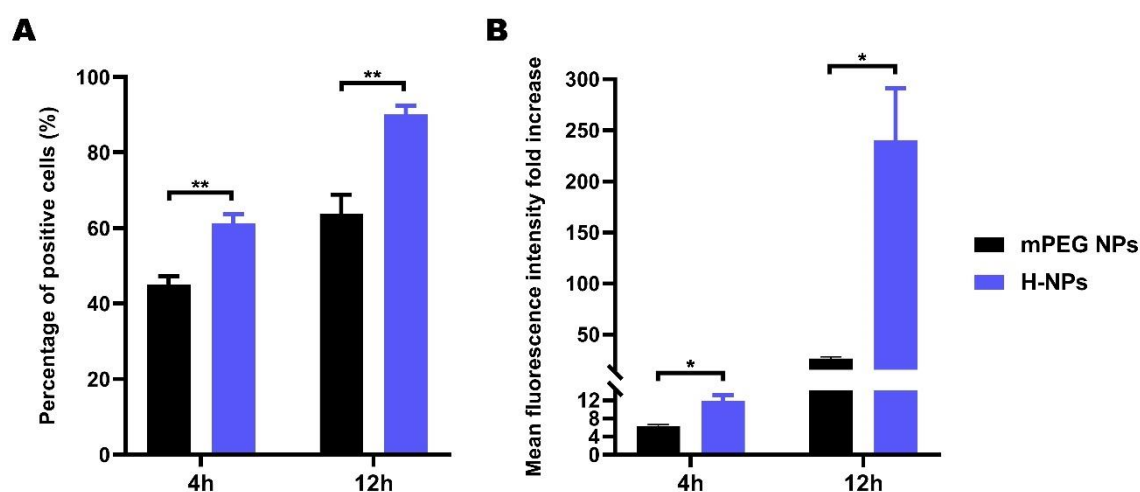


Figure 7. Flow cytometry analysis of cell internalization of mPEG NP or H-NP formulations into triple-culture GBM MCTMs cells. A) Percentage of positive cells. B) MFI values, normalized to the unstained control. Statistical comparison of H-NPs with mPEG NPs. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3.5 Anti-proliferative effect

The ability of DTX-loaded H-NPs or mPEG NPs to inhibit GBM MCTMs cell proliferation was assessed and compared to the non-nanoparticulate free DTX control.

GBM MCTMs metabolic activity was investigated after uninterrupted periods of treatment of 48 h (day 3) and 96 h (day 5) with DTX doses ranging from 0.0045 to 4.5 μM (**Figure 8A**, left panel). For both periods of treatment, the metabolic activity decreased in a dose-dependent manner and, from 0.045 to 4.5 μM DTX dose, 48 h of treatment already provided similar cytotoxicity levels as compared to 96 h. Overall, metabolic activity reached a minimum value of around 5% for all testing conditions. Interestingly, a more pronounced metabolic activity decrease was found for DTX-loaded H-NPs compared to

free DTX, after 48 h (25% *versus* 50% at 0.045 μ M; 10% *versus* 25% at 0.45 μ M) and 96 h (25% *versus* 50% at 0.045 μ M) of treatment. Whereas, the effect of DTX-loaded mPEG NPs was similar to free DTX for both periods and all drug concentrations. The more cytotoxic effect of DTX-loaded H-NPs was in accordance with the previous cell uptake results, and attributed to the ameliorated ability of H-NPs of being internalized by the cellular components of the MCTMs, especially due to the binding of surface His to the tumor cell-overexpressed LAT1. Thus, this resulted in a closer cell-drug interaction and, consequently, higher levels of inhibition of cell metabolic activity. It is important to note that these findings are based on static assay conditions, which can overestimate the effect of free DTX and underestimate the effect of DTX-loaded NP formulations, since free drug molecules are readily available to interact with cells, while nanoparticulated drug requires diffusion or erosion of the NP matrix to start exerting an anti-proliferative effect [32].

In an attempt to scrutinize the adjuvant effect of H-NP surface His on the cell proliferation of the GBM MCTMs, a 12 h dynamic-like boost treatment was performed [81]. Therefore, for the highest DTX dose, 4.5 μ M, MCTM cells were incubated with the testing formulations for only 12 h - an exposure time assessed in cell uptake studies -, and the metabolic activity was further analyzed at day 5 (**Figure 8A**, right panel). It was observed a significantly lower metabolic activity associated with DTX-loaded H-NPs (around 10%), corresponding to 4- and 3-times lower values compared to free DTX (around 40%) and DTX-loaded mPEG NPs (around 30%), respectively. Once again, this was attributed to the improvement of MCTM cell uptake provided by H-NPs, which resulted in a more pronounced chemotherapeutic effect of DTX. Over an exposure time of 12 h, H-NPs are hypothesized to present a higher retention into the cellular core of the GBM MCTMs, and even reach deeper areas of the microtissue, compared to mPEG NPs and the non-nanoparticulate free DTX control, as supported by key findings of other authors in the field [82-84]. Altogether, these findings evidenced the higher cytotoxicity sensitivity of the GBM MCTMs to the NP formulation modified with surface His (**Figure 8B**), ultimately leading to a clear DTX chemotherapeutic advantage.

Since NP surface His proved to enhance MCTM cell uptake and anti-proliferative effect, compared to mPEG NPs, the H-NP formulation was selected to proceed with further studies. Additional data regarding the impact of the unloaded NP formulations on GBM MCTMs metabolic activity can be found in **Figure S4**.

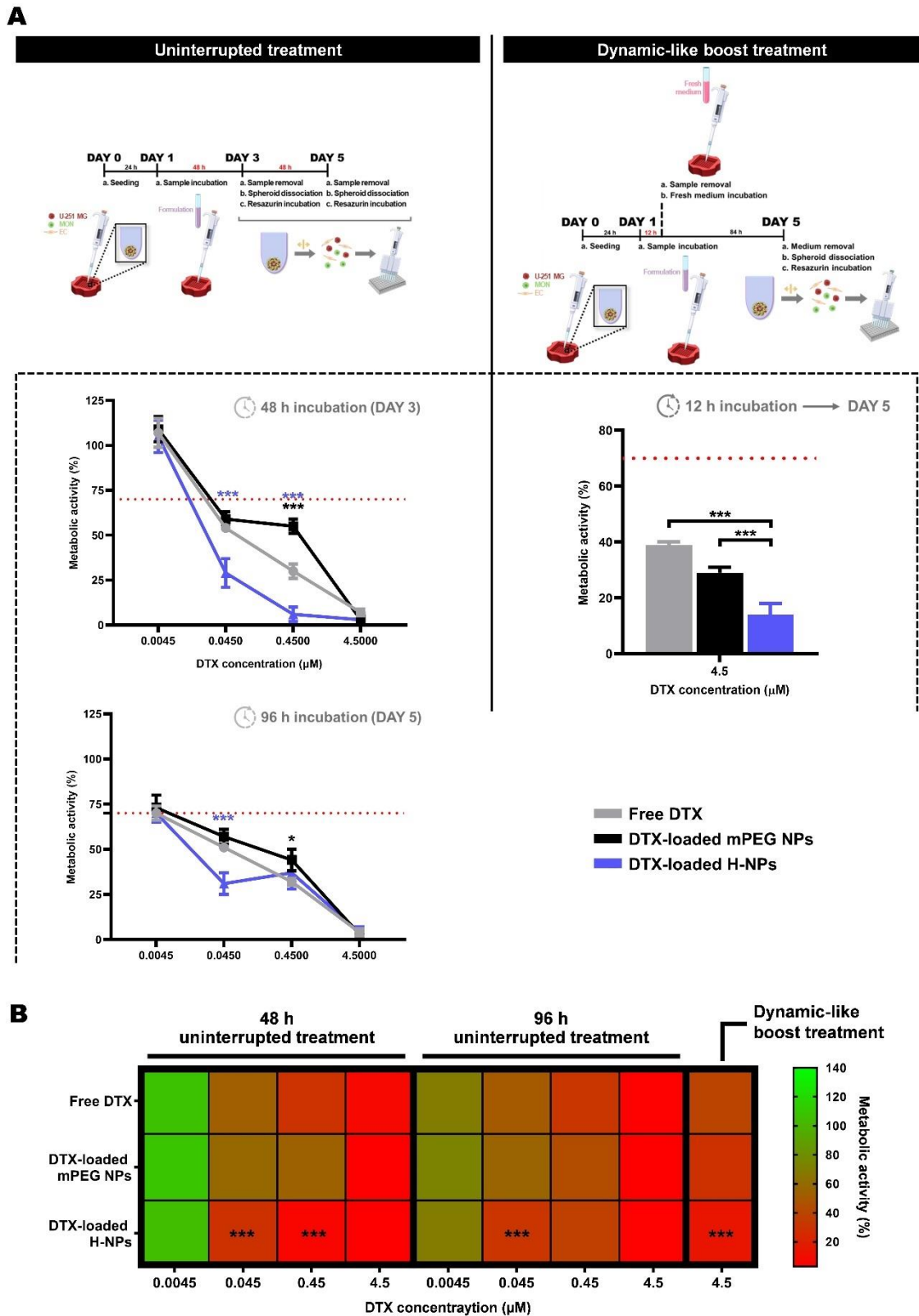


Figure 8. Evaluation of the effect of free DTX and DTX-loaded NP formulations on the proliferation of triple-culture GBM MCTMs cells. A) The left panel presents metabolic activity levels after uninterrupted periods of treatment of 48 h and 96 h. The right panel

presents a dynamic-like boost treatment of 12 h, integrated in an assessment period of incubation of 96 h. Statistical comparison of free DTX with the other drug-loaded groups, within each dose range. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. B) Heat-map representation of the anti-proliferative effect of free DTX and DTX-loaded NP formulations on triple-culture GBM MCTMs cells in the tested concentrations. Statistical comparison of DTX-loaded H-NPs with the other drug-loaded groups, within each dose range. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3.6 Macrophage polarization

Over the past years, there has been an increasing interest on the plasticity of TAMs within the TME, and their ability to either support or suppress the anti-tumor immunity. Nonetheless, TAMs are recognized as crucial to enhance tumor growth, mainly by fomenting angiogenesis and ECM remodeling, and hijacking pro-inflammatory networks [85]. Since macrophages constitute a major cellular component of the GBM MCTMs, we analyzed alterations in their polarization after a 48 h treatment with non-nanoparticulate free DTX, as well as unloaded and DTX-loaded H-NPs. Therefore, the lineage marker CD14⁺ macrophage population of MCTMs was analyzed regarding the expression of the mannose receptor CD206 and the scavenger receptor CD163 (both anti-inflammatory M2 markers), and the T cell co-stimulatory CD86 (a pro-inflammatory M1 marker), respectively, as illustrated in **Figure 9**.

For all testing groups - untreated (incubation with cell culture medium only), unloaded H-NPs, non-nanoparticulate free DTX, DTX-loaded H-NPs - 48 h after the triple-culture MCTM development, the CD14⁺ macrophage population (45.8 ± 10.6 , 47.5 ± 13.2 , 44.1 ± 10.3 , 47.1 ± 8.1 %, respectively) and total protein concentration (3.810 ± 0.093 , 3.784 ± 0.214 , 3.784 ± 0.139 , 3.761 ± 0.120 mg mL⁻¹, respectively; assuring maintenance of cell viability post-treatment) were assessed. Since human primary GBMs present TAMs populations that constitute approximately 30-50% of the cell bulk [3-5], this demonstrated a reliable *in vitro-in vivo* correlation for the GBM MCTMs. Regarding the polarization of the CD14⁺ macrophage population, for the untreated group, approximately 50% CD206, 15% CD163 and 20% CD86 positive cells were found. This indicated a higher percentage of M2-polarized macrophages within the MCTM constructs, which reflects the GBM *in vivo* scenario, suggesting cancer cell-mediated MON profile modulation and correlating with the disease poor survival [86]. TAMs tend to be polarized into a M2 phenotype in the GBM TME [87], suggesting that both U-251 MG tumor cells and ECs promoted the differentiation of naïve macrophages into a pro-tumor anti-inflammatory state. For the

non-nanoparticulate free DTX group, it was possible to observe a decreasing trend in the percentage of CD206 and CD163 positive cells compared to the untreated control (30% *versus* 50%, and 5% *versus* 15%, respectively). Whereas, the level of CD86 positive cells was similar to the untreated control (around 20%). This result was not unexpected since a very low 0.0045 μ M subcytotoxic DTX dose was used, alongside with a treatment period of only 48 h. Nevertheless, previous studies support our findings by reporting the polarization of human peripheral MONs-differentiated macrophages into a M1 phenotype after DTX treatment, although for a drug dose of at least 0.1 μ M [88]. Besides providing an immunomodulator effect through alterations in TAMs polarization, DTX is also reported to contribute to an anti-tumor immunity via suppression of myeloid-derived suppressor cells (MDSCs) [89], and regulation of the natural killer (NK) and T regulatory (Treg) TME cell populations [90]. DTX-loaded H-NPs, in turn, caused a decreasing trend in the percentage of CD206 and CD163 positive cells, and an increasing trend in the percentage of CD86 positive cells, compared to the untreated control (30% *versus* 50%, 5% *versus* 15%, and 30% *versus* 20%, respectively). The effect of DTX-loaded H-NPs on macrophage polarization was, thus, similar to the non-nanoparticulate free DTX group, and suggested immune cell modulation towards a more anti-tumor and pro-inflammatory macrophage phenotype. Interestingly, unloaded H-NPs seemed to also provide an effect on macrophage polarization *per se*. These unloaded NPs showed a decreasing trend in the percentage of CD206 and CD163 positive cells, and an increasing trend in the percentage of CD86 positive cells, with average values similar to DTX-loaded H-NPs (35% *versus* 30%, 5% *versus* 5%, and 20% *versus* 30%, respectively). However, no significant differences were found, thus, these results deserve careful interpretation and further research. Overall, these findings suggested that both DTX and H-NPs play a role in the polarization shift of the GBM MCTM naïve macrophages from a M2 anti-tumor to a M1 pro-tumor phenotype, despite the TME tumorigenic influence of co-cultured tumor cells and ECs.

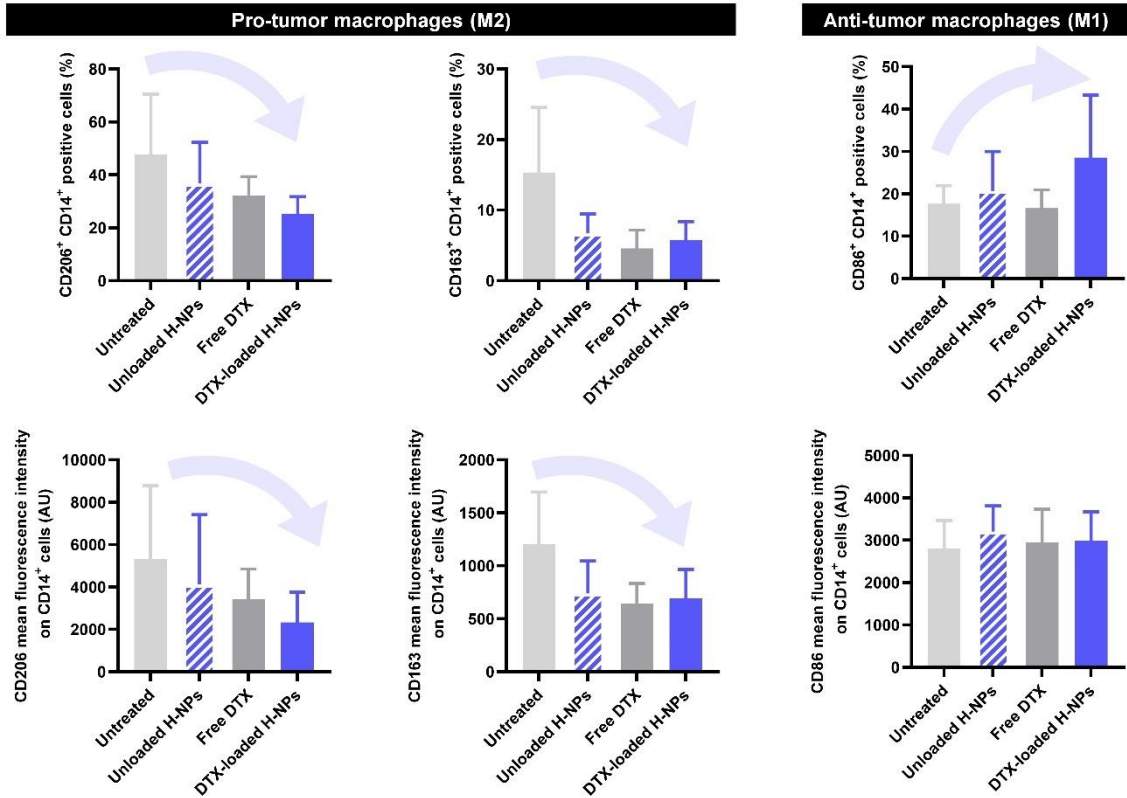


Figure 9. Macrophage polarization status of the triple-culture GBM MCTMs after treatment. The CD14⁺ macrophage population of MCTMs was analyzed for the expression of CD206 and CD163 (M2 markers), and CD86 (M1 marker), as percentage of positive cells and MFI, after incubating the triple culture MCTS with cell culture medium (untreated), unloaded H-NPs, free DTX and DTX-loaded H-NPs for 48 h.

3.7 Biomolecular signature

A complex signaling crosstalk between tumor, immune system and stromal cells is a hallmark of the GBM TME, leading to disease progression and invasion, immune evasion and resistance to therapy [91]. Since previous macrophage polarization data was partially inconclusive, we proceeded with a complementary assessment of the potential of the GBM MCTM to mimic the *in vivo* scenario. Thus, we investigated the biomolecular signature of GBM MCTMs after different treatments through analysis of secretion of an array of key signaling mediators that are involved in the tumor-macrophage-EC interplay, namely IFN- γ , IL-1 β , IL-1Ra, IL-6, IL-8, TGF- β , TNF- α and MCP-1. Although the GBM TME is vast and only partially represented by our MCTM, we will focus on the secretion and effect of these specific signaling mediators on the MCTM cellular components, and direct modulation of some of the principal TME immune cells (**Figure 10A**). The ability

of the MCTM to secrete these signaling mediators *per se* was validated (untreated control), and alterations in secretion levels after 48 h treatment with unloaded H-NPs, free DTX and DTX-loaded H-NPs were analyzed (**Figure 10B**).

Secretion levels of all cytokines - IFN- γ , IL-1 β , IL-1Ra, IL-6, IL-8, TGF- β , TNF- α and MCP-1 - were successfully detected in the supernatant of the GBM MCTM (**Figure 10B**). According to literature, GBM cells secrete IL-8 and MCP-1 to recruit and promote the infiltration of TAMs, a group of pro-tumor cells composed mainly by peripheral MONs, but also microglia [6, 92]. This leads to a local increase in the levels of TGF- β and IL-6, responsible for GBM invasion, progression and immune evasion [93]. Among other pro-tumor roles, these cytokines are involved in the glial-to-mesenchymal transition of GBM cells, which is a mechanism by which the tumor acquires chemotherapy and radiotherapy resistance [94-96]. On the other hand, TAMs secrete TNF- α in order to trigger the activation of GBM TME ECs, thus favoring tumor progression via neovascularization and angiogenesis, ultimately supporting tumor stemness [92]. As such, high levels of TNF- α in GBM are often considered a predictor of worse response to anti-angiogenic bevacizumab therapy. ECs, in turn, are a major source of angiocrine IL-6 within GBM, supporting the polarization of TME peripheral MONs and microglia into a M2 pro-tumor TAMs phenotype [97]. IL-6 is, moreover, directly associated with vascular formation in GBM via the PKC pathway [98, 99], and correlated with the VEGF and COX-2 angiogenic factors [100]. ECs also secrete IL-8 that acts on GBM cells as a critical mediator of tumor invasion in the perivascular niche via vascular sprouting, being involved in tumor growth and cancer stem cell upregulation [101]. As part of the immune system, NK cells are one of the most important anti-tumor key players in the GBM TME. These cells not only present a unique capacity to kill aberrant cells associated with oncogenic transformation independently of T cells, but also empower T cell responses against the tumor [102]. Due to the highly immunosuppressive nature of the GBM TME, several mechanisms of NK cell suppression are activated, such as through direct inhibition by TAMs or TGF- β secretion by tumor cells [103]. Another TME immunosuppressive mechanism is based on the stimulation and induction of Tregs by GBM cell-secreted TGF- β and MCP-1, alongside with TAMs. Importantly, Tregs constitute the predominant immunosuppressive cell population within GBM [103, 104]. Specifically, Tregs inhibit the antigen-presenting capacity of dendritic cells (DCs) and promote the suppression of T cell activity, hence resulting in a lower anti-tumor cytotoxicity of effector T cells (Teff) [103]. Teff are also inhibited by MDSCs, whose recruitment and expansion are triggered by TGF- β , IL-1 β , IL-6 and IFN- γ secreted by GBM cells [103]. Within the GBM TME, the infiltration and activation of tumor-associated neutrophils (TANs) in a process mediated by tumor cell-secreted MCP-1, IL-8 and TNF- α , is a marker of poor glioma prognosis – TANs seem to

interplay with TAMs to support tumor growth, although the exact mechanisms are still unclear [105]. Lastly, though its precise role also remains unclear, IL-1ra has been implicated in GBM progression due to its capacity to block growth-inhibiting autocrine loops of IL-1 [106] [107].

After 48 h treatment, at a drug dose of only 0.0045 μ M, both free DTX and DTX-loaded H-NPs caused a significant decrease in the secretion levels of IFN- α , IL-1 β and IL-6 compared to the untreated group, which was not observed for the unloaded H-NPs. This suggests an active role of the DTX in reducing the concentration of these tumor-stimulating cytokines within the MCTM, which presents promising insights regarding the anti-tumorigenic type of immune response that this drug is able to provide in the context of GBM. Interestingly, for IL-1Ra, IL-8 and TGF- β , significantly reduced secretion levels were observed for MCTMs treated with DTX-loaded H-NPs, which was not provided by free DTX. Moreover, for IFN- α , a more pronounced decrease in secretion levels of this cytokine was observed for DTX-loaded H-NPs, compared to free DTX. These results suggested the ability of H-NPs to serve as a vehicle to potentiate the anti-tumorigenic type of immune response associated with DTX. This was attributed to the higher DTX delivery efficiency to MCTMs provided by H-NPs, as evidenced by an enhancement of cell uptake by H-NPs (**Figure 7**), hence resulting in a faster modulation of cytokine secretion over a 48 h treatment period. In addition, for IL-1Ra, unloaded H-NPs reduced the cytokine levels similarly to DTX-loaded H-NPs, suggesting an active role of H-NPs on the modulation of this cytokine secretion *per se*. Future studies are required to understand the mechanism behind this, which still remains to be determined. Lastly, none of the treatment groups altered the secretion levels of TNF- α and MCP-1, which mainly act as chemoattractants within the GBM TME, thus indicating that these cytokines are not a DTX target *per se*. It of important note that the TME is orchestrated by a complex milieu of cytokines that present pleiotropy according to each tumor type, thus comparison of DTX effect on cytokine secretion levels is not appropriate if out of the GBM scope [108, 109]. Moreover, to the best of our knowledge, and since DTX does not belong to the GBM standard of care, no literature data is available for comparison regarding the effect of DTX on the secretion of the panel of cytokines herein analyzed. Nevertheless, our findings present promising insights on the immune response triggered by DTX chemotherapy in GBM, and evidence the capacity of H-NPs to boost this response, which might be useful for future GBM local therapies. Finally, the effect of DTX on cytokine secretion is highly dependent on the drug dose and exposure time, therefore, our findings should be taken into consideration for the herein specific experimental conditions [88, 110].

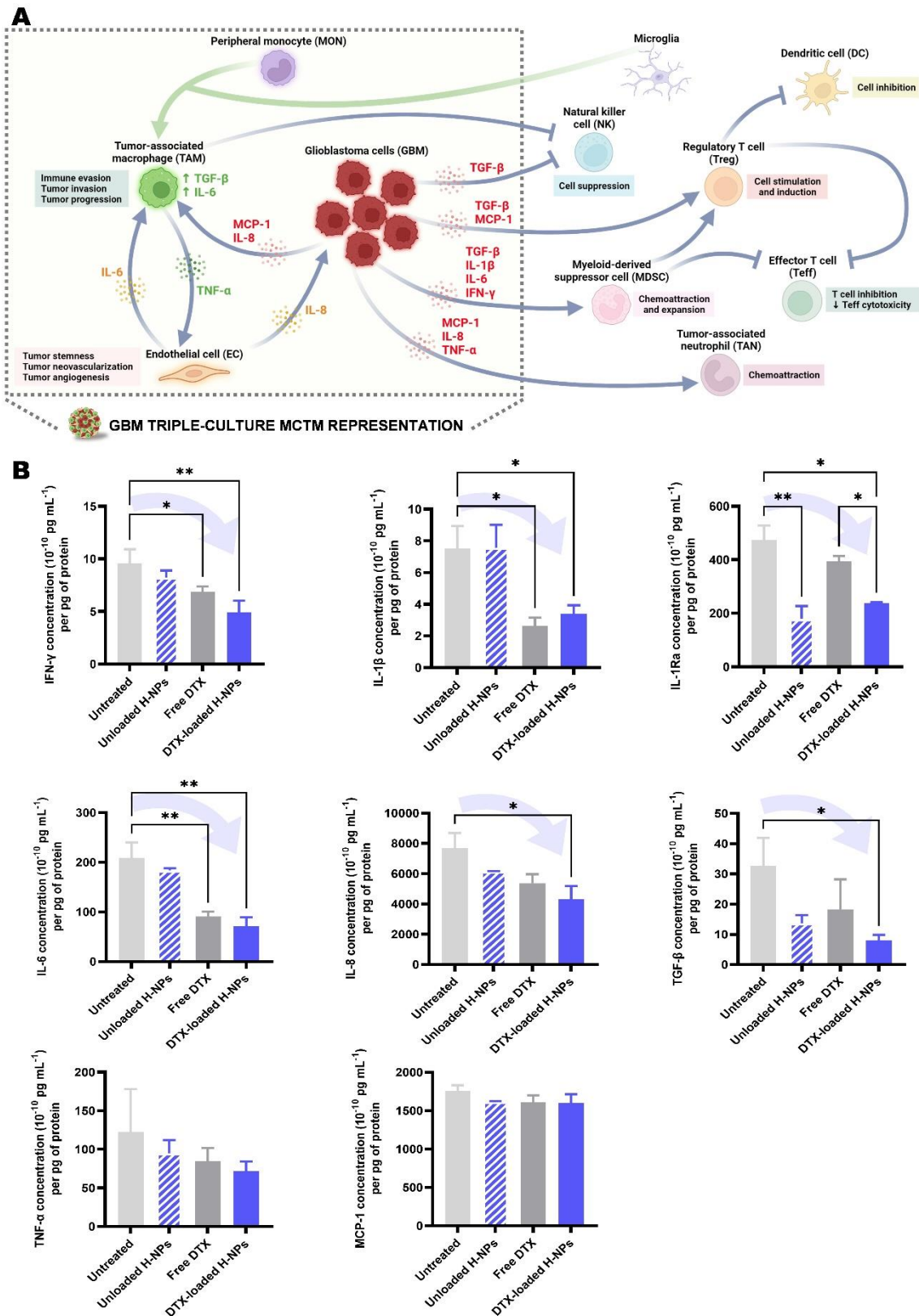


Figure 10. Analysis of the biomolecular signature of triple-culture GBM MCTMs after treatment with cell culture medium (untreated), unloaded H-NPs, free DTX and DTX-loaded H-NPs for 48 h. A) Schematics of the herein analyzed key regulatory mediators

(and cell-cell interactions) that interconnect tumor cells, macrophages and ECs within the GBM TME. B) Analysis of secretion of an array of signaling mediators that are involved in the tumor-macrophage-EC interplay. Statistical comparison between the different testing groups. * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

4. CONCLUSION

In this study, we have successfully developed a human GBM MCTM spheroid model constituted by three key cell populations of the GBM TME, namely U-251 MG tumor cells, macrophages differentiated *in situ* from blood peripheral MONs and brain microvascular primary ECs. The triple-culture GBM MCTMs demonstrated the capacity to mimic cellular and structural features of the tumor, such as cell spatial organization, intrinsic production of ECM, formation of necrosis areas, and a predominant pro-tumor and anti-inflammatory M2-like macrophage phenotype. The triple-culture GBM MCTM model was further exploited regarding its capacity to serve as an *in vitro* screening tool for drugs (DTX) and nano-therapeutics (DTX-loaded non-targeted mPEG NPs *versus* tumor-targeted H-NPs). The strategy used in the manufacture of tumor-targeted DTX-loaded H-NPs consisted of a straightforward surface functionalization of the particles with His, which is able to recognize and bind the tumor-overexpressed LAT1. DTX-loaded H-NPs provided 8- and 3-times higher MCTM cell uptake and anti-proliferative effect, respectively, compared to the non-targeted NP counterpart and free DTX. Moreover, DTX-loaded H-NPs reduced the expression of anti-inflammatory macrophage markers, while increasing the levels of expression of pro-inflammatory macrophage markers, suggesting reeducation of the tumor immune microenvironment. Accordingly, DTX-loaded H-NPs caused a reduction in the secretion of an array of cytokines by MCTMs, with a well-known role in the pro-tumor immunity (IFN- γ , IL-1 β , IL-1Ra, IL-6, IL-8, TGF- β). Altogether, the triple-culture GBM MCTM herein developed mimics, in a comprehensive manner, the tumor cell-macrophage-EC interplay within the GBM TME. By contributing to a better recapitulation of the bioarchitecture and composition of the native tumor, the triple-culture GBM MCTM model consists of a promising *in vitro* technology to dissect therapeutic resistance mechanisms of GBM tumors, and to ameliorate the robustness of preclinical findings and *in vivo* predictability potential.

5. Supporting information

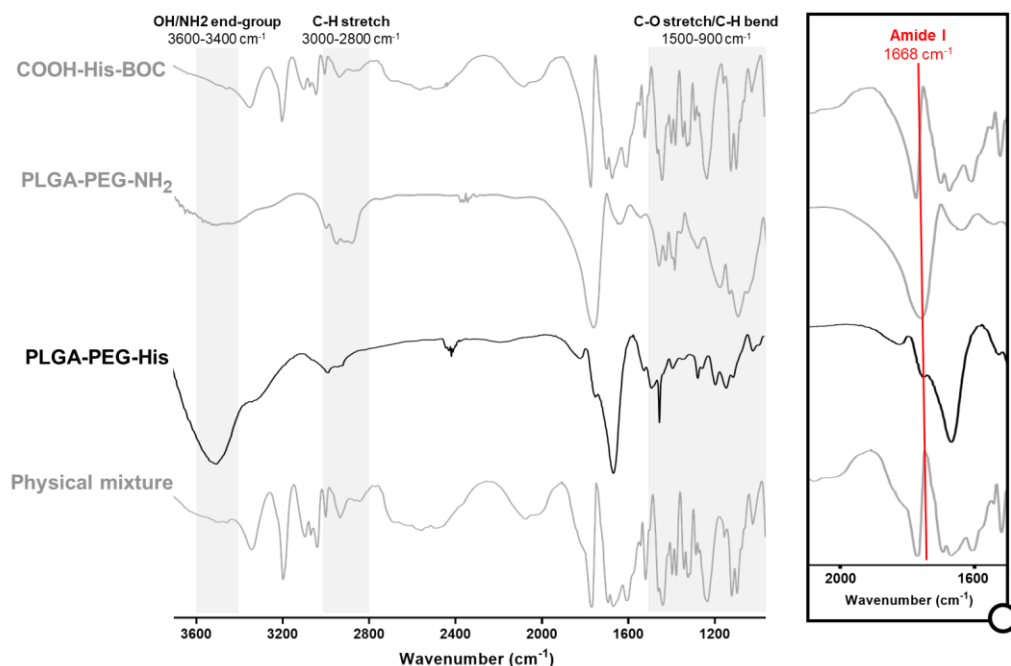


Figure S1. FTIR spectroscopy analysis of COOH-His-BOC, PLGA-PEG-NH₂, PLGA-PEG-His, and the physical mixture of COOH-His-BOC and PLGA-PEG-NH₂. The successful functionalization of PLGA-PEG-NH₂ with COOH-His-BOC, giving PLGA-PEG-His, was confirmed by a new band at 1668 cm⁻¹ (amide I).

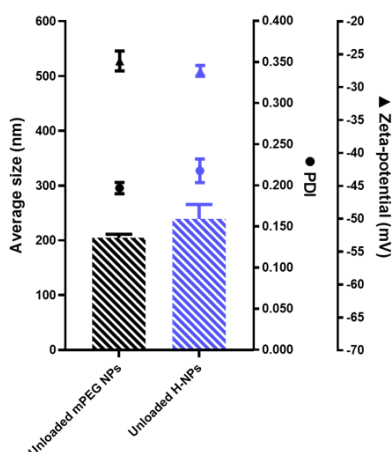


Figure S2. Characterization of average size, PDI and zeta-potential of unloaded mPEG NPs and H-NPs.

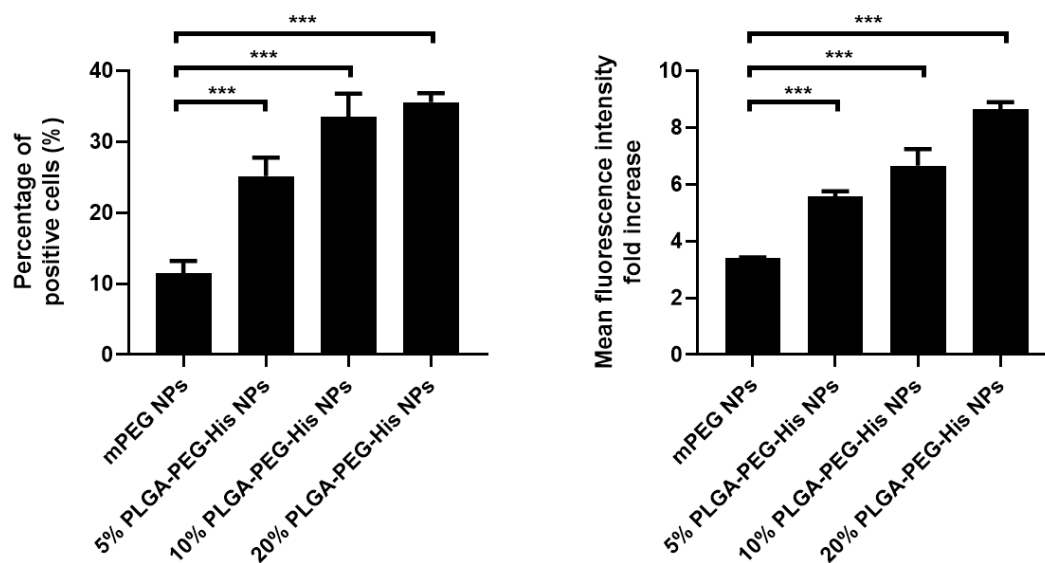


Figure S3. Flow cytometry analysis of cell internalization of mPEG NPs and H-NPs assembled from 5%, 10% or 20% PLGA-PEG-His into U-251 MG GBM cells. MFI values were normalized to the unstained control. Statistical comparison of H-NP groups with mPEG NPs. *** $p < 0.001$.

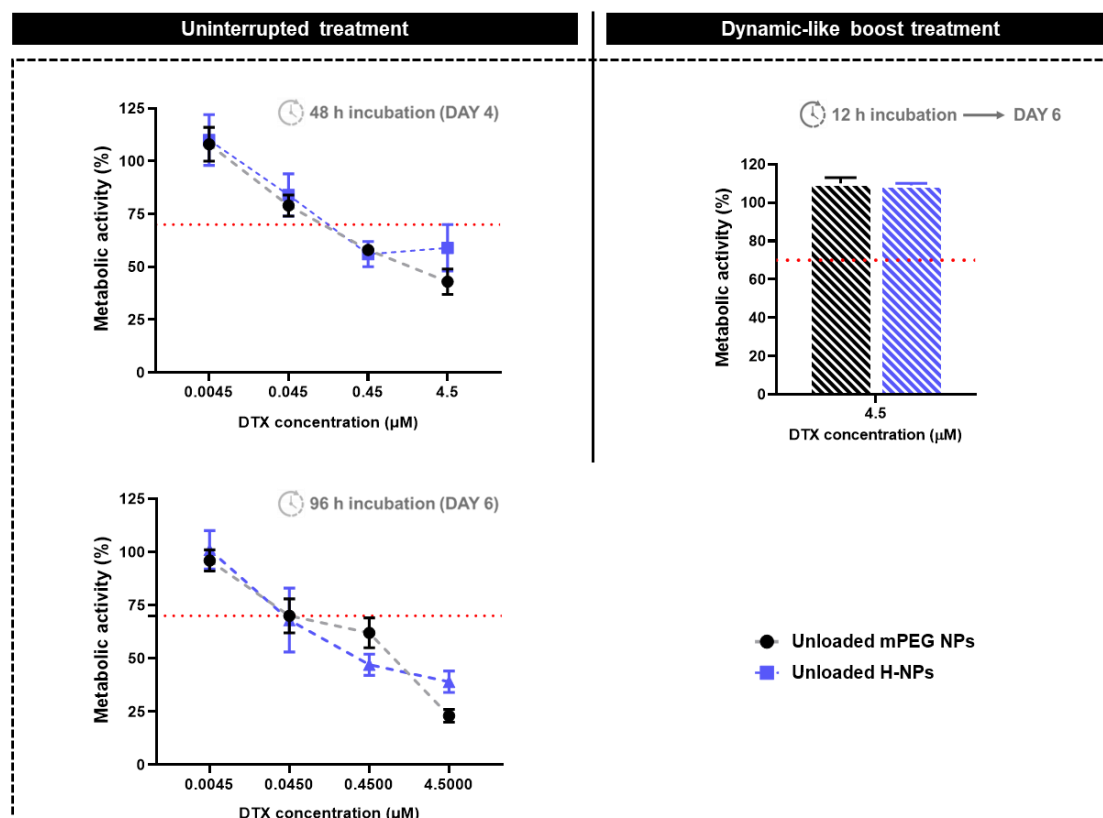


Figure S4. Evaluation of the effect of unloaded NP formulations on the proliferation of triple-culture GBM MCTMs cells. A) The left panel presents metabolic activity levels after uninterrupted periods of treatment of 48 h and 96 h. The right panel presents a dynamic-like boost treatment of 12 h, integrated in an assessment period of incubation of 96 h.

Table S1. AE and LD values for the developed DTX-loaded NP formulations.

DTX-loaded NP formulation	AE (%)	LD (%)
mPEG NPs	30.01 ± 5.09	1.55 ± 0.12
H-NPs	31.08 ± 4.01	1.58 ± 0.22

REFERENCES

- [1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J Clin*, 71 (2021) 209-249.
- [2] M.W. Yu, D.F. Quail, Immunotherapy for glioblastoma: Current progress and challenges, *Front Immunol*, 12 (2021) 676301.
- [3] D. Hambardzumyan, D.H. Gutmann, H. Kettenmann, The role of microglia and macrophages in glioma maintenance and progression, *Nat Neurosci*, 19 (2016) 20-27.
- [4] A.P. Landry, M. Balas, S. Alli, J. Spears, Z. Zador, Distinct regional ontogeny and activation of tumor associated macrophages in human glioblastoma, *Sci Rep*, 10 (2020) 19542.
- [5] R.A. Morantz, G.W. Wood, M. Foster, M. Clark, K. Gollahon, Macrophages in experimental and human brain tumors. Part 1: Studies of the macrophage content of experimental rat brain tumors of varying immunogenicity, *J Neurosurg*, 50 (1979) 298-304.
- [6] W. Zhou, S.Q. Ke, Z. Huang, W. Flavahan, X. Fang, J. Paul, L. Wu, A.E. Sloan, R.E. McLendon, X. Li, J.N. Rich, S. Bao, Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth, *Nat Cell Biol*, 17 (2015) 170-182.
- [7] R.S. Andersen, A. Anand, D.S.L. Harwood, B.W. Kristensen, Tumor-associated microglia and macrophages in the glioblastoma microenvironment and their implications for therapy, *Cancers*, 13 (2021) 4255.
- [8] A. Golebiewska, S. Bougnaud, D. Stieber, N.H. Brons, L. Vallar, F. Hertel, B. Klink, E. Schröck, R. Bjerkvig, S.P. Niclou, Side population in human glioblastoma is non-tumorigenic and characterizes brain endothelial cells, *Brain*, 136 (2013) 1462-1475.
- [9] M.L. Broekman, S.L.N. Maas, E.R. Abels, T.R. Mempel, A.M. Krichevsky, X.O. Breakefield, Multidimensional communication in the microenvirons of glioblastoma, *Nat Rev Neurol*, 14 (2018) 482-495.
- [10] D. Di Mascolo, A.L. Palange, R. Primavera, F. Macchi, T. Catelani, F. Piccardi, R. Spanò, M. Ferreira, R. Marotta, A. Armirotti, A.L. Gallotti, R. Galli, C. Wilson, G.A. Grant, P. Decuzzi, Conformable hierarchically engineered polymeric micromeshes enabling combinatorial therapies in brain tumours, *Nat Nanotechnol*, 16 (2021) 820-829.
- [11] Y. Guo, R. Liu, L. Zhou, H. Zhao, F. Lv, L. Liu, Y. Huang, H.-W. Zhang, C. Yu, S. Wang, Blood-brain-barrier penetrable thiolated paclitaxel-oligo (p-phenylene vinylene) nanomedicine with increased drug efficiency for glioblastoma treatment, *Nano Today*, 35 (2020) 100969.

- [12] Y. Fang, Y. Jiang, Y. Zou, F. Meng, J. Zhang, C. Deng, H. Sun, Z. Zhong, Targeted glioma chemotherapy by cyclic RGD peptide-functionalized reversibly core-crosslinked multifunctional poly(ethylene glycol)-b-poly(ϵ -caprolactone) micelles, *Acta Biomater*, 50 (2017) 396-406.
- [13] Y. Zou, Y. Wang, S. Xu, Y. Liu, J. Yin, D.B. Lovejoy, M. Zheng, X.-J. Liang, J.B. Park, Y.M. Efremov, I. Ulasov, B. Shi, Brain co-delivery of temozolomide and cisplatin for combinatorial glioblastoma chemotherapy, *Adv Mater*, 34 (2022) 2203958.
- [14] R. Jatyan, P. Singh, D.K. Sahel, Y.G. Karthik, A. Mittal, D. Chitkara, Polymeric and small molecule-conjugates of temozolomide as improved therapeutic agents for glioblastoma multiforme, *J Control Release*, 350 (2022) 494-513.
- [15] S. Quader, K. Kataoka, H. Cabral, Nanomedicine for brain cancer, *Adv Drug Deliv Rev*, 182 (2022) 114115.
- [16] S. Wilhelm, A.J. Tavares, Q. Dai, S. Ohta, J. Audet, H.F. Dvorak, W.C.W. Chan, Analysis of nanoparticle delivery to tumours, *Nat Rev Mater*, 1 (2016) 16014.
- [17] N. Pandey, P. Anastasiadis, C.P. Carney, P.P. Kanvinde, G.F. Woodworth, J.A. Winkles, A.J. Kim, Nanotherapeutic treatment of the invasive glioblastoma tumor microenvironment, *Adv Drug Deliv Rev*, 188 (2022) 114415.
- [18] F. Madani, S.S. Esnaashari, T.J. Webster, M. Khosravani, M. Adabi, Polymeric nanoparticles for drug delivery in glioblastoma: State of the art and future perspectives, *J Control Release*, 349 (2022) 649-661.
- [19] S. Đorđević, M.M. Gonzalez, I. Conejos-Sánchez, B. Carreira, S. Pozzi, R.C. Acúrcio, R. Satchi-Fainaro, H.F. Florindo, M.J. Vicent, Current hurdles to the translation of nanomedicines from bench to the clinic, *Drug Deliv Transl Res*, 12 (2022) 500-525.
- [20] G. Mehta, A.Y. Hsiao, M. Ingram, G.D. Luker, S. Takayama, Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy, *J Control Release*, 164 (2012) 192-204.
- [21] K.-H. Lee, T.-H. Kim, Recent advances in multicellular tumor spheroid generation for drug screening, *Biosensors (Basel)*, 11 (2021) 445.
- [22] S.J. Han, S. Kwon, K.S. Kim, Challenges of applying multicellular tumor spheroids in preclinical phase, *Cancer Cell Int*, 21 (2021) 152.
- [23] J. Mestas, C.C.W. Hughes, Of mice and not men: Differences between mouse and human immunology, *J Immunol*, 172 (2004) 2731.
- [24] M. Millard, I. Yakavets, V. Zorin, A. Kulmukhamedova, S. Marchal, L. Bezdetnaya, Drug delivery to solid tumors: The predictive value of the multicellular tumor spheroid model for nanomedicine screening, *Int J Nanomedicine*, 12 (2017) 7993-8007.
- [25] W. Yang, J. Soares, P. Greninger, E.J. Edelman, H. Lightfoot, S. Forbes, N. Bindal, D. Beare, J.A. Smith, I.R. Thompson, S. Ramaswamy, P.A. Futreal, D.A. Haber, M.R.

Stratton, C. Benes, U. McDermott, M.J. Garnett, Genomics of Drug Sensitivity in Cancer (GDSC): A resource for therapeutic biomarker discovery in cancer cells, *Nucleic Acids Res* 41 (2012) D955-D961.

[26] P.S. Yaraswi, K. Shetty, K.S. Yadav, Temozolomide nano enabled medicine: Promises made by the nanocarriers in glioblastoma therapy, *J Control Release*, 336 (2021) 549-571.

[27] R. Fan, D. Chuan, H. Hou, H. Chen, B. Han, X. Zhang, L. Zhou, A. Tong, J. Xu, G. Guo, Development of a hybrid nanocarrier-recognizing tumor vasculature and penetrating the BBB for glioblastoma multi-targeting therapy, *Nanoscale*, 11 (2019) 11285-11304.

[28] H. Gao, Z. Yang, S. Zhang, S. Cao, Z. Pang, X. Yang, X. Jiang, Glioma-homing peptide with a cell-penetrating effect for targeting delivery with enhanced glioma localization, penetration and suppression of glioma growth, *J Control Release*, 172 (2013) 921-928.

[29] Y. Zhu, Y. Jiang, F. Meng, C. Deng, R. Cheng, J. Zhang, J. Feijen, Z. Zhong, Highly efficacious and specific anti-glioma chemotherapy by tandem nanomicelles co-functionalized with brain tumor-targeting and cell-penetrating peptides, *J Control Release*, 278 (2018) 1-8.

[30] S. Ostermann, C. Csajka, T. Buclin, S. Leyvraz, F. Lejeune, L.A. Decosterd, R. Stupp, Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients, *Clin Cancer Res*, 10 (2004) 3728-3736.

[31] A.J. ten Tije, W.J. Loos, M. Zhao, S.D. Baker, R.H. Enting, H. van der Meulen, J. Verweij, A. Sparreboom, Limited cerebrospinal fluid penetration of docetaxel, *Anticancer Drugs*, 15 (2004) 715-718.

[32] C. Martins, F. Sousa, F. Araújo, B. Sarmiento, Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications, *Adv Healthc Mater*, 7 (2018) 1701035.

[33] M. Souri, M. Soltani, F. Moradi Kashkooli, M. Kiani Shahvandi, M. Chiani, F.S. Shariati, M.R. Mehrabi, L.L. Munn, Towards principled design of cancer nanomedicine to accelerate clinical translation, *Mater Today Bio*, 13 (2022) 100208.

[34] E. Nance, C. Zhang, T.Y. Shih, Q. Xu, B.S. Schuster, J. Hanes, Brain-penetrating nanoparticles improve paclitaxel efficacy in malignant glioma following local administration, *ACS Nano*, 8 (2014) 10655-10664.

[35] Z. Haining, N. Kawai, K. Miyake, M. Okada, S. Okubo, X. Zhang, Z. Fei, T. Tamiya, Relation of LAT1/4F2hc expression with pathological grade, proliferation and angiogenesis in human gliomas, *BMC Clin Pathol*, 12 (2012) 4.

- [36] H. Nawashiro, N. Otani, N. Shinomiya, S. Fukui, H. Ooigawa, K. Shima, H. Matsuo, Y. Kanai, H. Endou, L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors, *Int J Cancer*, 119 (2006) 484-492.
- [37] P. Häfliger, R.-P. Charles, The L-Type amino acid transporter LAT1 - an emerging target in cancer, *Int J Mol Sci*, 20 (2019) 2428.
- [38] K. Kobayashi, A. Ohnishi, J. Promsuk, S. Shimizu, Y. Kanai, Y. Shiokawa, M. Nagane, Enhanced tumor growth elicited by L-type amino acid transporter 1 in human malignant glioma cells, *Neurosurgery*, 62 (2008) 493-503.
- [39] M.J. Gattas, I.G. Estecho, M.A. Lago Huvelle, A.E. Errasti, E.A. Carrera Silva, M. Simian, A heterotypic tridimensional model to study the interaction of macrophages and glioblastoma in vitro, *Int J Mol Sci*, 22 (2021).
- [40] M. Tang, S.K. Tiwari, K. Agrawal, M. Tan, J. Dang, T. Tam, J. Tian, X. Wan, J. Schimelman, S. You, Q. Xia, T.M. Rana, S. Chen, Rapid 3D bioprinting of glioblastoma model mimicking native biophysical heterogeneity, *Small*, 17 (2021) 2006050.
- [41] M.A. Heinrich, R. Bansal, T. Lammers, Y.S. Zhang, R. Michel Schiffelers, J. Prakash, 3D-bioprinted mini-brain: A glioblastoma model to study cellular interactions and therapeutics, *Adv Mater*, 31 (2019) e1806590.
- [42] E.A. Akins, M.K. Aghi, S. Kumar, Incorporating tumor-associated macrophages into engineered models of glioma, *iScience*, 23 (2020) 101770.
- [43] H. Cai, W. Liu, X. Liu, Z. Li, T. Feng, Y. Xue, Y. Liu, Advances and prospects of vasculogenic mimicry in glioma: A potential new therapeutic target?, *Onco Targets Ther*, 13 (2020) 4473-4483.
- [44] D. Brevet, O. Hocine, A. Delalande, L. Raehm, C. Charnay, P. Midoux, J.O. Durand, C. Pichon, Improved gene transfer with histidine-functionalized mesoporous silica nanoparticles, *Int J Pharm*, 471 (2014) 197-205.
- [45] V. Jahed, E. Vasheghani-Farahani, F. Bagheri, A. Zarrabi, T. Fink, K. Lambertsen Larsen, Enhanced cellular uptake of phenamil through inclusion complex with histidine functionalized β -cyclodextrin as penetrative osteoinductive agent, *Int J Nanomedicine*, 14 (2019) 8221-8234.
- [46] C. Martins, F. Araújo, M.J. Gomes, C. Fernandes, R. Nunes, W. Li, H.A. Santos, F. Borges, B. Sarmento, Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy, *Eur J Pharm Biopharm*, 138 (2018) 111-124.
- [47] C. Martins, V.M. Chauhan, A.A. Selo, M. Al-Natour, J.W. Aylott, B. Sarmento, Modelling protein therapeutic co-formulation and co-delivery with PLGA nanoparticles continuously manufactured by microfluidics, *React Chem Eng*, 5 (2020) 308-319.

- [48] C. Martins, B. Sarmento, Microfluidic manufacturing of multitargeted PLGA/PEG nanoparticles for delivery of taxane chemotherapeutics, in: K.K. Jain (Ed.) Drug Delivery Systems, Springer New York, New York, NY, 2020, pp. 213-224.
- [49] A. ISO 10993-5: 2009 Biological Evaluation of Medical Devices-Part 5: Tests for *in Vitro* Cytotoxicity; ISO: Phoenix, USA, 2007 (2009) 1-11.
- [50] T. Bauleth-Ramos, T. Feijão, A. Gonçalves, M.-A. Shahbazi, Z. Liu, C. Barrias, M.J. Oliveira, P. Granja, H.A. Santos, B. Sarmento, Colorectal cancer triple co-culture spheroid model to assess the biocompatibility and anticancer properties of polymeric nanoparticles, *J Control Release*, 323 (2020) 398-411.
- [51] X. Gong, C. Lin, J. Cheng, J. Su, H. Zhao, T. Liu, X. Wen, P. Zhao, Generation of multicellular tumor spheroids with microwell-based agarose scaffolds for drug testing, *PLoS One*, 10 (2015) e0130348-e0130348.
- [52] L. Guo, Z. Zou, M. Freytag, R.E. Friedrich, P. Hartjen, M. Gosau, R. Smeets, L. Kluwe, Human dental pulp cells form spheroids in the presence of serum when seeded on a low-attachment cultural surface, *Processes*, 10 (2022) 1021.
- [53] K.S.P. Devi, D. Mishra, B. Roy, S.K. Ghosh, T.K. Maiti, Assessing the immunomodulatory role of heteroglycan in a tumor spheroid and macrophage co-culture model system, *Carbohydr Polym*, 127 (2015) 1-10.
- [54] F. Fehlauer, M. Muench, E. Richter, D. Rades, The inhibition of proliferation and migration of glioma spheroids exposed to temozolomide is less than additive if combined with irradiation, *Oncol Rep*, 17 (2007) 941-945.
- [55] R.J. Murphy, P.R. Buenzli, R.E. Baker, M.J. Simpson, Mechanical cell competition in heterogeneous epithelial tissues, *Bull Math Biol*, 82 (2020) 130.
- [56] M. Roy, S.D. Finley, Metabolic reprogramming dynamics in tumor spheroids: Insights from a multicellular, multiscale model, *PLoS Comput Biol*, 15 (2019) e1007053.
- [57] M. Mishkovsky, O. Gusyatiner, B. Lanz, C. Cudalbu, I. Vassallo, M.-F. Hamou, J. Bloch, A. Comment, R. Gruetter, M.E. Hegi, Hyperpolarized ¹³C-glucose magnetic resonance highlights reduced aerobic glycolysis *in vivo* in infiltrative glioblastoma, *Sci Rep*, 11 (2021) 5771.
- [58] Q. Yu, W. Xiao, S. Sun, A. Sohrabi, J. Liang, S.K. Seidlits, Extracellular matrix proteins confer cell adhesion-mediated drug resistance through integrin α_v in glioblastoma cells, *Front Cell Dev Biol*, 9 (2021).
- [59] M. Nakada, S. Nakada, T. Demuth, N.L. Tran, D.B. Hoelzinger, M.E. Berens, Molecular targets of glioma invasion, *Cell Mol Life Sci*, 64 (2007) 458-478.
- [60] V.P. Ferrer, V. Moura Neto, R. Mentlein, Glioma infiltration and extracellular matrix: key players and modulators, *Glia*, 66 (2018) 1542-1565.

- [61] C. Cannavà, R. Stancanelli, M.R. Marabeti, V. Venuti, C. Cascio, P. Guarneri, C. Bongiorno, G. Sortino, D. Majolino, A. Mazzaglia, S. Tommasini, C.A. Ventura, Nanospheres based on PLGA/amphiphilic cyclodextrin assemblies as potential enhancers of Methylene Blue neuroprotective effect, *RSC Adv*, 6 (2016) 16720-16729.
- [62] T.L. Moore, L. Rodriguez-Lorenzo, V. Hirsch, S. Balog, D. Urban, C. Jud, B. Rothen-Rutishauser, M. Lattuada, A. Petri-Fink, Nanoparticle colloidal stability in cell culture media and impact on cellular interactions, *Chem Soc Rev*, 44 (2015) 6287-6305.
- [63] S.-Y. Wu, H.-Y. Chou, H.-C. Tsai, R. Anbazhagan, C.-H. Yuh, J.M. Yang, Y.-H. Chang, Amino acid-modified PAMAM dendritic nanocarriers as effective chemotherapeutic drug vehicles in cancer treatment: A study using zebrafish as a cancer model, *RSC Adv*, 10 (2020) 20682-20690.
- [64] L. Li, X. Di, M. Wu, Z. Sun, L. Zhong, Y. Wang, Q. Fu, Q. Kan, J. Sun, Z. He, Targeting tumor highly-expressed LAT1 transporter with amino acid-modified nanoparticles: Toward a novel active targeting strategy in breast cancer therapy, *Nanomedicine*, 13 (2017) 987-998.
- [65] L. Li, X. Di, S. Zhang, Q. Kan, H. Liu, T. Lu, Y. Wang, Q. Fu, J. Sun, Z. He, Large amino acid transporter 1 mediated glutamate modified docetaxel-loaded liposomes for glioma targeting, *Colloids Surf B*, 141 (2016) 260-267.
- [66] Z. Wang, D. Chi, X. Wu, Y. Wang, X. Lin, Z. Xu, H. Liu, J. Sun, Z. He, Y. Wang, Tyrosine modified irinotecan-loaded liposomes capable of simultaneously targeting LAT1 and ATB(0,+) for efficient tumor therapy, *J Control Release*, 316 (2019) 22-33.
- [67] S. Okubo, H.-N. Zhen, N. Kawai, Y. Nishiyama, R. Haba, T. Tamiya, Correlation of l-methyl-11C-methionine (MET) uptake with l-type amino acid transporter 1 in human gliomas, *J Neurooncol*, 99 (2010) 217-225.
- [68] S. Nozaki, Y. Nakatani, A. Mawatari, N. Shibata, W.E. Hume, E. Hayashinaka, Y. Wada, H. Doi, Y. Watanabe, 18F-FIMP: A LAT1-specific PET probe for discrimination between tumor tissue and inflammation, *Sci Rep*, 9 (2019) 15718.
- [69] A. Habermeier, J. Graf, B.F. Sandhöfer, J.P. Boissel, F. Roesch, E.I. Closs, System l amino acid transporter LAT1 accumulates O-(2-fluoroethyl)-l-tyrosine (FET), *Amino Acids*, 47 (2015) 335-344.
- [70] L. Quan, R. Ohgaki, S. Hara, S. Okuda, L. Wei, H. Okanishi, S. Nagamori, H. Endou, Y. Kanai, Amino acid transporter LAT1 in tumor-associated vascular endothelium promotes angiogenesis by regulating cell proliferation and VEGF-A-dependent mTORC1 activation, *J Exp Clin Cancer Res*, 39 (2020) 266.
- [71] S. Fukumoto, K. Hanazono, D.-R. Fu, Y. Endo, T. Kadosawa, H. Iwano, T. Uchide, A new treatment for human malignant melanoma targeting L-type amino acid transporter

- 1 (LAT1): A pilot study in a canine model, *Biochem Biophys Res Commun*, 439 (2013) 103-108.
- [72] L. Cai, S.V. Kirchleitner, D. Zhao, M. Li, J.-C. Tonn, R. Glass, R.E. Kälin, Glioblastoma exhibits inter-individual heterogeneity of TSPO and LAT1 expression in neoplastic and parenchymal cells, *Int J Mol Sci*, 21 (2020) 612.
- [73] H. Pan, L.H. Xu, M.Y. Huang, Q.B. Zha, G.X. Zhao, X.F. Hou, Z.J. Shi, Q.R. Lin, D.Y. Ouyang, X.H. He, Piperine metabolically regulates peritoneal resident macrophages to potentiate their functions against bacterial infection, *Oncotarget*, 6 (2015) 32468-32483.
- [74] H. Basun, L.G. Forssell, O. Almkvist, R.F. Cowburn, R. Eklöf, B. Winblad, L. Wetterberg, Amino acid concentrations in cerebrospinal fluid and plasma in Alzheimer's disease and healthy control subjects, *J Neural Transm Park Dis Dement Sect*, 2 (1990) 295-304.
- [75] E. Calenoff, Interplaying factors that effect multiple sclerosis causation and sustenance, *ISRN Neurol*, 2012 (2012) 851541.
- [76] W. Hild, K. Pollinger, A. Caporale, C. Cabrele, M. Keller, N. Pluym, A. Buschauer, R. Rachel, J. Tessmar, M. Breunig, A. Goepferich, G protein-coupled receptors function as logic gates for nanoparticle binding and cell uptake, *Proc Natl Acad Sci USA*, 107 (2010) 10667-10672.
- [77] C.S. Schneider, J.G. Perez, E. Cheng, C. Zhang, P. Mastorakos, J. Hanes, J.A. Winkles, G.F. Woodworth, A.J. Kim, Minimizing the non-specific binding of nanoparticles to the brain enables active targeting of Fn14-positive glioblastoma cells, *Biomaterials*, 42 (2015) 42-51.
- [78] K. Rudolf, W. Eberlein, W. Engel, H.A. Wieland, K.D. Willim, M. Entzeroth, W. Wienen, A.G. Beck-Sickinger, H.N. Doods, The first highly potent and selective non-peptide neuropeptide Y Y1 receptor antagonist: BIBP3226, *Eur J Pharmacol*, 271 (1994) R11-13.
- [79] X. Montet, M. Funovics, K. Montet-Abou, R. Weissleder, L. Josephson, Multivalent effects of RGD peptides obtained by nanoparticle display, *J Med Chem*, 49 (2006) 6087-6093.
- [80] R.H. Kramer, J.W. Karpen, Spanning binding sites on allosteric proteins with polymer-linked ligand dimers, *Nature*, 395 (1998) 710-713.
- [81] M.A. Islam, Y. Xu, W. Tao, J.M. Ubellacker, M. Lim, D. Aum, G.Y. Lee, K. Zhou, H. Zope, M. Yu, W. Cao, J.T. Oswald, M. Dinarvand, M. Mahmoudi, R. Langer, P.W. Kantoff, O.C. Farokhzad, B.R. Zetter, J. Shi, Restoration of tumour-growth suppression in vivo via systemic nanoparticle-mediated delivery of PTEN mRNA, *Nat Biomed Eng*, 2 (2018) 850-864.

- [82] Y. Wang, Y. Xie, J. Li, Z.-H. Peng, Y. Sheinin, J. Zhou, D. Oupický, Tumor-penetrating nanoparticles for enhanced anticancer activity of combined photodynamic and hypoxia-activated therapy, *ACS Nano*, 11 (2017) 2227-2238.
- [83] S.M. Roy, V. Garg, S. Barman, C. Ghosh, A.R. Maity, S.K. Ghosh, Kinetics of nanomedicine in tumor spheroid as an *in vitro* model system for efficient tumor-targeted drug delivery with insights from mathematical models, *Front Bioeng Biotechnol*, 9 (2021) 785937.
- [84] F. Dilnawaz, S.K. Sahoo, Enhanced accumulation of curcumin and temozolomide loaded magnetic nanoparticles executes profound cytotoxic effect in glioblastoma spheroid model, *Eur J Pharm Biopharm*, 85 (2013) 452-462.
- [85] A. Christofides, L. Strauss, A. Yeo, C. Cao, A. Charest, V.A. Boussiotis, The complex role of tumor-infiltrating macrophages, *Nat Immunol*, 23 (2022) 1148-1156.
- [86] Z. Chen, D. Hambardzumyan, Immune microenvironment in glioblastoma subtypes, *Front Immunol*, 9 (2018) 1004.
- [87] Y. Komohara, K. Ohnishi, J. Kuratsu, M. Takeya, Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas, *J Pathol*, 216 (2008) 15-24.
- [88] C.R. Millrud, M. Mehmeti, K. Leandersson, Docetaxel promotes the generation of anti-tumorigenic human macrophages, *Exp Cell Res*, 362 (2018) 525-531.
- [89] K.N. Kodumudi, K. Woan, D.L. Gilvary, E. Sahakian, S. Wei, J.Y. Djeu, A novel chemoimmunomodulating property of docetaxel: Suppression of myeloid-derived suppressor cells in tumor bearers, *Clin Cancer Res*, 16 (2010) 4583-4594.
- [90] C.T. Garnett, J. Schlom, J.W. Hodge, Combination of docetaxel and recombinant vaccine enhances T-cell responses and antitumor activity: Effects of docetaxel on immune enhancement, *Clin Cancer Res*, 14 (2008) 3536-3544.
- [91] L. Barthel, M. Hadamitzky, P. Dammann, M. Schedlowski, U. Sure, B.K. Thakur, S. Hetze, Glioma: Molecular signature and crossroads with tumor microenvironment, *Cancer Metastasis Rev*, 41 (2022) 53-75.
- [92] Q. Wei, O. Singh, C. Ekinici, J. Gill, M. Li, Y. Mamatjan, S. Karimi, S. Bunda, S. Mansouri, K. Aldape, G. Zadeh, TNF α secreted by glioma associated macrophages promotes endothelial activation and resistance against anti-angiogenic therapy, *Acta Neuropathol Commun*, 9 (2021) 67.
- [93] T. Hori, T. Sasayama, K. Tanaka, Y.I. Koma, M. Nishihara, H. Tanaka, S. Nakamizo, H. Nagashima, M. Maeyama, Y. Fujita, H. Yokozaki, T. Hirose, E. Kohmura, Tumor-associated macrophage related interleukin-6 in cerebrospinal fluid as a prognostic marker for glioblastoma, *J Clin Neurosci*, 68 (2019) 281-289.

- [94] N. Tong, Z. He, Y. Ma, Z. Wang, Z. Huang, H. Cao, L. Xu, Y. Zou, W. Wang, C. Yi, Z. Yin, Q. Wang, Tumor associated macrophages, as the dominant immune cells, are an indispensable target for immunologically cold tumor-glioma therapy?, *Front Cell Dev Biol*, 9 (2021) 706286.
- [95] K.P.L. Bhat, V. Balasubramaniyan, B. Vaillant, R. Ezhilarasan, K. Hummelink, F. Hollingsworth, K. Wani, L. Heathcock, J.D. James, L.D. Goodman, S. Conroy, L. Long, N. Lelic, S. Wang, J. Gumin, D. Raj, Y. Kodama, A. Raghunathan, A. Olar, K. Joshi, C.E. Pelloski, A. Heimberger, S.H. Kim, D.P. Cahill, G. Rao, W.F.A. Den Dunnen, H. Boddeke, H.S. Phillips, I. Nakano, F.F. Lang, H. Colman, E.P. Sulman, K. Aldape, Mesenchymal differentiation mediated by NF- κ B promotes radiation resistance in glioblastoma, *Cancer Cell*, 24 (2013) 331-346.
- [96] Z. Azam, S.T. To, B.A. Tannous, Mesenchymal transformation: The rosetta stone of glioblastoma pathogenesis and therapy resistance, *Adv Sci* 7(2020) 2002015.
- [97] Q. Wang, Z. He, M. Huang, T. Liu, Y. Wang, H. Xu, H. Duan, P. Ma, L. Zhang, S.S. Zamvil, J. Hidalgo, Z. Zhang, D.M. O'Rourke, N. Dahmane, S. Brem, Y. Mou, Y. Gong, Y. Fan, Vascular niche IL-6 induces alternative macrophage activation in glioblastoma through HIF-2 α , *Nat Commun*, 9 (2018) 559.
- [98] J. Zhang, S. Sarkar, R. Cua, Y. Zhou, W. Hader, V.W. Yong, A dialog between glioma and microglia that promotes tumor invasiveness through the CCL2/CCR2/interleukin-6 axis, *Carcinogenesis*, 33 (2012) 312-319.
- [99] Q. Ma, W. Long, C. Xing, J. Chu, M. Luo, H.Y. Wang, Q. Liu, R.-F. Wang, Cancer stem cells and immunosuppressive microenvironment in glioma, *Front Immunol*, 9 (2018).
- [100] E. Codrici, I.-D. Popescu, C. Tanase, A.-M. Enciu, Friends with benefits: Chemokines, glioblastoma-associated microglia/macrophages, and tumor microenvironment, *Int J Mol Sci*, 23 (2022) 2509.
- [101] M.G. McCoy, D. Nyanyo, C.K. Hung, J.P. Goerger, W. R. Zipfel, R.M. Williams, N. Nishimura, C. Fischbach, Endothelial cells promote 3D invasion of GBM by IL-8-dependent induction of cancer stem cell properties, *Sci Rep*, 9 (2019) 9069.
- [102] M. Wang, Z. Zhou, X. Wang, C. Zhang, X. Jiang, Natural killer cell awakening: Unleash cancer-immunity cycle against glioblastoma, *Cell Death Dis*, 13 (2022) 588.
- [103] M.M. Grabowski, E.W. Sankey, K.J. Ryan, P. Chongsathidkiet, S.J. Lorrey, D.S. Wilkinson, P.E. Fecci, Immune suppression in gliomas, *J Neurooncol*, 151 (2021) 3-12.
- [104] Z. Amoozgar, J. Kloepper, J. Ren, R.E. Tay, S.W. Kazer, E. Kiner, S. Krishnan, J.M. Posada, M. Ghosh, E. Mamessier, C. Wong, G.B. Ferraro, A. Batista, N. Wang, M. Badeaux, S. Roberge, L. Xu, P. Huang, A.K. Shalek, D. Fukumura, H.-J. Kim, R.K. Jain,

Targeting Treg cells with GITR activation alleviates resistance to immunotherapy in murine glioblastomas, *Nat Commun*, 12 (2021) 2582.

[105] A. Buonfiglioli, D. Hambardzumyan, Macrophages and microglia: The cerberus of glioblastoma, *Acta Neuropathol Commun*, 9 (2021) 54.

[106] E. Oelmann, A. Kraemer, H. Serve, B. Reufi, D. Oberberg, S. Patt, H. Herbst, H. Stein, E. Thiel, W.E. Berdel, Autocrine interleukin-1 receptor antagonist can support malignant growth of glioblastoma by blocking growth-inhibiting autocrine loop of interleukin-1, *Int J Cancer*, 71 (1997) 1066-1076.

[107] H.J. Marcus, K.L.H. Carpenter, S.J. Price, P.J. Hutchinson, In vivo assessment of high-grade glioma biochemistry using microdialysis: A study of energy-related molecules, growth factors and cytokines, *J Neurooncol*, 97 (2010) 11-23.

[108] M. Murakami, D. Kamimura, T. Hirano, Pleiotropy and specificity: Insights from the interleukin 6 family of cytokines, *Immunity*, 50 (2019) 812-831.

[109] E.C.F. Yeo, M.P. Brown, T. Gargett, L.M. Ebert, The role of cytokines and chemokines in shaping the immune microenvironment of glioblastoma: Implications for immunotherapy, *Cells*, 10 (2021) 607.

[110] A.R. Harris, M.J. Perez, J.M. Munson, Docetaxel facilitates lymphatic-tumor crosstalk to promote lymphangiogenesis and cancer progression, *BMC Cancer*, 18 (2018) 718.

CHAPTER V. General discussion and future perspectives

1. GENERAL DISCUSSION

The lethal course of glioblastoma (GBM), characterized by a median survival time of only 15 months from diagnosis, urges the need to develop new therapeutic approaches with a higher efficacy, and new *in vitro* tools to accelerate the *in vitro* testing and translation of novel drugs. On the one hand, for the development of new therapeutic approaches, it is crucial to design systems with capacity to efficiently surpass the blood-brain barrier (BBB), which represents a significant hurdle for drug delivery to the brain. Moreover, it is also important to design systems with simultaneous capacity to precisely target the tumor cells of the brain in order to increase drug therapeutic indices. To accomplish both of these unmet needs, responsible for the failure of recent late-phase clinical trials, this work proposes BBB and tumor multi-targeted nanoparticles (NPs) with BBB-stimulus responsiveness to deliver docetaxel (DTX) to GBM as a model chemotherapy. By exploiting the overexpression of the low-density lipoprotein receptor (LDLR) and L-type amino acid transporter 1 (LAT1) in BBB and GBM cells, respectively, these stimulus-responsive NPs induce processes of drug intracellular trafficking and transport at the targeted sites. On the other hand, the development of new *in vitro* tools to support GBM drug testing requires high-throughput co-culture of tumor microenvironment (TME)-representative cell types with capacity to recapitulate the native features and complexity of the tumor. In sum, this doctoral thesis gathered together the areas of chemistry, materials science, pharmaceutical nanotechnology, cancer biology and cancer clinics, to create innovative anti-GBM strategies, based on (i) a stimulus-responsive intravenous nano-chemotherapy, and (ii) a 3D tumor microtissue-mimicking model.

Regarding the stimulus-responsive nano-chemotherapy (**CHAPTER III**), polymeric conjugates modified with either a L-Histidine amino acid (His) or acid-cleavable Angiopep-2 peptide (ANG2) moieties were chemically synthesized to serve as matrix of the proposed stimulus-sensitive NPs. Techniques such as ^1H NMR, FTIR and MALDI-TOF MS confirmed the successful chemical reactions, which presented conjugation efficiency values of more than 85%. NPs loaded with DTX were manufactured through a continuous-flow microfluidic technique, and presented a size range between 300 to 400 nm, 0.3 polydispersity index, and -35 to -25 mV zeta-potential. Although this size (>300 nm) is often seen as a limitation in what concerns BBB crossing ability, key literature references have demonstrated that this assumption is questionable. Studies conducted by Brown *et al.* [1], and also other authors [2-5], proved that, depending on the NP composition, 500 nm NPs might be actually able to transit across the BBB as efficiently as 100 nm NPs if undergoing receptor-mediated transport mechanisms. Thus, the

average size from 300 to 400 nm of the NPs herein produced was not foreseen as a potential drawback, since the NPs exploit the BBB-overexpressed LDLR to undergo blood-to-brain transport.

The first step of validation focused on the NPs functionalized only with His (H-NPs), for tumor targeting via LAT1. *In vitro* studies not only presented testing results in conventional GBM cell lines, but also in patient-derived GBM cells obtained from the tumor invasive margin of resected tissue. This GBM invasive margin (GIN) cells are crucial tools for *in vitro* testing since they are a molecular snapshot of the recurrent tumor, and highly resistant to chemotherapy. In U-87 MG, U-251 MG and U-373 MG cell lines, cell internalization of the NPs was increased by 3-fold through the use of the His targeting moiety. In GIN cells, this increase was even higher, of around 12-fold. As a control, non-tumor healthy astrocytes were used, and presented only basal levels of cell internalization of H-NPs. Then, GIN cells, in both 2D monolayer and 3D tumor spheroid settings, were assessed for metabolic activity inhibition. Here, H-NPs provided an enhance in metabolic activity inhibition by up to 2-fold. An *in vivo* trial was performed to confirm the therapeutic efficacy of H-NPs. A U-87 MG orthotopic GBM mice model was chosen, magnetic resonance imaging (MRI) assessments performed to evaluate appropriate tumor development, and animals were intratumorally administered (corresponding to 2 µg DTX per brain). The median survival was considerably improved by DTX-loaded H-NPs (46.5 days) compared with the untreated (30 days), DTX-loaded non-functionalized NPs (33.5 days), and free DTX (38 days) groups. Moreover, H-NPs resulted in a higher number of long-term survivors at 60 days (37.5% *versus* 0% for non-functionalized NPs) and 120 days (12.5% *versus* 0% for both non-functionalized NPs and free DTX). These findings offered robust evidence that, once inside the brain parenchyma, H-NPs boost the chemotherapeutic effect of DTX and provide an *in vivo* therapeutic benefit.

Apart from the His surface modification for tumor targeting, the second step of validation consisted of adding an acid-cleavable ANG2 surface moiety for enhanced NP shuttling from blood to brain (H-CL-ANG2-NPs). In a conventional hCMEC/D3 BBB *in vitro* model, H-CL-ANG2-NPs doubled the BBB permeation ability compared with H-NPs. *In vivo*, in a healthy mice model with an intact BBB, brain accumulation of H-CL-ANG2-NPs reached approximately 4-times higher values than H-NPs. Another *in vivo* trial was performed to confirm the therapeutic efficacy of H-CL-ANG2-NPs. A U-87 MG orthotopic GBM mice model was chosen, MRI assessments evaluated appropriate tumor development, and animals were intravenously administered (2 mg kg⁻¹, every 3 days, total of 5 injections). The median survival of the animals (54 days) presented a significant improvement compared with the untreated group (37 days), free DTX (43 days), DTX-

loaded non-functionalized NPs (38 days), DTX-loaded H-NPs (40.5 days), and DTX-loaded NPs functionalized with His and a non-cleavable ANG2 control moiety (41 days). Moreover, H-CL-ANG2-NPs presented an increase of more than 50% in the number of long-term survivors (at 60 days). Regarding the evaluation of systemic toxicity caused by the treatment, no significant alterations were found in mice body weight, organ weight coefficients, histological analysis (heart, liver, lungs, spleen, kidneys), hematologic parameters (blood total cell count), and blood biochemistry markers (blood urea nitrogen, creatinine, albumin, aspartate aminotransferase, alanine aminotransferase, total bilirubin and total protein), in comparison with the untreated animals.

Overall, these findings presented strong evidence that both the acid-cleavable properties of the ANG2 moiety and the presence of the His moiety in the stimuli-responsive multifunctional NPs herein proposed are paramount to achieve higher BBB permeation and targeting of GBM cells, respectively, resulting in a potent anti-cancer effect. Although the DTX dose used in *in vivo* studies were considerably lower (2 mg kg^{-1}) than the doses reported by other authors (7.5 to 10 mg kg^{-1} [6-8]), the final NP prototype was still able to provide a prominent anti-GBM effect. In order to understand the therapeutic advantage of this final NP prototype compared with nanosystems previously reported by other authors, a review of literature of the past 10 years was performed. By pre-selecting works based on the use of the same GBM animal model herein assessed (U-87 MG) and dual-targeting (BBB and GBM), the maximum values of median survival were 45 days [9], 25 days [10], 35 days [11-14] and 40 days [15-17], significantly lower than the median survival obtained for the proposed NP prototype (54 days). The BBB and GBM dual-targeted system (lacking BBB responsiveness) developed by Zhu *et al.* reached 53 days median survival, but using a DTX dose 4-times higher than the one herein administered (2 versus 7.5 mg kg^{-1}) [8]. This literature review confirmed the therapeutic advantage of the proposed NP prototype, and the promising potential of this nano-chemotherapy to increase the survival of GBM patients in a clinical setting in the future.

Regarding the 3D tumor-mimicking model (**CHAPTER IV**), a GBM multicellular tumor microtissue (MCTM) was developed by co-culturing in high-throughput agarose micro-molds, U-251 MG GBM cells, macrophages derived from hospital buffy coats-isolated peripheral monocytes (MONs), and brain microvascular primary endothelial cells (ECs). The MCTM presented a spheroid-like structure of around $500 \mu\text{m}$ diameter, maintenance of cell viability *per se* over 7 days, areas of necrosis reassembling the native tumor, and endogenous production of extracellular matrix (ECM). H-NPs loaded with DTX, previously reported, were used as a model nanomedicine to assess the ability of the GBM MCTM to respond to chemotherapy. Compared with non-functionalized NPs and

the free drug, H-NPs provided an enhancement in MCTM cell uptake and cytotoxicity of up to approximately 9- and 4-fold, respectively. The immune environment of the GBM MCTM was characterized by a predominant pro-tumor M2-macrophage phenotype. The treatment with DTX-loaded H-NPs tended to slightly decrease the pro-tumor M2-macrophage markers within the MCTM, while increasing the anti-tumor M1 counterparts. Through analysis of the secretion of a panel of medium cytokines, it was possible to observe that the treatment with DTX-loaded H-NPs also provided a slight decrease in the levels of cytokines associated with a pro-tumor role (e.g. IL-1 β , IL-6, IL-8 and TGF- β).

Altogether, this work allowed the development of a novel triple-culture GBM MCTM with ability to mimic, in a comprehensive manner, the tumor cell-macrophage-EC interplay within the GBM TME. Compared with literature data, the model herein proposed presents an upgrade towards more humanized *in vitro* GBM technologies regarding the human and primary cell sources, as well as the inclusion of three of the most representative GBM cell types - previously reported 3D cell culture systems only combined GBM cells with either macrophage cell lines or ECs [18-21]. Therefore, the proposed GBM 3D *in vitro* model recapitulates in a more biologically relevant manner the bioarchitecture and composition of the native tumor, presenting a promising disease modeling tool to dissect therapeutic resistance mechanisms, ground robust preclinical findings and offer a reliable *in vivo* predictability.

2. FUTURE PERSPECTIVES

Regarding the stimulus-responsive NPs, future perspectives include to corroborate the *in vivo* efficacy studies using a new cohort of animals. Moreover, due to the challenges of histological analysis faced in *in vivo* studies presented in **Chapter III**, a later endpoint should be chosen for brain excision and analysis of key makers of tumor progression and treatment effectiveness (e.g. cleaved caspase-3 and Ki67). It is also envisaged to perform quantification studies of DTX in brain and other major organs through mass spectrometry techniques to map drug biodistribution with higher precision. As another method of *in vivo* validation, different models need to be exploited to test the efficacy of the stimulus-responsive NPs. Firstly, even though an immunocompromised mice model was selected due to the possibility of development of a human tumor xenograft, a shift to an immunocompetent mice model should be considered. Immunocompetent models present a more accurate representation of the clinical scenario, including the impact of immune cells on the efficacy of the NPs [22]. An example of a well-reported

immunocompetent mice model is the GL261 [23, 24]. Another interesting *in vivo* model consists of canine spontaneous GBM, which presents a higher level of similarity with the human disease compared with small rodent models, and provides a large animal model system for a more realistic validation of the NPs [25]. To foster translation, future studies should also address the amelioration of the loading degree of the developed NPs (current value of only approximately 1.5%) to reduce productions costs. Although the NPs were manufactured through a microfluidic approach, which is known to possess capacity to scale up the batch size and provide high-throughput production of nanosystems [26], production parameters should be tuned and optimized to improve the final physicochemical characteristics of the formulation. In light of this and to prevent the possibility of future manufacture constraints in-house, a collaboration has been established with MyBiotech (Germany), a contract development and manufacturing organization (CDMO), to benefit from specialized microreactor technologies to achieve a highly reproducible up-scaling, cost- and environmental-friendly production of the DTX-loaded NPs, while still maintaining the quality of the existing lab-scale formulation [27-29]. To reduce production costs and considering the versatility of the herein used chemical strategies, other BBB and GBM targeting ligands might be screened for targeting efficiency in order to endow NPs with even a more powerful therapeutic effect. The T7 peptide is an example of an alternative ligand for the BBB-overexpressed transferrin receptor, whereas a fucoidan surface decoration could be explored to target the GBM-overexpressed P-selectin [30, 31]. Versatility also characterizes the loading ability of the proposed stimulus-responsive NPs – herein, DTX was used as a model chemotherapy, but several other therapeutic cargos might be exploited in the context of GBM therapies. These cargos can include the standard of care temozolomide chemotherapy, or prospective antibody drugs [32] and RNA therapeutics [33] – **Appendix II** presents an exploratory study that was already pursued to preliminarily assess the suitability of the developed NPs to encapsulate protein-based therapeutics in future approaches. Lastly, as the proposed stimuli-responsive NPs presented an enhanced blood-to-brain trafficking and tumor targeting, it is foreseen that the NP prototype might also be therapeutically advantageous in clinical cases of lower aggressiveness tumor models, such as brain metastasis and lower-grade gliomas.

In what concerns the 3D tumor-mimicking model, the first logical future step is the implantation of the GBM MCTM *in vivo*, in order to unravel the dynamics of tumor growth and evaluate the retention of its architectural features over time [34]. Since *in vivo* mice models present limited capacity of mimicking human physiology, the implantation of this humanized MCTM could better capture the human GBM scenario. In the perspective of

amelioration of the GBM MCTM, one of the limitations of the model is its inability to use the cultured ECs to form vasculature tubular structures as the highly angiogenic tumor native tissue. Thus, it would be of great importance to embed the GBM MCTM within a hydrogel with capacity to stimulate and support EC sprouting [35, 36], hence upgrading the model to a prevascularized tissue. For a more reliable representation of the human GBM, future work should focus on the inclusion of the GBM MCTM into a continuous-flow blood-mimicking model with structural and physical influence of the BBB. To achieve this, one possible approach is to recreate the BBB-GBM TME in a fluid-dynamic microfluidic chip where the GBM MCTM could be cultured in close contact with a BBB endothelial cell layer [37]. Lastly, the developed GBM MCTM grounds the basis for more complex 3D cellular structures, with co-culture of additional cell populations. For instance, the inclusion of neurons would be of great benefit to study the hijacking of neuronal mechanisms by GBM cells to fuel brain invasion [38]. Pericytes consist of another interesting cell population to be included in the GBM MCTM since these cells contribute to tumor progression through a variety of mechanisms, including the support of angiogenesis, and have been seen as potential targets for therapeutic interventions [39].

As a concluding remark, the work developed under the scope of this doctoral thesis paved the way towards the transition from bench to the bedside of two distinct and powerful tools to manage the GBM burden: (i) a cutting-edge stimulus-responsive nanomedicine to efficiently deliver therapeutic cargos across the BBB and target tumor cells, and (ii) a novel GBM 3D *in vitro* model to accelerate the translation of newly developed anti-cancer nanomedicines.

REFERENCES

- [1] T.D. Brown, N. Habibi, D. Wu, J. Lahann, S. Mitragotri, Effect of nanoparticle composition, size, shape, and stiffness on penetration across the blood–brain barrier, *ACS Biomater Sci Eng*, 6 (2020) 4916-4928.
- [2] T. Tsuji, H. Yoshitomi, J. Usukura, Endocytic mechanism of transferrin-conjugated nanoparticles and the effects of their size and ligand number on the efficiency of drug delivery, *Microscopy*, 62 (2013) 341-352.
- [3] J. Key, A.L. Palange, F. Gentile, S. Aryal, C. Stigliano, D. Di Mascolo, E. De Rosa, M. Cho, Y. Lee, J. Singh, P. Decuzzi, Soft discoidal polymeric nanoconstructs resist macrophage uptake and enhance vascular targeting in tumors, *ACS Nano*, 9 (2015) 11628-11641.
- [4] Z. Wang, J. Ding, X. Ma, S. Luo, Selective ultrasound contrast enhancement in the tumor by nanocapsules with perfluorooctylbromide: Effect of PLGA–PEG proportion, *RSC Adv*, 8 (2018) 17958-17966.
- [5] L. Ribovski, E. de Jong, O. Mergel, G. Zu, D. Keskin, P. van Rijn, I.S. Zuhorn, Low nanogel stiffness favors nanogel transcytosis across an in vitro blood–brain barrier, *Nanomedicine*, 34 (2021) 102377.
- [6] R. Fan, D. Chuan, H. Hou, H. Chen, B. Han, X. Zhang, L. Zhou, A. Tong, J. Xu, G. Guo, Development of a hybrid nanocarrier-recognizing tumor vasculature and penetrating the BBB for glioblastoma multi-targeting therapy, *Nanoscale*, 11 (2019) 11285-11304.
- [7] H. Gao, Z. Yang, S. Zhang, S. Cao, Z. Pang, X. Yang, X. Jiang, Glioma-homing peptide with a cell-penetrating effect for targeting delivery with enhanced glioma localization, penetration and suppression of glioma growth, *J Control Release*, 172 (2013) 921-928.
- [8] Y. Zhu, Y. Jiang, F. Meng, C. Deng, R. Cheng, J. Zhang, J. Feijen, Z. Zhong, Highly efficacious and specific anti-glioma chemotherapy by tandem nanomicelles co-functionalized with brain tumor-targeting and cell-penetrating peptides, *J Control Release*, 278 (2018) 1-8.
- [9] Z. Jin, L. Piao, G. Sun, C. Lv, Y. Jing, R. Jin, Dual functional nanoparticles efficiently across the blood–brain barrier to combat glioblastoma via simultaneously inhibit the PI3K pathway and NKG2A axis, *J Drug Target*, 29 (2021) 323-335.
- [10] X. Wang, Y. Zhao, S. Dong, R.J. Lee, D. Yang, H. Zhang, L. Teng, Cell-penetrating peptide and transferrin co-modified liposomes for targeted therapy of glioma, *Molecules*, 24 (2019) 3540.

- [11] S. Lakkadwala, B. dos Santos Rodrigues, C. Sun, J. Singh, Dual functionalized liposomes for efficient co-delivery of anti-cancer chemotherapeutics for the treatment of glioblastoma, *J Control Release*, 307 (2019) 247-260.
- [12] C. Liu, Z. Zhao, H. Gao, I. Rostami, Q. You, X. Jia, C. Wang, L. Zhu, Y. Yang, Enhanced blood-brain-barrier penetrability and tumor-targeting efficiency by peptide-functionalized poly(amidoamine) dendrimer for the therapy of gliomas, *Nanotheranostics*, 3 (2019) 311-330.
- [13] Z. Belhadj, C. Zhan, M. Ying, X. Wei, C. Xie, Z. Yan, W. Lu, Multifunctional targeted liposomal drug delivery for efficient glioblastoma treatment, *Oncotarget*, 8 (2017) 66889-66900.
- [14] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, Glioma targeting and blood-brain barrier penetration by dual-targeting doxorubicin liposomes, *Biomaterials*, 34 (2013) 5628-5639.
- [15] X. Zhu, H. Zhou, Y. Liu, Y. Wen, C. Wei, Q. Yu, J. Liu, Transferrin/aptamer conjugated mesoporous ruthenium nanosystem for redox-controlled and targeted chemo-photodynamic therapy of glioma, *Acta Biomater*, 82 (2018) 143-157.
- [16] C. Chen, Z. Duan, Y. Yuan, R. Li, L. Pang, J. Liang, X. Xu, J. Wang, Peptide-22 and cyclic RGD functionalized liposomes for glioma targeting drug delivery overcoming BBB and BBTB, *ACS Appl Mater Interfaces*, 9 (2017) 5864-5873.
- [17] P. Zhang, L. Hu, Q. Yin, L. Feng, Y. Li, Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy, *Mol Pharm*, 9 (2012) 1590-1598.
- [18] M.J. Gattas, I.G. Estecho, M.A. Lago Huvelle, A.E. Errasti, E.A. Carrera Silva, M. Simian, A heterotypic tridimensional model to study the interaction of macrophages and glioblastoma in vitro, *Int J Mol Sci*, 22 (2021).
- [19] M. Tang, S.K. Tiwari, K. Agrawal, M. Tan, J. Dang, T. Tam, J. Tian, X. Wan, J. Schimelman, S. You, Q. Xia, T.M. Rana, S. Chen, Rapid 3D bioprinting of glioblastoma model mimicking native biophysical heterogeneity, *Small*, 17 (2021) 2006050.
- [20] M.A. Heinrich, R. Bansal, T. Lammers, Y.S. Zhang, R. Michel Schiffelers, J. Prakash, 3D-bioprinted mini-brain: A glioblastoma model to study cellular interactions and therapeutics, *Adv Mater*, 31 (2019) e1806590.
- [21] E.A. Akins, M.K. Aghi, S. Kumar, Incorporating tumor-associated macrophages into engineered models of glioma, *iScience*, 23 (2020) 101770.
- [22] S. Đorđević, M.M. Gonzalez, I. Conejos-Sánchez, B. Carreira, S. Pozzi, R.C. Acúrcio, R. Satchi-Fainaro, H.F. Florindo, M.J. Vicent, Current hurdles to the translation of nanomedicines from bench to the clinic, *Drug Deliv Transl Res*, 12 (2022) 500-525.

- [23] M. Bausart, K. Vanvarenberg, B. Ucakar, A. Lopes, G. Vandermeulen, A. Malfanti, V. Pr  at, Combination of DNA vaccine and immune checkpoint blockades improves the immune response in an orthotopic unresectable glioblastoma model, *Pharmaceutics*, 14 (2022) 1025.
- [24] A. Malfanti, G. Catania, Q. Degros, M. Wang, M. Bausart, V. Pr  at, Design of bio-responsive hyaluronic acid-doxorubicin conjugates for the local treatment of glioblastoma, *Pharmaceutics*, 14 (2022) 124.
- [25] P.J. Dickinson, R.A. LeCouteur, R.J. Higgins, J.R. Bringas, R.F. Larson, Y. Yamashita, M.T. Krauze, J. Forsayeth, C.O. Noble, D.C. Drummond, D.B. Kirpotin, J.W. Park, M.S. Berger, K.S. Bankiewicz, Canine Spontaneous glioma: A translational model system for convection-enhanced delivery, *Neuro Oncol*, 12 (2010) 928-940.
- [26] A. Gdowski, K. Johnson, S. Shah, I. Gryczynski, J. Vishwanatha, A. Ranjan, Optimization and scale up of microfluidic nanolipomer production method for preclinical and potential clinical trials, *J Nanobiotechnology*, 16 (2018) 12-12.
- [27] N. G  nday T  reli, A. Torge, J. Juntke, B.C. Schwarz, N. Schneider-Daum, A.E. T  reli, C.-M. Lehr, M. Schneider, Ciprofloxacin-loaded PLGA nanoparticles against cystic fibrosis *P. aeruginosa* lung infections, *Eur J Pharm Biopharm*, 117 (2017) 363-371.
- [28] N. G  nday T  reli, A.E. T  reli, M. Schneider, Optimization of ciprofloxacin complex loaded PLGA nanoparticles for pulmonary treatment of cystic fibrosis infections: Design of experiments approach, *Int J Pharm*, 515 (2016) 343-351.
- [29] N. G  nday T  reli, A.E. T  reli, M. Schneider, Counter-ion complexes for enhanced drug loading in nanocarriers: Proof-of-concept and beyond, *Int J Pharm*, 511 (2016) 994-1001.
- [30] Y. Shamay, M. Elkabets, H. Li, J. Shah, S. Brook, F. Wang, K. Adler, E. Baut, M. Scaltriti, P.V. Jena, E.E. Gardner, J.T. Poirier, C.M. Rudin, J. Baselga, A. Haimovitz-Friedman, D.A. Heller, P-selectin is a nanotherapeutic delivery target in the tumor microenvironment, *Sci Transl Med*, 8 (2016) 345ra387.
- [31] D.P. Anthony, M. Hegde, S.S. Shetty, T. Rafic, S. Mutalik, B.S.S. Rao, Targeting receptor-ligand chemistry for drug delivery across blood-brain barrier in brain diseases, *Life Sci* 274 (2021) 119326.
- [32] N. Pandey, P. Anastasiadis, C.P. Carney, P.P. Kanvinde, G.F. Woodworth, J.A. Winkles, A.J. Kim, Nanotherapeutic treatment of the invasive glioblastoma tumor microenvironment, *Adv Drug Deliv Rev*, 188 (2022) 114415.
- [33] K. Melnick, F. Dastmalchi, D. Mitchell, M. Rahman, E.J. Sayour, Contemporary RNA therapeutics for glioblastoma, *Neuromolecular Med*, 24 (2022) 8-12.

- [34] M. Devarasetty, A. Dominijanni, S. Herberg, E. Shelkey, A. Skardal, S. Soker, Simulating the human colorectal cancer microenvironment in 3D tumor-stroma co-cultures in vitro and in vivo, *Sci Rep*, 10 (2020) 9832.
- [35] I.V. Vakhrushev, E.K. Nezhurina, P.A. Karalkin, A.V. Tsvetkova, N.S. Sergeeva, A.G. Majouga, K.N. Yarygin, Heterotypic multicellular spheroids as experimental and preclinical models of sprouting angiogenesis, *Biology (Basel)*, 11 (2021).
- [36] A. Wang, L.A. Madden, V.N. Paunov, Fabrication of angiogenic sprouting coculture of cell clusteroids using an aqueous two-phase pickering emulsion system, *ACS Applied Bio Materials*, 5 (2022) 1804-1816.
- [37] J.P. Straehla, C. Hajal, H.C. Safford, G.S. Offeddu, N. Boehnke, T.G. Dacoba, J. Wyckoff, R.D. Kamm, P.T. Hammond, A predictive microfluidic model of human glioblastoma to assess trafficking of blood–brain barrier-penetrant nanoparticles, *Proc Natl Acad Sci USA*, 119 (2022) e2118697119.
- [38] V. Venkataramani, Y. Yang, M.C. Schubert, E. Reyhan, S.K. Tetzlaff, N. Wißmann, M. Botz, S.J. Soyka, C.A. Beretta, R.L. Pramatarov, L. Fankhauser, L. Garofano, A. Freudenberg, J. Wagner, D.I. Tanev, M. Ratliff, R. Xie, T. Kessler, D.C. Hoffmann, L. Hai, Y. Dörflinger, S. Hoppe, Y.A. Yabo, A. Golebiewska, S.P. Niclou, F. Sahm, A. Lasorella, M. Slowik, L. Döring, A. Iavarone, W. Wick, T. Kuner, F. Winkler, Glioblastoma hijacks neuronal mechanisms for brain invasion, *Cell*, 185 (2022) 2899-2917.e2831.
- [39] A. Sattiraju, A. Mintz, Pericytes in glioblastomas: Multifaceted role within tumor microenvironments and potential for therapeutic interventions, *Adv Exp Med Biol*, 1147 (2019) 65-91.

APPENDIX I. Supplementary data of Chapter I (Literature review)

This appendix was based on the following publication:

- **C Martins**, B Sarmiento. “Multi-ligand functionalized blood-to-tumor sequential targeting strategies in the field of glioblastoma nanomedicine” (manuscript under review)

Table 1 – A) Nanomedicines approved by regulatory agencies for cancer therapy (discontinued products such as Depocyt were avoided). Antibody-drug conjugates and engineered proteins (e.g. fusion proteins) were not included. B) Examples of targeted cancer nanomedicines undergoing clinical trials.

Product designation	Carrier type	Cargo	Administration route	Disease indication	Year and region of first approval	References
A. Clinically-approved cancer nanomedicines						
SMANCS	Poly(styrene-co-maleic acid)-conjugated drug	Neocarzinostatin	Trans-arterial chemoembolization	Hepatocellular carcinoma	1994, Japan	[1]
Doxil/Caelyx/Lipodox	Liposomes	DOX	Intravenous	Kaposi sarcoma, multiple myeloma, metastatic breast and ovarian cancer	1995, US	[2, 3]
DaunoXome	Liposomes	Daunorubicin	Intravenous	Kaposi sarcoma	1996, US	[4]
Myocet	Liposomes	DOX	Intravenous	Metastatic breast cancer	2000, Europe	[5]
Lipusu	Liposomes	PTX	Intravenous	Metastatic gastric cancer	2003, China	[6]
Abraxane/Pazenir	Albumin NPs	PTX	Intravenous	Advanced non-small cell lung, metastatic breast and metastatic pancreatic cancer	2005, US	[7, 8]
Oncaspar	PEG-conjugated drug	L-asparaginase	Intravenous, intramuscular	Acute lymphoblastic leukemia	2006, US	[9, 10]
Nanoxel	Micelles of pH-sensitive co-polymer of N-isopropyl acrylamide and vinylpyrrolidone monomers	PTX	Intravenous	Non-small cell lung and breast cancer	2006, India	[11]
Genexol-PM	Micelles of PEG and PLA	PTX	Intravenous	Non-small cell lung, breast, ovarian and gastric cancer	2007, Korea	[12]
Mepact	Liposomes	Mifamurtide	Intravenous	Osteosarcoma	2009, Europe	[13]
NanoTherm	Iron oxide inorganic NPs	-	Intratumoral	Recurrent GBM	2010, Europe	[14, 15]
Marqibo	Liposomes	Vincristine	Intravenous	Philadelphia chromosome-negative acute lymphoblastic leukaemia and non-small-cell lung cancer	2012, US	[6, 16]

PICN	Liposomal nanosuspension (stabilized with polymer and lipids)	PTX	Intravenous	Metastatic breast cancer	2014, India	[17, 18]
Onivyde (MM-398)	Liposomes	Irinotecan	Intravenous	Metastatic pancreatic cancer	2015, US	[19]
Paclical/Apealea	Micelles of isoforms of N-retinoyl-cysteic acid methyl esters	PTX	Intravenous	Peritoneal, ovarian and fallopian tube cancer	2015, Russian Federation	[9, 20]
Liporaxel (DHP-107)	Lipid/emulsion-based	PTX	Oral	Advanced gastric cancer	2016, Korea	[21]
Vyxeos	Liposomes	Cytarabine and daunorubicin (5:1)	Intravenous	High-risk acute myeloid leukemia	2017, US	[22]
Hensify	Hafnium oxide inorganic NPs	-	Intratumoral	Locally-advanced soft tissue sarcoma	2019, Europe	[23]
B. Targeted cancer nanomedicines undergoing clinical trials						
MCC-465	Liposomes (GAH antibody fragment targeting moiety)	DOX	Intravenous	Stomach cancer	Phase I	[24, 25]
C225-ILS-DOX	Liposomes (cetuximab antibody fragment targeting moiety)	DOX	Intravenous	Solid tumors (EGFR target)	Phase I	[26]
MBP-426	Liposomes (Tf targeting moiety)	Oxaliplatin	Intravenous	Solid tumors (transferrin receptor (TfR) target)	Phase I	[27, 28]
CALAA-01	Cyclodextrin and PEG polymeric NPs (Tf targeting moiety)	siRNA against ribonucleotide reductase M2	Intravenous	Solid tumors (TfR target)	Phase I	[29]
89Zr-DFO-cRGDY-PEG-Cy5-C' dots	Silica inorganic NPs (RGD peptide targeting moiety)	Zirconium-89 positron emission tomography label and dasatinib prototype small molecule inhibitor	Intravenous	High-grade gliomas (tumor and tumor vasculature α_v integrins target)	Phase I	[30]
2B3-101	Liposomes (glutathione tripeptide targeting moiety)	DOX	Intravenous	Brain tumors (unknown transporter target at the BBB)	Phase II	[28, 31]

SGT-53	Liposomes (TfR antibody fragment targeting moiety)	Wild-type p53 DNA	Intravenous	Solid tumors (TfR target)	Phase II	[32]
MM-302	Liposomes (HER2 antibody targeting moiety)	DOX	Intravenous	Breast cancer (HER2 target)	Phase III	[33]

Table 2 – Multi-ligand functionalized, BBB and tumor sequential- and dual-targeted nanosystems reported in literature for the management of GBM. The presented nanosystems were designed for intravenous administration. The table does not include nanosystems presenting multi-ligand functionalization only for the purpose of higher BBB crossing and not envisaging tumor targeting; functionalization moieties can be therapeutic; single-ligand functionalization strategies based on fusion proteins or chimeric peptides were not included; studies presenting *in vivo* data were only included if providing GBM orthotopic testing models.

Carrier type	Cargo(s)	BBB/ neovasculature target(s)	BBB- /neovasculature- directed moiety(ies)	GBM target(s)	GBM- directed moiety(ies)	Key findings	References
Lipid NPs	Bortezomib	nAChRs	D8 peptide	GRP78	RI-VAP peptide	Median survival of GL261 GBM orthotopic mice model increased to 46 days (16, 26, 30, 36 and 29 days for the untreated group, free drug, non-functionalized NPs, D8-NPs and RI-VAP-NPs, respectively)	[34]
Liposomes	-	LDLR	Polysorbate 80 (P80) coating	Folate receptor Cell mitochondria	Folic acid (acid-cleavable) Berberine isoquinoline plant alkaloid	Similar brain accumulation to P80-coated liposomes in a C6 orthotopic GBM mice model, and superior brain accumulation compared with non-functionalized liposomes, berberine-liposomes, folic acid-liposomes, and folic acid plus berberine liposomes	[35]
Liposomes	DOX and Iridamine	Dopamine and sigma receptors	p-HA (acid-cleavable)	Sigma receptors	p-HA (acid-cleavable)	Median survival of C6 GBM orthotopic mice model increased to 41.5 days (16.5, 22, 23.5, 24.5 and 33.5 days	[36]

	Cell mitochondria	Triphenylphosphonium (TPP, endosomal escape)	for the untreated group, free drugs cocktail, non-functionalized liposomes, TPP-liposomes, and TPP plus non-cleavable p-HA liposomes, respectively)				
PEG monomethacrylate polymeric NPs	Choline transporter	Choline analogue	PD-1 protein (cytotoxic T cells)	PD-L1 antibody (acid-cleavable)	Median survival of LCPN GBM orthotopic mice model increased to 19 days (8, 8 and 12 days for the untreated group, free PD-L1 antibody and non-functionalized NPs, respectively)	[37]	
Dendrimers	LSINCT5 siRNA	-	tlp-1 CPP	NKG2A receptor (T and NK cells)	NKG2A antibody (acid-cleavable)	Median survival of U-87 MG GBM orthotopic mice model increased to around 45 days (20, 40 and 30 days for the non-functionalized NPs, tlyp-1-NPs and NKG2A antibody-NPs, respectively)	[38]
Erythrocyte membrane-enveloped PLGA polymeric NPs	Euphorbia factor L1	-	DWSW bacterial quorum sensing peptide	CD13 receptor	NGR peptide	Median survival of C6 GBM orthotopic mice model increased to 36 days (20, 22.5, 24, 27.7 and 30 days for the untreated group, free cargo, non-functionalized NPs, DWSW-NPs and NGR-NPs, respectively)	[39]
Dendrimers	Arsenic trioxide	Not mentioned	TGN peptide	NRP1R	RGD peptide	Median survival of glioma-bearing mice (model unspecified) increased to 24.3 days (14.5, 14.25, 18.5 and 16.4 days for the untreated group, free cargo, RGD-NPs and TGN-NPs, respectively)	[40]
Liposomes	DOX	TfR	Tf protein	TfR	Tf protein	Median survival of U-87 MG GBM orthotopic mice model	[41]

									increased to 25 days (20, 22 and 24 days for the untreated group, free drug and non-functionalized liposomes, respectively)
								R8 CPP	
									Median survival of U-87 MG GBM orthotopic mice model increased to 36 days (22, 25, 25.5, 30 and 27.5 days for the untreated group, free drugs cocktail, non-functionalized liposomes, Tf-liposomes and Pen-liposomes, respectively)
Liposomes	DOX and erlotinib	TfR	Tf protein	-				Pen CPP	[42]
Dendrimers	DOX	LDLR	ANG2 peptide	EGFR				EP-1 peptide	[43]
									Median survival of U-87 MG GBM orthotopic mice model increased to 34 days (16, 19, 24, 31 and 27 days for the untreated group, free DOX, non-functionalized NPs, ANG2-NPs and EP-1-NPs, respectively)
Erythrocyte membrane-enveloped lipid NPs	Vincristine	TfR	T7 peptide	CD13 receptor				NGR peptide	[44]
									Median survival of C6 GBM orthotopic mice model increased to 36 days (18, 19, 20, 29 and 21 days for the untreated group, free drug, non-functionalized NPs, T7-NPs and NGR-NPs, respectively)
Liposomes	5-fluorouracil	TfR	Tf protein	-				Pen CPP	[45]
									Lower cell viability (50-60%) in a 3D <i>in vitro</i> tumor model compared with the free drug (90%), non-functionalized liposomes (80%), Tf-liposomes (70%) and Pen-liposomes (80%)
PLGA polymeric NPs	DOX	LDLR	ANG2 peptide	Nucleolin				AS1411 aptamer	[46]
									BBB <i>in vitro</i> crossing capacity similar to ANG2-NPs, and higher than free DOX, non-

functionalized NPs and AS1411-NPs					
Dithiolane trimethylene carbonate and PEG micelles	DTX	LDLR	ANG2 peptide	LDLR	ANG2 peptide
				-	TAT CPP
Mesoporous ruthenium inorganic NPs	RBT (attached via glutathione-cleavable bonds)	TfR	Tf protein	Nucleolin	AS1411 aptamer
Liposomes	DOX	Dopamine receptors	pHA	$\alpha v \beta 3$ integrins	RGD peptide
		$\alpha v \beta 3$ integrins	RGD peptide		
Liposomes	DOX	LDLR	Pep-22 peptide	$\alpha v \beta 3 / \alpha v \beta 5$ integrins	RGD peptide
		$\alpha v \beta 3 / \alpha v \beta 5$ integrins	RGD peptide		
PLGA and PEG polymeric NPs	PTX	Heparan sulfate	CGKRK peptide	IL-13 $\alpha 2$ receptor	Pep-1 peptide

Lipid NPs	Model siRNA	LDLR	ANG2 peptide	LDLR		U-87 MG GBM cells and bEnd.3 BBB cells <i>in vitro</i> uptake similar to non-functionalized NPs and MMP-activable glutamic acid residues-NPs	[52]
				-	MMP-activable glutamic acid residues (endosomal escape)		
PCL and PEG polymeric NPs	DTX	LDLR	ANG2 peptide	-	MMP-activable R8 CPP	Median survival of C6 GBM orthotopic mice model increased to 32 days (19, 20, 26 and 27 days for the untreated group, non-functionalized NPs, R8-NPs and ANG2-NPs, respectively)	[53]
PCL and PEG polymeric NPs	-	$\alpha v\beta 3$ integrins	RGD peptide	IL-13 $\alpha 2$ receptor	IL-13 peptide	Slightly higher brain accumulation in a C6 orthotopic GBM mice model compared with non-functionalized NPs, RGD-NPs and IL-13 peptide-NPs	[54]
PCL and PEG polymeric NPs	DTX	$\alpha v\beta 3$ integrins	RGD peptide	IL-13 $\alpha 2$ receptor	IL-13 peptide	Median survival of C6 GBM orthotopic mice model increased to 35 days (17, 20, 22, 27 and 26 days for the untreated group, free DTX, non-functionalized NPs, RGD-NPs and IL-13 peptide-NPs, respectively)	[55]
Liposomes	DOX	TfR	T7 peptide	TfR	T7 peptide	Median survival of C6 GBM orthotopic mice model increased to 43 days (17, 22, 26, 35 and 28 days for the untreated group, free DOX, non-functionalized NPs, T7-NPs and TAT-NPs, respectively)	[56]
Liposomes	DOX	TfR	Tf protein	Folate receptor	Folate	Median survival of U-87 MG GBM orthotopic mice model increased to 30 days (20, 24 and 27 days for the untreated group, free DOX and non-	[57]

functionalized NPs, respectively)					
PCL and PEG polymeric NPs	DTX	Not mentioned	TGN peptide	Nucleolin	AS1411 aptamer
Median survival of C6 GBM orthotopic mice model increased to 32 days (17, 18, 17, 25 and 25 days for the untreated group, free DTX, non-functionalized NPs, TGN-NPs and AS1411-NPs, respectively)					
PCL and poly(ethyl ethylene phosphate) micelles	PTX	TfR	Tf protein	$\alpha\beta 3$ integrins	RGD peptide
Median survival of U-87 MG GBM orthotopic mice model increased to 42.8 days (34.5, 33.5, 34.8 and 39.5 days for the untreated group, free PTX, non-functionalized NPs and Tf-NPs, respectively)					

[58, 59]

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References

- [1] R. Duncan, Polymer conjugates as anticancer nanomedicines, *Nat Rev Cancer*, 6 (2006) 688-701.
- [2] P.P. Wibroe, D. Ahmadvand, M.A. Oghabian, A. Yaghmur, S.M. Moghimi, An integrated assessment of morphology, size, and complement activation of the PEGylated liposomal doxorubicin products Doxil®, Caelyx®, DOXOrubicin, and SinaDoxosome, *J Control Release*, 221 (2016) 1-8.
- [3] M.D. Al-Amin, F. Bellato, F. Mastrotto, M. Garofalo, A. Malfanti, S. Salmaso, P. Caliceti, Dexamethasone loaded liposomes by thin-film hydration and microfluidic procedures: Formulation challenges, *Int J Mol Sci*, 21 (2020) 1611.
- [4] J. Fox, FDA Advisers Okay NeXstars DaunoXome, *Bio/Technology*, 13 (1995) 635–636.
- [5] R.C.F. Leonard, S. Williams, A. Tulpule, A.M. Levine, S. Oliveros, Improving the therapeutic index of anthracycline chemotherapy: Focus on liposomal doxorubicin (Myocet™), *Breast*, 18 (2009) 218-224.
- [6] I. de Lázaro, D.J. Mooney, Obstacles and opportunities in a forward vision for cancer nanomedicine, *Nat Mater*, 20 (2021) 1469-1479.
- [7] E. Miele, G.P. Spinelli, E. Miele, F. Tomao, S. Tomao, Albumin-bound formulation of paclitaxel (Abraxane® ABI-007) in the treatment of breast cancer, *Int J Nanomed*, 4 (2009) 99-105.
- [8] D.M. Zagalo, S. Simões, J. Sousa, Regulatory science approach in pharmaceutical development of follow-on versions of non-biological complex drug products, *J Pharm Sci*, 111 (2022) 2687-2713.
- [9] H. He, L. Liu, E.E. Morin, M. Liu, A. Schwendeman, Survey of clinical translation of cancer nanomedicines-lessons learned from successes and failures, *Acc Chem Res*, 52 (2019) 2445-2461.
- [10] P.A. Dinndorf, J. Gootenberg, M.H. Cohen, P. Keegan, R. Pazdur, FDA drug approval summary: Pegaspargase (Oncaspar®) for the first-Line treatment of children with acute lymphoblastic leukemia (ALL), *Oncologist*, 12 (2007) 991-998.
- [11] R.D.A. Miguel, A.S. Hirata, P.C. Jimenez, L.B. Lopes, L.V. Costa-Lotufo, Beyond formulation: Contributions of nanotechnology for translation of anticancer natural products into new drugs, *Pharmaceutics*, 14 (2022).
- [12] D.W. Kim, S.Y. Kim, H.K. Kim, S.W. Kim, S.W. Shin, J.S. Kim, K. Park, M.Y. Lee, D.S. Heo, Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell

lung cancer, *Ann Oncol*, 18 (2007) 2009-2014.

[13] K. Ando, K. Mori, N. Corradini, F. Redini, D. Heymann, Mifamurtide for the treatment of nonmetastatic osteosarcoma, *Expert Opin Pharmacother* 12 (2011) 285-292.

[14] K. Maier-Hauff, F. Ulrich, D. Nestler, H. Niehoff, P. Wust, B. Thiesen, H. Orawa, V. Budach, A. Jordan, Efficacy and safety of intratumoral thermotherapy using magnetic iron-oxide nanoparticles combined with external beam radiotherapy on patients with recurrent glioblastoma multiforme, *J Neurooncol*, 103 (2011) 317-324.

[15] F. Soetaert, P. Korangath, D. Serantes, S. Fiering, R. Ivkov, Cancer therapy with iron oxide nanoparticles: Agents of thermal and immune therapies, *Adv Drug Deliv Rev*, 163-164 (2020) 65-83.

[16] J.A. Silverman, S.R. Deitcher, Marqibo® (vincristine sulfate liposome injection) improves the pharmacokinetics and pharmacodynamics of vincristine, *Cancer Chemother Pharmacol*, 71 (2013) 555-564.

[17] T. Stylianopoulos, R.K. Jain, Design considerations for nanotherapeutics in oncology, *Nanomedicine*, 11 (2015) 1893-1907.

[18] A.M. Sofias, M. Dunne, G. Storm, C. Allen, The battle of “nano” paclitaxel, *Adv Drug Deliv Rev*, 122 (2017) 20-30.

[19] J. Carnevale, A.H. Ko, MM-398 (nanoliposomal irinotecan): Emergence of a novel therapy for the treatment of advanced pancreatic cancer, *Future Oncol*, 12 (2016) 453-464.

[20] E. Bernabeu, M. Cagel, E. Lagomarsino, M. Moretton, D.A. Chiappetta, Paclitaxel: What has been done and the challenges remain ahead, *Int J Pharm*, 526 (2017) 474-495.

[21] Y.K. Kang, M.H. Ryu, S.H. Park, J.G. Kim, J.W. Kim, S.H. Cho, Y.I. Park, S.R. Park, S.Y. Rha, M.J. Kang, J.Y. Cho, S.Y. Kang, S.Y. Roh, B.Y. Ryoo, B.H. Nam, Y.W. Jo, K.E. Yoon, S.C. Oh, Efficacy and safety findings from DREAM: a phase III study of DHP107 (oral paclitaxel) versus i.v. paclitaxel in patients with advanced gastric cancer after failure of first-line chemotherapy, *Ann Oncol*, 29 (2018) 1220-1226.

[22] M. Alfayez, H. Kantarjian, T. Kadia, F. Ravandi-Kashani, N. Daver, CPX-351 (vyxeos) in AML, *Leuk Lymphoma*, 61 (2020) 288-297.

[23] S. Bonvalot, P.L. Rutkowski, J. Thariat, S. Carrère, A. Ducassou, M.P. Sunyach, P. Agoston, A. Hong, A. Mervoyer, M. Rastrelli, V. Moreno, R.K. Li, B. Tiangco, A.C. Herraéz, A. Gronchi, L. Mangel, T. Sy-Ortin, P. Hohenberger, T. de Baère, A. Le Cesne, S. Helfre, E. Saada-Bouza, A. Borkowska, R. Anghel, A. Co, M. Gebhart, G. Kantor, A. Montero, H.H. Loong, R. Vergés, L. Lapeire, S. Dema, G. Kacso, L. Austen, L. Moureau-Zabotto, V. Servois, E. Wardelmann, P. Terrier, A.J. Lazar, J.V.M.G. Bovée, C. Le Péchoux, Z. Papai, NBTXR3, a first-in-class radioenhancer hafnium oxide nanoparticle,

plus radiotherapy versus radiotherapy alone in patients with locally advanced soft-tissue sarcoma (Act.In.Sarc): A multicentre, phase 2–3, randomised, controlled trial, *Lancet Oncol*, 20 (2019) 1148-1159.

[24] S. Hosokawa, T. Tagawa, H. Niki, Y. Hirakawa, K. Nohga, K. Nagaike, Efficacy of immunoliposomes on cancer models in a cell-surface-antigen-density-dependent manner, *Br J Cancer*, 89 (2003) 1545-1551.

[25] Y. Matsumura, M. Gotoh, K. Muro, Y. Yamada, K. Shirao, Y. Shimada, M. Okuwa, S. Matsumoto, Y. Miyata, H. Ohkura, K. Chin, S. Baba, T. Yamao, A. Kannami, Y. Takamatsu, K. Ito, K. Takahashi, Phase I and pharmacokinetic study of MCC-465, a doxorubicin (DXR) encapsulated in PEG immunoliposome, in patients with metastatic stomach cancer, *Ann Oncol*, 15 (2004) 517-525.

[26] C. Mamot, R. Ritschard, A. Wicki, G. Stehle, T. Dieterle, L. Bubendorf, C. Hilker, S. Deuster, R. Herrmann, C. Rochlitz, Tolerability, safety, pharmacokinetics, and efficacy of doxorubicin-loaded anti-EGFR immunoliposomes in advanced solid tumours: a phase 1 dose-escalation study, *Lancet Oncol*, 13 (2012) 1234-1241.

[27] N.N. Senzer, K. Matsuno, N. Yamagata, T. Fujisawa, E. Wasserman, W. Sutherland, S. Sharma, A. Phan, Abstract C36: MBP-426, a novel liposome-encapsulated oxaliplatin, in combination with 5-FU/leucovorin (LV): Phase I results of a Phase I/II study in gastro-esophageal adenocarcinoma, with pharmacokinetics, *Mol Cancer Ther*, 8 (2009) C36.

[28] W. Gu, F. Meng, R. Haag, Z. Zhong, Actively targeted nanomedicines for precision cancer therapy: Concept, construction, challenges and clinical translation, *J Control Release*, 329 (2021) 676-695.

[29] M.E. Davis, J.E. Zuckerman, C.H.J. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas, Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles, *Nature*, 464 (2010) 1067-1070.

[30] S. Quader, K. Kataoka, H. Cabral, Nanomedicine for brain cancer, *Adv Drug Deliv Rev*, 182 (2022) 114115.

[31] P.J. Gaillard, C.C.M. Appeldoorn, R. Dorland, J. van Kregten, F. Manca, D.J. Vugts, B. Windhorst, G.A.M.S. van Dongen, H.E. de Vries, D. Maussang, O. van Tellingen, Pharmacokinetics, brain delivery, and efficacy in brain tumor-bearing mice of glutathione pegylated liposomal doxorubicin (2B3-101), *PLoS One*, 9 (2014) e82331.

[32] N. Senzer, J. Nemunaitis, D. Nemunaitis, C. Bedell, G. Edelman, M. Barve, R. Nunan, K.F. Pirollo, A. Rait, E.H. Chang, Phase I study of a systemically delivered p53 nanoparticle in advanced solid tumors, *Mol Ther*, 21 (2013) 1096-1103.

[33] P. Munster, I.E. Krop, P. LoRusso, C. Ma, B.A. Siegel, A.F. Shields, I. Molnár, T.J. Wickham, J. Reynolds, K. Campbell, B.S. Hendriks, B.S. Adiwijaya, E. Geretti, V. Moyo, K.D. Miller, Safety and pharmacokinetics of MM-302, a HER2-targeted antibody–

liposomal doxorubicin conjugate, in patients with advanced HER2-positive breast cancer: a phase 1 dose-escalation study, *Br J Cancer*, 119 (2018) 1086-1093.

[34] M. Farshbaf, S. Mojarad-Jabali, S. Hemmati, A.Y. Khosroushahi, H. Motasadizadeh, A. Zarebkohan, H. Valizadeh, Enhanced BBB and BBTB penetration and improved anti-glioma behavior of bortezomib through dual-targeting nanostructured lipid carriers, *J Control Release*, 345 (2022) 371-384.

[35] C. Yang, Z. Yang, S. Wang, J. Chen, Q. Liu, H. Tianle, L. Hai, R. Lu, Y. Wu, Berberine and folic acid co-modified pH-sensitive cascade-targeted PTX-liposomes coated with Tween 80 for treating glioma, *Bioorg Med Chem*, 69 (2022) 116893.

[36] J. Lu, R. Li, B. Mu, Y. Peng, Y. Zhao, Y. Shi, L. Guo, L. Hai, Y. Wu, Multiple targeted doxorubicin-lonidamine liposomes modified with p-hydroxybenzoic acid and triphenylphosphonium to synergistically treat glioma, *Eur J Med Chem*, 230 (2022) 114093.

[37] H. Wang, Y. Chao, H. Zhao, X. Zhou, F. Zhang, Z. Zhang, Z. Li, J. Pan, J. Wang, Q. Chen, Z. Liu, Smart nanomedicine to enable crossing blood–brain barrier delivery of checkpoint blockade antibody for immunotherapy of glioma, *ACS Nano*, 16 (2022) 664-674.

[38] Z. Jin, L. Piao, G. Sun, C. Lv, Y. Jing, R. Jin, Dual functional nanoparticles efficiently across the blood–brain barrier to combat glioblastoma via simultaneously inhibit the PI3K pathway and NKG2A axis, *J Drug Target*, 29 (2021) 323-335.

[39] Y. Cui, J. Sun, W. Hao, M. Chen, Y. Wang, F. Xu, C. Gao, Dual-target peptide-modified erythrocyte membrane-enveloped PLGA nanoparticles for the treatment of glioma, *Front Oncol*, 10 (2020) 563938.

[40] X. Shi, R. Ma, Y. Lu, Y. Cheng, X. Fan, J. Zou, H. Zheng, F. Li, J.-G. Piao, iRGD and TGN co-modified PAMAM for multi-targeted delivery of ATO to gliomas, *Biochem Biophys Res Commun*, 527 (2020) 117-123.

[41] X. Wang, Y. Zhao, S. Dong, R.J. Lee, D. Yang, H. Zhang, L. Teng, Cell-penetrating peptide and transferrin co-modified liposomes for targeted therapy of glioma, *Molecules*, 24 (2019) 3540.

[42] S. Lakkadwala, B. dos Santos Rodrigues, C. Sun, J. Singh, Dual functionalized liposomes for efficient co-delivery of anti-cancer chemotherapeutics for the treatment of glioblastoma, *J Control Release*, 307 (2019) 247-260.

[43] C. Liu, Z. Zhao, H. Gao, I. Rostami, Q. You, X. Jia, C. Wang, L. Zhu, Y. Yang, Enhanced blood-brain-barrier penetrability and tumor-targeting efficiency by peptide-functionalized poly(amidoamine) dendrimer for the therapy of gliomas, *Nanotheranostics*, 3 (2019) 311-330.

[44] S. Fu, M. Liang, Y. Wang, L. Cui, C. Gao, X. Chu, Q. Liu, Y. Feng, W. Gong, M.

- Yang, Z. Li, C. Yang, X. Xie, Y. Yang, C. Gao, Dual-modified novel biomimetic nanocarriers improve targeting and therapeutic efficacy in glioma, *ACS Appl Mater Interfaces*, 11 (2019) 1841-1854.
- [45] S. Lakkadwala, J. Singh, Dual functionalized 5-fluorouracil liposomes as highly efficient nanomedicine for glioblastoma treatment as assessed in an in vitro brain tumor model, *J Pharm Sci*, 107 (2018) 2902-2913.
- [46] S. Wang, C. Zhao, P. Liu, Z. Wang, J. Ding, W. Zhou, Facile construction of dual-targeting delivery system by using lipid capped polymer nanoparticles for anti-glioma therapy, *RSC Adv*, 8 (2018) 444-453.
- [47] Y. Zhu, Y. Jiang, F. Meng, C. Deng, R. Cheng, J. Zhang, J. Feijen, Z. Zhong, Highly efficacious and specific anti-glioma chemotherapy by tandem nanomicelles co-functionalized with brain tumor-targeting and cell-penetrating peptides, *J Control Release*, 278 (2018) 1-8.
- [48] X. Zhu, H. Zhou, Y. Liu, Y. Wen, C. Wei, Q. Yu, J. Liu, Transferrin/aptamer conjugated mesoporous ruthenium nanosystem for redox-controlled and targeted chemo-photodynamic therapy of glioma, *Acta Biomater*, 82 (2018) 143-157.
- [49] Z. Belhadj, C. Zhan, M. Ying, X. Wei, C. Xie, Z. Yan, W. Lu, Multifunctional targeted liposomal drug delivery for efficient glioblastoma treatment, *Oncotarget*, 8 (2017) 66889-66900.
- [50] C. Chen, Z. Duan, Y. Yuan, R. Li, L. Pang, J. Liang, X. Xu, J. Wang, Peptide-22 and cyclic RGD functionalized liposomes for glioma targeting drug delivery overcoming BBB and BBTB, *ACS Appl Mater Interfaces*, 9 (2017) 5864-5873.
- [51] L. Lv, Y. Jiang, X. Liu, B. Wang, W. Lv, Y. Zhao, H. Shi, Q. Hu, H. Xin, Q. Xu, Z. Gu, Enhanced antiglioblastoma efficacy of neovasculature and glioma cells dual targeted nanoparticles, *Mol Pharm*, 13 (2016) 3506-3517.
- [52] J. Bruun, T.B. Larsen, R.I. Jølk, R. Eliassen, R. Holm, T. Gjetting, T.L. Andresen, Investigation of enzyme-sensitive lipid nanoparticles for delivery of siRNA to blood-brain barrier and glioma cells, *Int J Nanomed*, 10 (2015) 5995-6008.
- [53] H. Gao, S. Zhang, S. Cao, Z. Yang, Z. Pang, X. Jiang, Angiopep-2 and activatable cell-penetrating peptide dual-functionalized nanoparticles for systemic glioma-targeting delivery, *Mol Pharm*, 11 (2014) 2755-2763.
- [54] H. Gao, Y. Xiong, S. Zhang, Z. Yang, S. Cao, X. Jiang, RGD and interleukin-13 peptide functionalized nanoparticles for enhanced glioblastoma cells and neovasculature dual targeting delivery and elevated tumor penetration, *Mol Pharm*, 11 (2014) 1042-1052.
- [55] H. Gao, Z. Yang, S. Cao, Y. Xiong, S. Zhang, Z. Pang, X. Jiang, Tumor cells and neovasculature dual targeting delivery for glioblastoma treatment, *Biomaterials*, 35

(2014) 2374-2382.

[56] T. Zong, L. Mei, H. Gao, W. Cai, P. Zhu, K. Shi, J. Chen, Y. Wang, F. Gao, Q. He, Synergistic dual-ligand doxorubicin liposomes improve targeting and therapeutic efficacy of brain glioma in animals, *Mol Pharm*, 11 (2014) 2346-2357.

[57] J.-Q. Gao, Q. Lv, L.-M. Li, X.-J. Tang, F.-Z. Li, Y.-L. Hu, M. Han, Glioma targeting and blood–brain barrier penetration by dual-targeting doxorubicin liposomes, *Biomaterials*, 34 (2013) 5628-5639.

[58] H. Gao, J. Qian, S. Cao, Z. Yang, Z. Pang, S. Pan, L. Fan, Z. Xi, X. Jiang, Q. Zhang, Precise glioma targeting of and penetration by aptamer and peptide dual-functioned nanoparticles, *Biomaterials*, 33 (2012) 5115-5123.

[59] H. Gao, Z. Yang, S. Zhang, Z. Pang, Q. Liu, X. Jiang, Study and evaluation of mechanisms of dual targeting drug delivery system with tumor microenvironment assays compared with normal assays, *Acta Biomater*, 10 (2014) 858-867.

[60] P. Zhang, L. Hu, Q. Yin, L. Feng, Y. Li, Transferrin-modified c[RGDfK]-paclitaxel loaded hybrid micelle for sequential blood-brain barrier penetration and glioma targeting therapy, *Mol Pharm*, 9 (2012) 1590-1598.

APPENDIX II. Polymeric nanoparticles for protein therapeutic co-formulation and co-delivery: an exploratory model study

This appendix was based on the following publication:

- **C Martins**, VM Chauhan, AA Selo, M Al-Natour, JW Aylott, B Sarmento. “Modelling protein therapeutic co-formulation and co-delivery with PLGA nanoparticles continuously manufactured by microfluidics”. Reaction Chemistry & Engineering, 5, 308-319 (2020)

ABSTRACT

Formulating protein therapeutics into nanoparticles (NPs) of poly(lactic-co-glycolic acid) (PLGA) provides key features such as protection against clearance, sustained release and less side effects by possible attachment of targeting ligands. These NPs also offer the potential for protein combination therapy, which is expected to exploit synergetic bioresponses, avoid multiple dosage regimens and consequent mis-dosing. Since the conventional manufacture of protein-loaded PLGA NPs is still associated with low-throughput, new continuous manufacturing methods such as microfluidics have been established. Herein, PLGA NPs continuously manufactured through microfluidics are proposed for co-formulation and -delivery of two model proteins consisting of bovine serum albumin (BSA) conjugated to either fluorescein (FITC) or tetramethylrhodamine (TRITC) isothiocyanates. Protein co-formulated NPs of 100 nm, monodispersed and with 70% of association efficiency were obtained. The microfluidic setup allowed a production rate of around 7 g of particles per day and demonstrated scale-up capacity. Model proteins were released in a controlled manner and without significant changes in their secondary structure. Studies in macrophage-like cells proved that protein co-formulated PLGA NPs did not impair the metabolic activity of these cells (>70%). This work has demonstrated the effectiveness of continuously manufactured PLGA NPs for co-formulating and co-delivering model proteins, providing a proof-of-concept foundation for future protein combination nanotherapies.

KEYWORDS

Co-delivery; Co-formulation; Microfluidics; PLGA nanoparticles; Protein combination therapy; Protein therapeutics.

1. INTRODUCTION

The protein therapeutic market is an important segment of the pharmaceutical industry and is anticipated to be worth \$230 billion by 2021 [1]. The exponential growth of this market is correlated with the superior therapeutic performance over small-molecule drugs, which is due to higher bioactivity and specificity [2]. Protein-based therapeutics have therefore been widely investigated for the treatment of various diseases, with more than 200 Food and Drug Administration (FDA)-approvals for clinical use since 1980 [3]. Although proteins hold great potential as therapeutics, their delivery poses a significant challenge. Protein therapeutics are highly affected by physiological conditions, which may compromise their stability and trigger their proteolytic degradation, opsonization, as well as complexation with biologic products leading to conformational changes and consequent loss of function [4]. Moreover, lipophilic cell membranes restrict the passage of proteins with intracellular targets, which are subject to challenges in interacting with cells and accessing sub-cellular compartments [5]. As a result, protein therapeutics can be diverted away from their target, resulting in side effects emerging from off-site accumulation and the need for repeated administrations. Polymer-based nanoparticles (NPs) have been identified as a promising strategy to circumvent these drawbacks by offering a protective vehicle for the formulation of protein therapeutics. This is because, they have been found to prevent the rapid clearance of the protein therapeutics whilst providing their controlled release over time [6, 7]. The FDA-approved poly(lactic-co-glycolic acid) (PLGA) polymer is the most widely utilized polymer for protein therapeutic-loaded NPs with biodegradable and biocompatible properties [8]. PLGA is a surface-functionalizable polymer that allows further attachment of a variety of targeting ligands, which are key to inducing higher accumulation of the NPs at the target site, therefore reducing potential side effects [9]. However, conventional bulk processes for synthesis of protein therapeutic-loaded PLGA NPs are often related to slow- and low-throughput, unsuccessful association efficiency and lack of batch-to-batch consistency [10, 11]. These pitfalls are being overcome by the rapid development of continuous manufacturing methods such as microfluidics that offer the possibility of precisely control the particle-formation environment and enable the production of PLGA NPs with tunable physicochemical characteristics and higher drug loading capacity, as well as monodisperse batches [12-14]. Microfluidics also allows a reproducible, time-saving up-scale manufacturing of NPs, which is crucial to reach industrial and clinical batch sizes [15].

Another challenge with manufacturing protein therapeutic-loaded PLGA NPs is the harsh

environment encountered when employing the double emulsion systems commonly used in the formulation process. The shear stress inherent to the immiscibility of the phases involved in these systems leads to the interfacial adsorption of proteins, which is the major cause of their denaturation [16, 17]. Typical nanoprecipitation-based processes would be more appropriate due to the absence of oily-aqueous interfaces, although they are mostly designed for the formulation of highly hydrophobic cargos [18]. Some authors have introduced modifications in the nanoprecipitation process to accomplish the formulation of hydrophilic compounds into hydrophobic polymeric matrices, however, these modifications generally require more complex co-solvent systems [19-24].

The development of PLGA NPs for protein delivery has made notable advances, while their potential to co-formulate protein therapeutics and perform combination therapy is yet to be fully investigated. Co-formulation of multiple therapeutics into polymeric NPs is expected to simplify delivery to patients by reducing multiple dosage regimens, achieve synergetic bioresponses by tuning the ratio of the loaded cargos, as well as to provide a simultaneous targeting to multiple therapeutic active sites [25]. Combination therapy may be particularly useful for multi-cytokine treatments, since immune responses trigger several biochemical cascades in which many molecular networks need to be targeted simultaneously [26].

Bovine serum albumin (BSA) is an ideal protein candidate for preliminary model studies due to its relatively high molecular weight (around 66.5 kDa) and economical price point [27]. The conjugation of fluorochromes as either fluorescein (FITC) or tetramethylrhodamine (TRITC) isocyanates with BSA is well known to significantly change its physicochemical properties, which produces fluorochrome-dependent protein profiles [28]. For example, protein solubility can be considerably altered by the labelling fluorochrome, due to a net-increase in the total number of attractive sites in the protein-fluorochrome system, as reported by Quinn et al. [29]. Therefore, BSA-FITC and BSA-TRITC can be distinguished from each other and from the unlabelled BSA with respect to their physiochemical characteristics and used as two different model proteins, to study continuous manufacturing and encapsulation into polymeric NPs.

This study reports PLGA NPs for all-in-one co-formulation and -delivery of two models of protein therapeutics consisting of BSA-FITC and BSA-TRITC. The study addresses continuous manufacturing by investigating a microfluidic modified-nanoprecipitation technique for the production of protein co-formulated PLGA NPs, with further assessment of their impact on the viability of macrophage-like cells.

2. MATERIALS AND METHODS

2.1 Materials

The X-junction microfluidic device (Quartz Droplet Junction Chip, 190 μm etch depth) was acquired from Dolomite (Royston, UK). BSA-FITC, BSA-TRITC, Tween[®]-80, sodium chloride, phosphate buffered saline (PBS), phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, USA); acetone from Thermo Fisher Scientific (Waltham, MA, USA); high glucose with ultraglutamine Dulbecco's Modified Eagle medium (DMEM) from Lonza (Basel, Switzerland); Triton X-100 from Spi-Chem (West Chester, PA, USA); and fetal bovine serum (FBS) and penicillin-streptomycin from Gibco (Waltham, MA, USA). Acid-terminated PLGA polymer (50:50 lactic to glycolic ratio, molecular weight of 10 kDa) was kindly provided by Dr Mohammad Al-Natour and its synthesis was described elsewhere [30].

The human monocytic leukaemia THP-1 cell line was purchased from ATCC (Manassas, VA, USA).

2.2 Nanoparticles manufacturing and process optimization

The organic phase used for the microfluidic manufacturing of the NPs consisted of a mixture of PLGA in acetone, for which the concentration was optimized between 0.25% and 1% w/v. The aqueous phase, in turn, was kept as a 2% w/v solution of Tween[®]-80, and both BSA-FITC and BSA-TRITC were added to this phase to obtain protein-loaded PLGA NPs. For the BSA-FITC and BSA-TRITC individual formulation, a concentration of 33 $\mu\text{g/mL}$ and 67 $\mu\text{g/mL}$ of BSA-FITC and BSA-TRITC in the 2% w/v solution of Tween[®]-80, respectively, was used. Whereas, for the BSA-FITC and BSA-TRITC co-formulation, a concentration of 25 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$ of BSA-FITC and BA-TRITC in the 2% w/v solution of Tween[®]-80, respectively, was used. The microfluidic chip displayed a three-inlets X-layout, in which the organic and aqueous phases were injected through the central and the two outer inlets, respectively (**Figure 1**). The total flow rate was varied from 25:75 to 500:1500 $\mu\text{L/min}$ regarding the organic:aqueous phases. Each experiment was conducted until 10 mg of PLGA NPs were produced (equivalent to 2 min run time using the final flow rate conditions). NPs were washed three times with ultrapure water and recovered by ultrafiltration using Amicon Ultra-15 Centrifugal Filter units (Merck Millipore, Billerica, MA, USA) with a molecular weight cut-off (MWCO) of 100 kDa.

2.2.1 Scale-up experiment

A scale-up experiment was performed in relation to the mass of PLGA in the final batch of unloaded NPs. The batch size was scaled 5- and 25-times, corresponding to a final mass of PLGA of 50 mg and 250 mg, respectively.

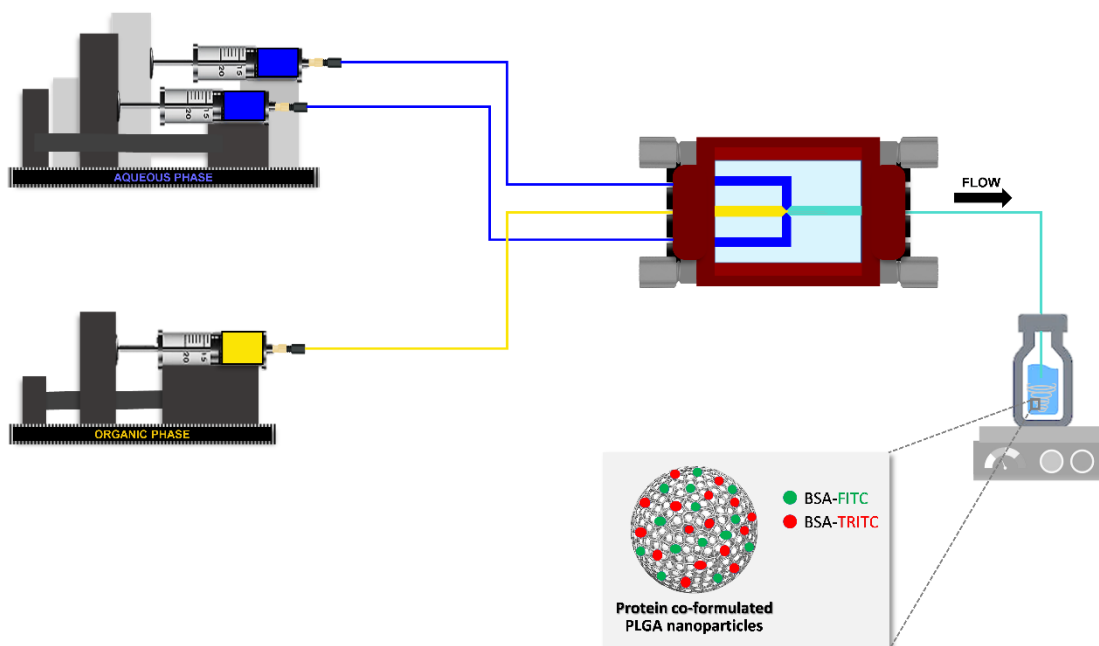


Figure 1. Schematic representation of the microfluidic setup.

2.3 Characterization of nanoparticles

2.3.1 Mean particle size, size distribution and surface charge

NPs were characterized for their average size (Z-average) and polydispersity index (PDI) by dynamic light scattering (DLS), and zeta-potential (ζ -potential) through laser Doppler anemometry (LDA), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). For these measurements, samples were diluted (1:100) in an ionic solution of 10 mM sodium chloride.

2.3.2 Association efficiency

The amount of loaded BSA-FITC and BSA-TRITC was directly quantified in order to

determine the association efficiency (AE) of the developed nanosystems. The calculation of AE followed Equation (1) [31].

$$AE_{BSA} (\%) = \frac{\text{Quantified loaded mass of BSA}}{\text{Initial mass of BSA}} \times 100 \quad (1)$$

The amount of loaded BSA-FITC and BSA-TRITC was spectrophotometrically quantified after the extraction of the proteins from the matrix of the NPs according to a protocol adapted from [32]. Briefly, the protein-loaded NPs were treated with a solution of 3 M NaOH for 24 h at 100 rpm in an orbital shaker (Thermo Scientific, Madison, WI, USA), to promote the disaggregation of the polymeric matrix and release of the associated BSA-FITC and BSA-TRITC. The suspension was then sonicated for 1 h and centrifuged at 14,000 rpm for 15 min (Hermle Z 300, Hermle Labortechnik GMBH, Wehingen, DE). The supernatant was filtered through a 0.22 µm syringe filter (Millex® GP, Millipore, Bedford, MA, USA). The absorbance of the filtrate was read in a Varian Cary® 50 Bio UV-VIS Spectrophotometer (Agilent Technologies, Stockport, UK) at 488 nm and 543 nm for the detection of BSA-FITC and BSA-TRITC, respectively.

2.3.3 Morphology

The morphological features of the NPs were analyzed by transmission electron microscopy (TEM) using a Philips Tecnai BioTwin-12 microscope (FEI, Eindhoven, NL) at an accelerating voltage of 120 kV. Images were recorded using a Gatan SIS Megaview IV digital camera (Gatan Inc., Warrendale, PA). Samples were prepared by dropping 20 µL of the suspension of the NPs onto a 300-mesh copper grid and negatively-stained with 20 µL of 1% w/v uranyl acetate.

2.4 *In vitro* release study

To investigate the *in vitro* protein release profile, PLGA NPs formulated with BSA-FITC (55 mg) and BSA-TRITC (55 mg) or co-formulated with BSA-FITC and BSA-TRITC (140 mg) were dispersed in PBS, pH 7.4, to reach the total volume of 2 mL. The samples were then stirred at 37 °C and 100 rpm in an orbital shaker incubator (Incu-Shake MINI, SciQuip, Staffordshire, UK). Aliquots of 1 mL were collected at specific time points (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5 and 24 h) during the assay, and the withdrawn volume

was replaced with pre-heated PBS, pH 7.4, to maintain sink conditions. All the collected aliquots were centrifuged at 14,000 rpm for 15 min, and the spectrophotometric absorbance of the supernatant was read at 488 nm and 543 nm for the detection of BSA-FITC and BSA-TRITC, respectively.

2.5 Structural stability of the released proteins

Alterations in the secondary structure of the total protein content released from PLGA NPs were assessed by circular dichroism (CD) spectroscopy. PLGA NPs individually formulated with BSA-FITC (55 mg) and BSA-TRITC (55 mg) or co-formulated with BSA-FITC and BSA-TRITC (140 mg) were dispersed in PBS (pH 7.4, 2 mL). The samples were then stirred at 37 °C and 100 rpm in an orbital shaker incubator (Incu-Shake MINI, SciQuip, Staffordshire, UK) for 24 h. After centrifugation at 14,000 rpm for 15 min, the supernatant was concentrated to 240 µg/mL of protein by ultrafiltration using Amicon Ultra-15 Centrifugal Filter units (Merck Millipore, Billerica, MA, USA) with a MWCO of 10 kDa. The spectral profile of each sample was compared with the respective native and denatured (90 °C, 1 h) controls. The concentration of native and denatured controls was defined as 1 mg/mL and 200 µg/mL, respectively. The ellipticity value of proteins was scanned over 200–260 nm wavelength using an Aviv Model 400 spectrometer (Aviv Biomedical Inc, Lakewood, New Jersey, USA). A quartz cell was used, and the bandwidth and time-per-point were 1 nm and 1 s, respectively. The mean residue ellipticity (MRE) was calculated according to Equation (3) [33].

$$\text{MRE} = \frac{(\theta_{\text{sample}} - \theta_{\text{media}}) \times \text{MW}}{(N-1) \times l \times C} \quad (3)$$

where θ is the raw ellipticity value (mdeg), MW is the molecular weight of BSA (66.5 kDa), N is the number of residues of BSA (583), l is the cuvette path length (1 mm), and C is the protein concentration in mg/mL. The α -helix percentage was also calculated according to Equation (4) [34].

$$\alpha\text{-helix (\%)} = \frac{-\text{MRE}_{208 \text{ nm}} - 4000}{33,000 - 4000} \quad (4)$$

2.6 Cell culturing

THP-1 cells were grown in suspension in tissue culture flasks (passage 14–25). The cells

were maintained in DMEM medium containing FBS (10%, v/v) and penicillin-streptomycin (1%, v/v). Cultures were kept in an incubator (CellCulture CO₂ incubator, ESCO GB Ltd., Downton, UK) at 37 °C with 5% CO₂, in a water saturated atmosphere. After reaching confluency, cells were centrifuged and split into new tissue culture flasks.

2.7 Cellular metabolic activity assay

The influence of samples on metabolic activity was assessed in macrophage-like cells differentiated from the THP-1 cell line using the MTT assay. THP-1 cells were seeded in a 96-well plate (1×10^5 cells/well) in 200 μ L complete DMEM medium at a PMA concentration of 10 ng/mL in order to induce their differentiation into macrophage-like cells [35]. After 24 h of incubation, medium was removed and cells were washed twice with 200 μ L PBS, pH 7.4. Then, cells were incubated with the samples in concentrations of 0.03 μ M, 0.003 μ M and 0.0003 μ M (in medium) determined in relation to the proteins, for 24 h. Afterwards, solutions were discarded, cells were washed with 200 μ L PBS, pH 7.4, and treated with 200 μ L of MTT solution (0.5 mg/mL, in medium), during 4 h in the dark. Formazan crystals, resulting from the reduction of MTT by viable cells, were solubilized with 100 μ L of DMSO, with of 20 min gentle shaking at 100 rpm at room temperature. The absorbance was measured at 590 nm and 630 nm. A negative control (NC), consisting of cells incubated with 1 % of Triton X-100 in medium solution (0 % of metabolic activity), and a positive control (PC), consisting of cells incubated only with medium (100 % of metabolic activity), were also prepared and treated similarly to the sample wells. Metabolic activity was expressed as a percentage compared to the controls according to Equation (5).

$$\text{Metabolic activity (\%)} = \frac{\text{Experimental value} - \text{NC}}{\text{PC} - \text{NC}} \times 100 \quad (5)$$

2.8 Statistical analysis

The results were represented as mean $\pm$ standard deviation from a minimum of three independent experiments. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a post hoc test (Tukey's honestly significant difference). Differences were considered significant at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. The statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

3. RESULTS AND DISCUSSION

In this work, PLGA NPs were co-formulated with BSA-FITC and BSA-TRITC via a microfluidic modified-nanoprecipitation technique. The manufacturing of these NPs was performed using a X-junction microfluidic chip. An acetone organic phase consisting of PLGA polymer and an aqueous solution of Tween[®]-80 were used. The standard nanoprecipitation methodology was first described by Fessi *et al.* [36] who detailed how materials for delivery could be solubilized in the organic phase. In this article, it is shown that the BSA-FITC and BSA-TRITC model proteins can also be included in the aqueous Tween[®]-80 solution.

The continuous manufacturing of polymeric NPs using microfluidics is a highly versatile technique in which many parameters must be optimized in order to produce the desired particulate system. Therefore, to clarify the manufacturing method, the influence of PLGA concentration in the organic phase and total flow rate of the system was investigated, whilst maintaining the ratio of organic:aqueous solvent phase and aqueous phase concentration of Tween[®]-80.

An increase in the PLGA concentration of the organic phase from 0.25% w/v to 1% w/v produced a significant increase ($*p<0.05$) in the Z-average of NPs from around 110 nm to 140 nm, respectively (**Figure 2A**). This increase in size can be explained by the rise in the number and consequent fusion of PLGA chains within the vicinity of nucleation sites during particle formation [37]. PDI values lower than 0.2 across the same range of concentrations (**Figure 2B**) demonstrated size distribution uniformity of all NP populations, which is a typical characteristic of microfluidic-produced particles. This is because, the manufacturing device allows the controlled separation of the nucleation and growth stages through the distance away from the location where the initial mixing of the organic and aqueous phase occurs [11, 38]. The ζ -potential, in turn, significantly dropped ($**p<0.01$) from around -9 mV to -17 mV with increasing concentrations of PLGA (**Figure 2C**) as a result of the higher density of polymer carboxyl end-groups exposed on the surface of the NPs [39]. Increasing concentrations of PLGA also resulted in a considerable increase in the production rate of NPs (**Figure 2D**) from around 0.2 g/day to 0.7 g/day. The production rate is usually an undervalued parameter in most bulk and even continuous manufacturing methods used for PLGA NPs production. However, this parameter has attracted a greater focus in recent years. This is because, the production rate directly correlates with the capability of the manufacturing method to address the throughput required for the application in industrial and clinical settings [40, 41].

These physicochemical properties of NPs are ideal for systemic administration and have been previously shown to permit transit through and escape from the spleen and liver [42, 43]. Furthermore, these properties also permit the application of these particles for cancer nanomedicine. This is because, their Z-average enables enhanced permeability and retention (EPR) effect by migrating through the vascular fenestrations of tumours [44]. More particularly, the size dimension of the developed NPs could be also useful in the field of brain drug delivery since it is generally accepted that it facilitates the transport of particles through the vascular endothelial cells of the blood-brain barrier [45, 46]. PLGA at 1% w/v in acetone solvent was defined as the organic phase to proceed with further studies, as it provided the highest possible polymer concentration and consequent NPs production rate, whilst maintaining homogeneous monodistributed NPs [47].

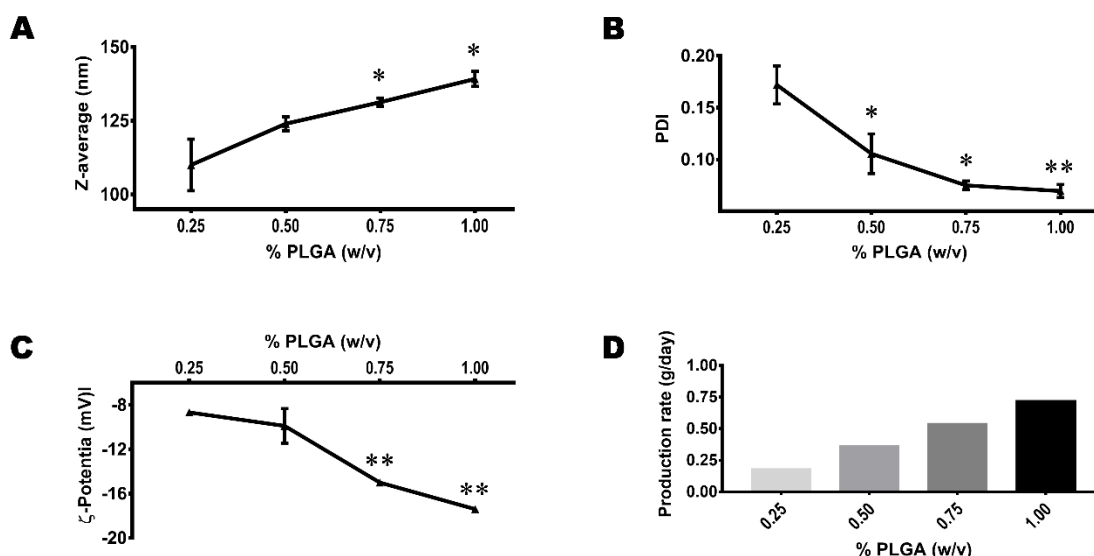


Figure 2. Optimization of PLGA concentration for the manufacturing of unloaded NPs, using an organic:aqueous flow rate of 50:150 μ L/min. NP properties of (A) Z-average, (B) PDI and (C) ζ -potential were investigated, as well as the (D) manufacturing productivity. Final conditions were defined as 1% w/v PLGA. Error bars represent the mean $\pm$ SD (n=3) and the significance levels were assigned at * p <0.05 and ** p <0.01.

The optimization of the manufacturing process involved an increase in the total flow rate from 100 μ L/min to 2000 μ L/min. These flow rates corresponded to an increase in the organic phase flow rate from 25 μ L/min to 75 μ L/min and aqueous flow rate 500 μ L/min to 1500 μ L/min. Higher total flow rates led to a significant decrease in the Z-average of the PLGA NPs (** p <0.01, **Figure 3A**), which is in accordance with previously published

literature by Zhang et al. [48]. They have explained that the faster diffusion of the organic phase into the aqueous phase enabled higher nucleation rates and the formation of multiple, smaller spatial supersaturation zones. This places the nucleation stage in a privileged position compared to the growth one, since more nuclei are formed and only limited amount of polymer is left to grow them. Therefore, lower diameter NPs can be obtained. The PDI (**Figure 3B**) and ζ -potential (**Figure 3C**) did not change significantly across the same range of total flow rate, keeping values less than 0.2 and around -20 mV, respectively. Increasing total flow rates resulted in a greater increase in the production rate of the PLGA NPs (**Figure 3D**) from around 0.4 g/day to 7 g/day. It is important to highlight that the obtained maximum production rate of 7 g/day demonstrated superior yields to previously reported literature data for polymeric nanosystems, that were manufactured using a single microfluidic device, e.g. 0.0072-0.0144 g/day [49], 0.012-0.079 g/day [50], 0.156 g/day [51] and 0.252 g/day [52]. Based on the data presented in **Figure 3**, the final total flow rate was defined as 2000 $\mu\text{L}/\text{min}$, which corresponded to an organic:aqueous flow rate of 500:1500 $\mu\text{L}/\text{min}$. These greater flow rates provided the highest productivity, while maintaining homogeneous monodisperse PLGA NPs.

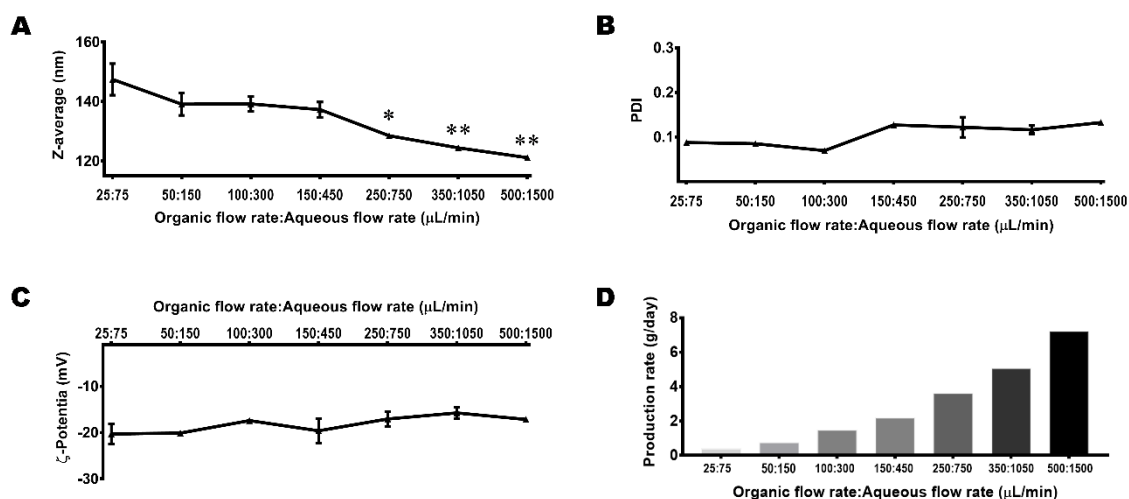


Figure 3. Optimization of the total flow rate when manufacturing unloaded NPs. NP properties of (A) Z-average, (B) PDI and (C) ζ -potential were investigated, as well as the (D) manufacturing productivity. Final conditions were defined as an organic:aqueous flow rate of 500:1500 $\mu\text{L}/\text{min}$. Error bars represent the mean $\pm$ SD ($n=3$) and the significance levels were assigned as * $p<0.05$ and ** $p<0.01$.

To ensure the controlled, reproducible continuous manufacturing of PLGA NPs using microfluidics, the scale of the manufacturing method was investigated. Specifically, a scale-up of the final yield by x5 (50 mg) and x25 (250 mg) was conducted. The Z-average, PDI and ζ -potential properties did not change significantly and demonstrated to be independent on the scale-up (**Table 1**), which indicated the strength of the herein proposed continuous microfluidic manufacturing process. This successful up-production in a continuous-flow setup offers potential to speed up the translation of the developed nanomedicines from laboratory to industry and clinical scales [53].

Table 1 – Evaluation of the impact of a scale-up manufacturing on the physicochemical properties of NPs of Z-average, PDI and ζ -potential.

Scaling	Z-average (nm)	PDI	ζ -Potential (mV)
Standard scale	116.9 $\pm$ 1.7	0.105 $\pm$ 0.006	- 17.2 $\pm$ 1.1
5-times scale-up	121.9 $\pm$ 3.8	0.109 $\pm$ 0.015	- 16.6 $\pm$ 2.8
25-times scale-up	120.9 $\pm$ 2.3	0.107 $\pm$ 0.013	- 16.5 $\pm$ 3.6

PLGA NPs were manufactured unloaded, individually formulated with BSA-FITC or BSA-TRITC, as well as co-formulated with BSA-FITC and BSA-TRITC. The Z-average (around 120 nm), PDI (around 0.1) and ζ -potential (around 15 mV) of all protein-loaded formulations did not exhibit significant changes compared to unloaded NPs (**Table 2**). PLGA nanosystems individually formulated with either BSA-FITC or BSA-TRITC presented an AE of around 80%. Although there is limited information in the literature highlighting the loading of BSA-TRITC into PLGA NPs, the obtained BSA-FITC AE value was far higher than the ones reported by other authors, approximately around 30-45% [32, 34, 54]. Moreover, the co-formulated nanosystem also presented high AE for both BSA-FITC and BSA-TRITC, corresponding to values of around 70% and 80%, respectively. It was hypothesized that the selected concentration of Tween[®]-80 endowed the protein-containing aqueous phase with an optimal viscosity to promote its diffusion through the organic phase. This provides enhanced dispersion of BSA-FITC and/or BSA-TRITC molecules through the PLGA matrix. It was furthermore envisaged that any residual Tween[®]-80 coating the surface of the NPs could act as an entrapment layer for BSA-FITC and/or BSA-TRITC molecules residing at the polymer-surfactant interface.

Table 2 – Evaluation of the physicochemical properties of NPs of Z-average, PDI, ζ -

potential and AE after individual formulation with BSA-FITC or BSA-TRITC, and co-formulation with BSA-FITC and BSA-TRITC.

NPs	Z-average (nm)	PDI	ζ -Potential (mV)	AE (%)	
				BSA-FITC	BSA-TRITC
Unloaded	116.9 $\pm$ 1.7	0.105 $\pm$ 0.006	- 17.2 $\pm$ 1.1	-	-
BSA-FITC	117.4 $\pm$ 2.1	0.102 $\pm$ 0.012	- 14.1 $\pm$ 2.3	77.7 $\pm$ 4.0	-
BSA-TRITC	121.3 $\pm$ 2.6	0.104 $\pm$ 0.002	- 15.7 $\pm$ 2.1	-	79.9 $\pm$ 3.2
BSA-FITC and BSA-TRITC	118.7 $\pm$ 4.1	0.106 $\pm$ 0.002	-14.6 $\pm$ 2.7	66.3 $\pm$ 4.5	77.5 $\pm$ 5.7

TEM imaging was used as a tool to provide more insight into the size and morphology of the developed NPs. Monodisperse particles with a homogenous surface and spherical morphology were observed (**Figure 4**). All formulations presented a size of NPs of around 100 nm, which is in accordance with DLS measurements.

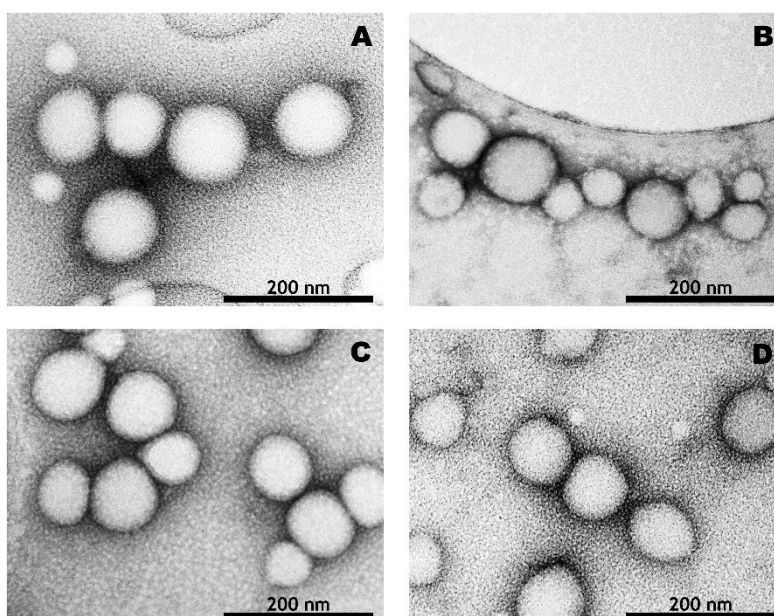


Figure 4. TEM images for (A) unloaded NPs, individually loaded (B) BSA-FITC and (C) BSA-TRITC NPs, and (D) BSA-FITC and BSA-TRITC co-formulated NPs.

Protein release was evaluated *in vitro* in buffered saline (pH 7.4), to represent an intravenous environment. PLGA NPs individually loaded with BSA-FITC were characterized by an initial rapid burst effect, with around 80% of the protein being

recovered in the first 2 h (**Figure 5A**). In comparison, PLGA NPs individually loaded with BSA-TRITC, significantly less of the protein was recovered (around 50%) over the same time period and the overall release was more sustained (**Figure 5B**). This results suggested that BSA-FITC may have a tendency to be located close or on the surface of the NPs, whereas BSA-TRITC could be dispersed into the polymeric PLGA matrix [55]. Since the TRITC fluorochrome is more hydrophobic than FITC [56], it also rendered BSA-TRITC protein more hydrophobicity than BSA-FITC. The PLGA polymer is known to present a hydrophobic backbone and, thus, it is prone to retain hydrophobic and hydrophilic cargos into its matrix and close to its surface, respectively, which can explain the previous release results [55].

Co-formulated BSA-FITC and BSA-TRITC PLGA NPs exhibited a release profile of BSA-TRITC comparable to the BSA-TRITC individual formulation, with also around 50% of the protein recovered over the first 2 h (**Figure 5C**). However, the release profile of BSA-FITC underwent significant alterations compared to the individual formulation, with only around 20% of the protein recovered over 2h (**Figure 5C**). It was hypothesized that this difference was related to the opposite net charge values of FITC and TRITC fluorochromes. FITC is negatively charged under neutral pH and presents a net value of -1.02, whereas TRITC possesses a net positive charge of +0.99 [57]. BSA conjugated with the positively charged TRITC, which is hypothesised to be distributed through the polymeric matrix of PLGA NPs, may exert electrostatic interactions on BSA conjugated with the negatively charged FITC, which would be expected to be located closer to the particles' surface. This electrostatic attraction could therefore be responsible for retaining the BSA-FITC for prolonged time periods, sustaining its release, in comparison to the BSA-FITC individual formulation.

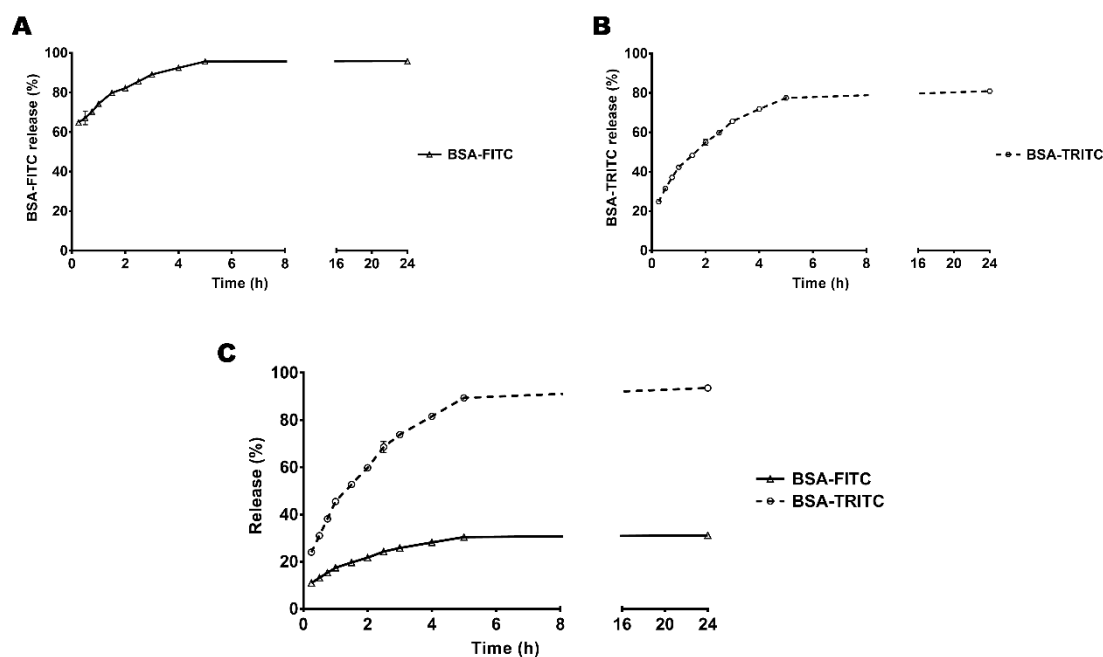


Figure 5 – *In vitro* release profile of individually loaded (A) BSA-FITC and (B) BSA-TRITC NPs, and (C) BSA-FITC and BSA-TRITC co-formulated NPs. Error bars represent the mean $\pm$ SD (n=3).

Protein structure is important since alterations in the secondary structure of proteins are likely to be related to their denaturation, which ultimately culminates in aggregation and loss of biologic function [34, 58]. One of the major disadvantages of the loading of protein therapeutics into polymeric NPs is their denaturation after the manufacturing process, mainly due to the deposition of these molecules in the organic-aqueous phase of emulsions and/or the contact of the organic solvents with their nonpolar amino acids [59]. Therefore, maintenance of the secondary structure of proteins after the release from PLGA NPs was studied through CD, where controls of each protein type in its native and denatured form were used for comparison purposes.

All CD spectra presented double minima at around 220 nm and 210 nm, which is characteristic of the α -helical structure of BSA [60, 61] (**Figure 6**). The spectra of BSA-FITC and BSA-TRITC released from the individually and co-formulated PLGA NPs were comparable to the native forms of the proteins. They were also distinguishable from the denatured control, which produced diminished absorbance bands. The α -helix content was calculated in order to confirm the structural similarities between the released samples and native controls. The calculated values were found in the interval from 50-60% as literature data reports for BSA [34, 62], with variations in the α -helix content relative to the control of only around 0%, 4% and 3% for individually loaded BSA-FITC

NPs (**Figure 6A**) and BSA-TRITC PLGA NPs (**Figure 6B**), and co-formulated BSA-FITC and BSA-TRITC PLGA NPs (**Figure 6C**). The CD results confirmed that the proposed continuous microfluidic manufacturing method for production of polymeric PLGA NPs was successfully able to encapsulate model proteins, either individually or as a co-formulation, in the absence of deviations in the secondary structure of the released proteins.

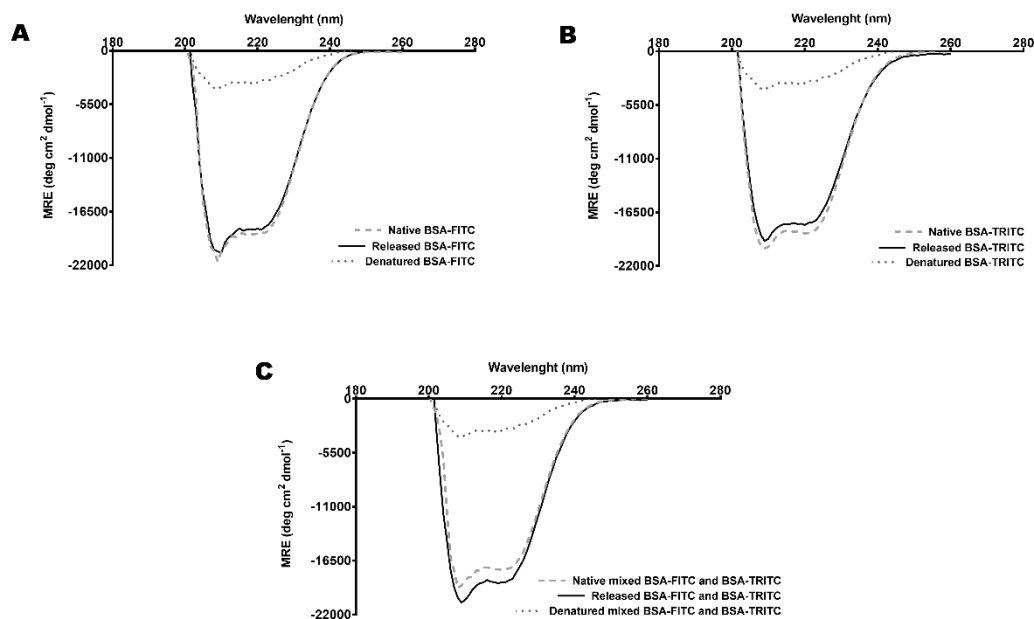


Figure 6 – CD spectral profile of the total released protein from individually formulated (A) BSA-FITC NPs and (B) BSA-TRITC NPs, and (C) BSA-FITC and BSA-TRITC co-formulated NPs. Spectral profile of the respective native and thermally denatured total protein is also represented. Results are presented as mean (n=3).

The influence of the developed nanosystems on metabolic activity was accessed through a MTT assay in macrophage-like cells differentiated from the THP-1 cell line (**Figure 7**). This cell line was selected only as a model due to its well described, easy and relatively inexpensive culture. Protein concentrations ranging from 0.0003-0.03 μ M were investigated for their effects on metabolic activity. The metabolic activity of macrophage-like cells was found to be always above 70% when in contact with the free BSA-FITC, BSA-TRITC, and mixed BSA-FITC and BSA-TRITC at all evaluated concentrations. This demonstrated the absence of cytotoxicity associated with the free proteins and was expected since the major molarity contribution from the protein-fluorochrome complex

originates from the BSA, for which the normal physiologic concentration is much higher than the maximum one herein tested (around 0.76 M in blood serum) [63, 64]. Unloaded NPs also exhibited cell metabolic activity greater than 70% for all tested concentrations, as well as NPs individually loaded with BSA-FITC or BSA-TRITC and co-formulated with BSA-FITC and BSA-TRITC. The polymeric matrix of NPs did not induce cytotoxicity and did not compromise cell viability. This observation was also true for the physical mixture of NPs individually loaded with BSA-FITC or BSA-TRITC in amounts equivalent to the co-formulated NPs. Therefore, it is important to note, according to the 10993-5 norm from the International Organization for Standardization (ISO) for *in vitro* pre-clinical evaluation of the cytotoxicity of medical devices, these PLGA NP formulations may be considered non-toxic and potentially safe [65].

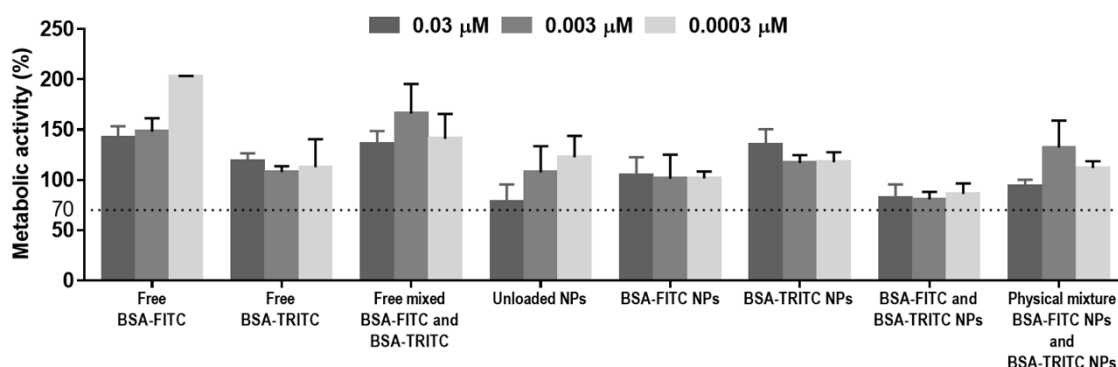


Figure 7 – Metabolic activity of macrophage-like THP-1 cells after 3 h exposure to the free and nanoformulated proteins. Sample data were normalized to the positive (buffer, 100%) and negative (lysis buffer, 0%) controls. Error bars represent the mean $\pm$ SD (n=3).

4. CONCLUSION

A continuous microfluidic manufacturing method that enabled the engineering of monodisperse BSA-FITC and BSA-TRITC co-formulated PLGA NPs with a high association efficiency is reported herein. The enhanced production rate and scale-up potential of the demonstrated microfluidic method indicated the capacity to manufacture monodisperse PLGA NPs in industrial and clinical batch sizes. The microfluidic manufacture process preserved the protein secondary structure and did not appear to induce denaturation upon release. Co-formulated BSA-FITC and BSA-TRITC PLGA NPs

did not present cytotoxicity in macrophage-like cells compared to the free mixed form of the proteins and even the physical mixture of NPs individually loaded with each protein type. Therefore, it is anticipated that this model study will pave the way towards novel combination therapies based on PLGA nanosystems that require the co-delivery of protein therapeutics to a range of pathologies. Perspectives for future work include the co-formulation of other proteins and investigation of the preservation of bioactivity post-release, as well as studies of the efficacy of the nanosystem *in vivo*.

REFERENCES

- [1] S.S. Dewan, Global markets for bioengineered protein drugs, BCC Research, (2017).
- [2] W. Gao, M. Sun, Drug nanorods are potential new nanocarriers for intracellular protein delivery, *Theranostics*, 8 (2018) 3872-3873.
- [3] S.S. Usmani, G. Bedi, J.S. Samuel, S. Singh, S. Kalra, P. Kumar, A.A. Ahuja, M. Sharma, A. Gautam, G.P.S. Raghava, THPdb: Database of FDA-approved peptide and protein therapeutics, *PLoS One*, 12 (2017) e0181748.
- [4] L.R. Brown, Commercial challenges of protein drug delivery, *Expert Opin Drug Deliv*, 2 (2005) 29-42.
- [5] V. Torchilin, Intracellular delivery of protein and peptide therapeutics, *Drug Discov Today Technol*, 5 (2008) e95-e103.
- [6] M.S. Akash, K. Rehman, S. Chen, Polymeric-based particulate systems for delivery of therapeutic proteins, *Pharm Dev Technol*, 21 (2016) 367-378.
- [7] S. Mohammadi-Samani, B. Taghipour, PLGA micro and nanoparticles in delivery of peptides and proteins: Problems and approaches, *Pharm Dev Technol*, 20 (2015) 385-393.
- [8] J. Hajavi, M. Ebrahimian, M. Sankian, M.R. Khakzad, M. Hashemi, Optimization of PLGA formulation containing protein or peptide-based antigen: recent advances, *J Biomed Mater Res A*, 106 (2018) 2540-2551.
- [9] C. Martins, F. Sousa, F. Araújo, B. Sarmiento, Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications, *Adv Healthc Mater*, 7 (2018) 1701035.
- [10] R. Juliano, Nanomedicine: Is the wave cresting?, *Nat Rev Drug Discov*, 12 (2013) 171-172.
- [11] J. Ma, S.M.-Y. Lee, C. Yi, C.-W. Li, Controllable synthesis of functional nanoparticles by microfluidic platforms for biomedical applications – A review, *Lab Chip*, 17 (2017) 209-226.

- [12] S. Rezvantab, M. Keshavarz Moraveji, Microfluidic assisted synthesis of PLGA drug delivery systems, *RSC Adv*, 9 (2019) 2055-2072.
- [13] X. Li, X. Jiang, Microfluidics for producing poly (lactic-co-glycolic acid)-based pharmaceutical nanoparticles, *Adv Drug Deliv Rev*, 128 (2018) 101-114.
- [14] C. Martins, F. Araújo, M.J. Gomes, C. Fernandes, R. Nunes, W. Li, H.A. Santos, F. Borges, B. Sarmento, Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy, *Eur J Pharm Biopharm*, 138 (2018) 111-124.
- [15] A. Gdowski, K. Johnson, S. Shah, I. Gryczynski, J. Vishwanatha, A. Ranjan, Optimization and scale up of microfluidic nanolipomer production method for preclinical and potential clinical trials, *J Nanobiotechnology*, 16 (2018) 12-12.
- [16] M. Witting, K. Obst, W. Friess, S. Hedtrich, Recent advances in topical delivery of proteins and peptides mediated by soft matter nanocarriers, *Biotechnol Adv*, 33 (2015) 1355-1369.
- [17] M.M. Pakulska, S. Miersch, M.S. Shoichet, Designer protein delivery: from natural to engineered affinity-controlled release systems, *Science*, 351 (2016) aac4750.
- [18] D. Duchêne, G. Ponchel, D. Wouessidjewe, Cyclodextrins in targeting: Application to nanoparticles, *Adv Drug Deliv Rev*, 36 (1999) 29-40.
- [19] K.S. Yadav, K.K. Sawant, Modified nanoprecipitation method for preparation of cytarabine-loaded PLGA nanoparticles, *AAPS PharmSciTech*, 11 (2010) 1456-1465.
- [20] U. Bilati, E. Allémann, E. Doelker, Nanoprecipitation versus emulsion-based techniques for the encapsulation of proteins into biodegradable nanoparticles and process-related stability issues, *AAPS PharmSciTech*, 6 (2005) E594-E604.
- [21] L. Peltonen, J. Aitta, S. Hyvonen, M. Karjalainen, J. Hirvonen, Improved entrapment efficiency of hydrophilic drug substance during nanoprecipitation of poly(l)lactide nanoparticles, *AAPS PharmSciTech*, 5 (2004) E16.
- [22] J. Xu, S. Zhang, A. Machado, S. Lecommandoux, O. Sandre, F. Gu, A. Colin, Controllable microfluidic production of drug-loaded PLGA nanoparticles using partially water-miscible mixed solvent microdroplets as a precursor, *Sci Rep*, 7 (2017) 4794.
- [23] U. Bilati, E. Allémann, E. Doelker, Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles, *Eur J Pharm Sci*, 24 (2005) 67-75.
- [24] L. Peltonen, P. Koistinen, M. Karjalainen, A. Hakkinen, J. Hirvonen, The effect of cosolvents on the formulation of nanoparticles from low-molecular-weight poly(l)lactide, *AAPS PharmSciTech*, 3 (2002) E32.
- [25] L. Wu, D. Leng, D. Cun, C. Foged, M. Yang, Advances in combination therapy of lung cancer: rationales, delivery technologies and dosage regimens, *J Control Release*, 260 (2017) 78-91.

- [26] E. Garaci, F. Pica, P. Sinibaldi-Vallebona, P. Pierimarchi, A. Mastino, C. Matteucci, G. Rasi, Thymosin α 1 in combination with cytokines and chemotherapy for the treatment of cancer, *Int Immunopharmacol*, 3 (2003) 1145-1150.
- [27] P. Li, L.-W. Qi, E.H. Liu, J.-L. Zhou, X.-D. Wen, Analysis of chinese herbal medicines with holistic approaches and integrated evaluation models, *TrAC-Trend Anal Chem*, 27 (2008) 66-77.
- [28] S. Bingaman, V.H. Huxley, R.E. Rumbaut, Fluorescent dyes modify properties of proteins used in microvascular research, *Microcirculation*, 10 (2003) 221-231.
- [29] M.K. Quinn, N. Gnan, S. James, A. Ninarello, F. Sciortino, E. Zaccarelli, J.J. McManus, How fluorescent labelling alters the solution behaviour of proteins, *Phys Chem Chem Phys*, 17 (2015) 31177-31187.
- [30] M. Al-Natour, Polymer nanomedicine: Understanding activity at the cellular and molecular level using metabolomics, University of Nottingham, 2019.
- [31] J. das Neves, B. Sarmento, Precise engineering of dapivirine-loaded nanoparticles for the development of anti-HIV vaginal microbicides, *Acta Biomater*, 18 (2015) 77-87.
- [32] A.S. Guedj, A.J. Kell, M. Barnes, S. Stals, D. Goncalves, D. Girard, C. Lavigne, Preparation, characterization, and safety evaluation of poly(lactide-co-glycolide) nanoparticles for protein delivery into macrophages, *Int J Nanomedicine*, 10 (2015) 5965-5979.
- [33] M.E. Fealey, B.P. Binder, V.N. Uversky, A. Hinderliter, D.D. Thomas, Structural impact of phosphorylation and dielectric constant variation on synaptotagmin's IDR, *Biophys J*, 114 (2018) 550-561.
- [34] S.Y. Lee, H.J. Cho, Dopamine-conjugated poly(lactic-co-glycolic acid) nanoparticles for protein delivery to macrophages, *J Colloid Interface Sci*, 490 (2017) 391-400.
- [35] P.B. Aldo, V. Craveiro, S. Guller, G. Mor, Effect of culture conditions on the phenotype of THP-1 monocyte cell line, *Am J Reprod Immunol* 70 (2013) 80-86.
- [36] H. Fessi, F. Puisieux, J.P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, *Int J Pharm*, 55 (1989) R1-R4.
- [37] J. Aubry, F. Ganachaud, J.P. Cohen Addad, B. Cabane, Nanoprecipitation of polymethylmethacrylate by solvent shifting: 1. Boundaries, *Langmuir*, 25 (2009) 1970-1979.
- [38] Q. Guo, Q. Zhu, T. Miao, J. Tao, X. Ju, Z. Sun, H. Li, G. Xu, H. Chen, L. Han, LRP1-upregulated nanoparticles for efficiently conquering the blood-brain barrier and targetedly suppressing multifocal and infiltrative brain metastases, *J Control Release*, 303 (2019) 117-129.

- [39] R. Nicolete, D.F.d. Santos, L.H. Faccioli, The uptake of PLGA micro or nanoparticles by macrophages provokes distinct in vitro inflammatory response, *Int Immunopharmacol*, 11 (2011) 1557-1563.
- [40] I.U. Khan, C.A. Serra, N. Anton, T.F. Vandamme, Production of nanoparticle drug delivery systems with microfluidics tools, *Expert Opin Drug Deliv*, 12 (2015) 547-562.
- [41] S. Yadavali, H.-H. Jeong, D. Lee, D. Issadore, Silicon and glass very large scale microfluidic droplet integration for terascale generation of polymer microparticles, *Nat Commun*, 9 (2018) 1222.
- [42] D.H. Jo, J.H. Kim, T.G. Lee, J.H. Kim, Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases, *Nanomedicine*, 11 (2015) 1603-1611.
- [43] N. Hoshyar, S. Gray, H. Han, G. Bao, The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction, *Nanomedicine* 11 (2016) 673-692.
- [44] E. Blanco, H. Shen, M. Ferrari, Principles of nanoparticle design for overcoming biological barriers to drug delivery, *Nat Biotechnol*, 33 (2015) 941-951.
- [45] R.P. Moura, C. Martins, S. Pinto, F. Sousa, B. Sarmiento, Blood-brain barrier receptors and transporters: An insight on their function and how to exploit them through nanotechnology, *Expert Opin Drug Deliv*, 16 (2019) 271-285.
- [46] T.T. Zhang, W. Li, G. Meng, P. Wang, W. Liao, Strategies for transporting nanoparticles across the blood-brain barrier, *Biomater Sci*, 4 (2016) 219-229.
- [47] R.H. Fang, K.N.H. Chen, S. Aryal, C.-M.J. Hu, K. Zhang, L. Zhang, Large-scale synthesis of lipid-polymer hybrid nanoparticles using a multi-inlet vortex reactor, *Langmuir*, 28 (2012) 13824-13829.
- [48] H.-X. Zhang, J.-X. Wang, L. Shao, J.-F. Chen, Microfluidic fabrication of monodispersed pharmaceutical colloidal spheres of atorvastatin calcium with tunable sizes, *Ind Eng Chem Res*, 49 (2010) 4156-4161.
- [49] P.M. Valencia, E.M. Pridgen, M. Rhee, R. Langer, O.C. Farokhzad, R. Karnik, Microfluidic platform for combinatorial synthesis and optimization of targeted nanoparticles for cancer therapy, *ACS Nano*, 7 (2013) 10671-10680.
- [50] R. Othman, G.T. Vladislavljević, H.C. Hemaka Bandulasena, Z.K. Nagy, Production of polymeric nanoparticles by micromixing in a co-flow microfluidic glass capillary device, *Chem Eng J*, 280 (2015) 316-329.
- [51] J. Sun, L. Zhang, J. Wang, Q. Feng, D. Liu, Q. Yin, D. Xu, Y. Wei, B. Ding, X. Shi, X. Jiang, Tunable rigidity of (polymeric core)-(lipid shell) nanoparticles for regulated cellular uptake, *Adv Mater*, 27 (2015) 1402-1407.

- [52] J.-M. Lim, N. Bertrand, P.M. Valencia, M. Rhee, R. Langer, S. Jon, O.C. Farokhzad, R. Karnik, Parallel microfluidic synthesis of size-tunable polymeric nanoparticles using 3D flow focusing towards in vivo study, *Nanomedicine*, 10 (2014) 401-409.
- [53] T. Baby, Y. Liu, A.P.J. Middelberg, C.-X. Zhao, Fundamental studies on throughput capacities of hydrodynamic flow-focusing microfluidics for producing monodisperse polymer nanoparticles, *Chem Eng Sci*, 169 (2017) 128-139.
- [54] N. Rescignano, L. Tarpani, R. Tiribuzi, S. Montesano, S. Martino, L. Latterini, J.M. Kenny, I. Armentano, Protein encapsulation in biodegradable polymeric nanoparticles: Morphology, fluorescence behaviour and stem cell uptake, *Macromol Biosci*, 13 (2013) 1204-1212.
- [55] H.S. Yoo, T.G. Park, Biodegradable polymeric micelles composed of doxorubicin conjugated PLGA-PEG block copolymer, *J Control Release*, 70 (2001) 63-70.
- [56] J.W. Goding, Immunofluorescence, in: J.W. Goding (Ed.) *Monoclonal Antibodies* (Third Edition), Academic Press, London, 1996, pp. 352-399.
- [57] L. Yin, W. Wang, S. Wang, F. Zhang, S. Zhang, N. Tao, How does fluorescent labeling affect the binding kinetics of proteins with intact cells?, *Biosens Bioelectron*, 66 (2015) 412-416.
- [58] H. Sah, Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation, *J Control Release*, 58 (1999) 143-151.
- [59] R. Gaudana, V. Khurana, A. Parenky, A.K. Mitra, Encapsulation of protein-polysaccharide HIP complex in polymeric nanoparticles, *J Drug Deliv*, 2011 (2011) 458128-458128.
- [60] N.K. Kunda, I.M. Alfagih, S.R. Dennison, H.M. Tawfeek, S. Somavarapu, G.A. Hutcheon, I.Y. Saleem, Bovine serum albumin adsorbed PGA-co-PDL nanocarriers for vaccine delivery via dry powder inhalation, *Pharm Res*, 32 (2015) 1341-1353.
- [61] S. Vakilian, S. Mashayekhan, I. Shabani, M. Khorashadizadeh, A. Fallah, M. Soleimani, Structural stability and sustained release of protein from a multilayer nanofiber/nanoparticle composite, *Int J Biol Macromol*, 75 (2015) 248-257.
- [62] S. Rawat, C. Raman Suri, D.K. Sahoo, Molecular mechanism of polyethylene glycol mediated stabilization of protein, *Biochem Biophys Res Commun*, 392 (2010) 561-566.
- [63] R.E. Wang, L. Tian, Y.-H. Chang, A homogeneous fluorescent sensor for human serum albumin, *J Pharm Biomed Anal*, 63 (2012) 165-169.
- [64] S. Choi, E.Y. Choi, D.J. Kim, J.H. Kim, T.S. Kim, S.W. Oh, A rapid, simple measurement of human albumin in whole blood using a fluorescence immunoassay (I), *Clin Chim Acta*, 339 (2004) 147-156.
- [65] K. Gellynck, V. Kodeck, E. Van De Walle, K. Kersemans, F. De Vos, H. Declercq, P. Dubruel, L. Vlamincx, M. Cornelissen, First step toward near-infrared continuous

glucose monitoring: In vivo evaluation of antibody coupled biomaterials, *Exp Biol Med*,
240 (2015) 446-457.

APPENDIX III. Doctoral thesis supplementary documents

The full version of publications that were partially reproduced in this doctoral thesis will be presented as appendix information in the following pages. These publications include:

- **C Martins**, VM Chauhan, M Araújo, A Abouselo, C Barrias, JW Aylott, B Sarmento. "Advanced polymeric nanotechnology to augment therapeutic delivery and disease diagnosis". *Nanomedicine*, 15, 2287-2309 (2020) (**CHAPTER I**)
- **C Martins**, VM Chauhan, AA Selo, M Al-Natour, JW Aylott, B Sarmento. "Modelling protein therapeutic co-formulation and co-delivery with PLGA nanoparticles continuously manufactured by microfluidics". *Reaction Chemistry & Engineering*, 5, 308-319 (2020) (**APPENDIX II**)
- **C Martins**, B Sarmento. "Microfluidic-manufacturing of multi-targeted PLGA/PEG nanoparticles for delivery of taxane chemotherapeutics", in *Methods in Molecular Biology*, Ed. K Jain, 2059:213-224, Springer, 2020 (**CHAPTER III**)

In the following pages, it will also be included the permission granted for reproduction and integration of the **CHAPTER IV**-based publication (full article use) in this doctoral thesis. **CHAPTER IV** was based on the following publication:

- **C Martins**, C Pacheco, C Moreira-Barbosa, A Marques-Magalhães, S Dias, M Araújo, MJ Oliveira, B Sarmento. "Glioblastoma immuno-endothelial multicellular microtissue as a 3D in vitro evaluation tool of anti-cancer nano-therapeutics". *Journal of Controlled Release*, 353, 77-95 (2023)



Advanced polymeric nanotechnology to augment therapeutic delivery and disease diagnosis

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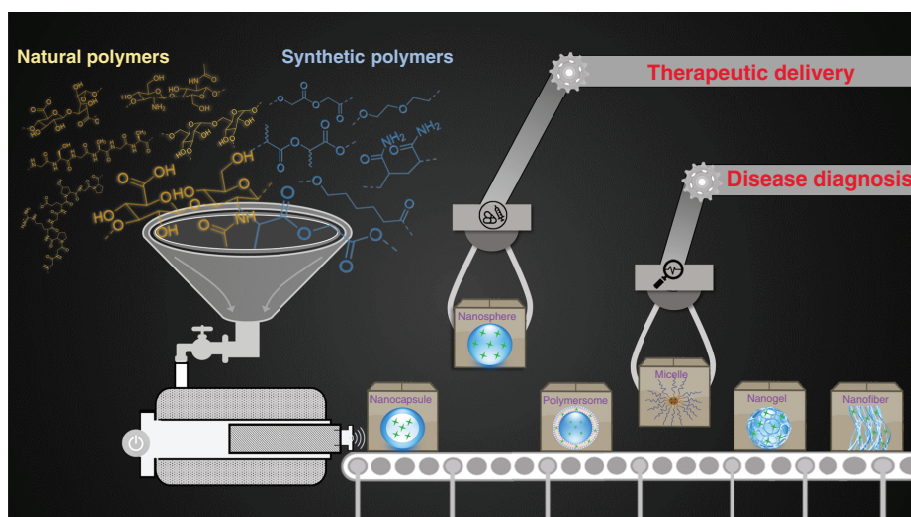
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Therapeutic and diagnostic payloads are usually associated with properties that compromise their efficacy, such as poor aqueous solubility, short half-life, low bioavailability, nonspecific accumulation and diverse side effects. Nanotechnological solutions have emerged to circumvent some of these drawbacks, augmenting therapeutic and/or diagnostic outcomes. Nanotechnology has benefited from the rise in polymer science research for the development of novel nanosystems for therapeutic and diagnostic purposes. Polymers are a widely used class of biomaterials, with a considerable number of regulatory approvals for application in clinics. In addition to their versatility in production and functionalization, several synthetic and natural polymers demonstrate biocompatible properties that dictate their successful biological performance. This article highlights the physicochemical characteristics of a variety of natural and synthetic biocompatible polymers, as well as their role in the manufacture of nanotechnology-based systems, state-of-art applications in disease treatment and diagnosis, and current challenges in finding a way to clinics.

Graphical abstract:



First draft submitted: 10 April 2020; Accepted for publication: 1 July 2020; Published online: 18 September 2020

Keywords: biocompatibility • diagnosis • nanotechnology • polymers • therapeutic delivery

Biomaterials research has witnessed an exponential growth over the last 30 years and has demonstrated strong economical and societal benefit indicators, which include improvements in the efficacy and reduction in cost of therapeutic interventions [1]. Polymers are one of the most multifaceted class of biomaterials with common use in the biomedical field for a range of applications. The interest in polymer science has ascended due to the versatility and ease of production of polymeric materials, which position them in between the mechanical strength of metals and the favorable biological properties of ceramics [2].

One of the most important requirements of a polymer for application in the biomedical area is the biocompatibility. This concept was defined by Williams as the ability of a material to accomplish the desired function at the cellular or tissue level in a clinically relevant manner, without eliciting any undesirable local or systemic host response [3]. Natural polymers are of a similar composition to tissues and thus biologically recognizable. However, their intrinsic variability and poor tunability permit application of synthetic polymers [4]. Biocompatible natural polymers used in biomedical applications include saccharide- or protein-based materials as chitosan (CHI), alginate (ALG), dextran (DEX) and hyaluronic acid (HA), or silk fibroin (SF) and gelatin (GEL), respectively. Whereas biocompatible synthetic polymers include esters as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) and poly(caprolactone) (PCL), acrylamides as poly(acrylamide) (PAM), or ethers as poly(ethylene glycol) (PEG).

The application of biocompatible polymers for the production of nanomaterials is at the forefront of the hot research topics of nanotechnology for therapeutic delivery and diagnosis purposes. This is because the loading of therapeutic and diagnostic cargos into polymeric nanomaterials has been shown to present enhanced physicochemical profile and biological performance. The advantages of these polymeric nanomaterials include: precise and targeted cargo delivery due to the prospect of surface functionalization, hence reducing cargo-related side effects; enhanced pharmacokinetic and pharmacodynamic properties of associated cargos, resulting in a higher therapeutic and/or diagnostic efficacy; elimination of noncontrolled release of cargos; and increase in the half-life of cargo molecules due to the polymeric protection against clearance [5,6]. As a result, the number of applications for biocompatible polymeric nanomaterials in the areas of therapeutic delivery and disease diagnosis is rapidly increasing, with increasing requirements for novel materials with enhanced functionality.

In this article, the physicochemical properties of different natural and synthetic biocompatible polymers will be explored, as well as their assembly in nanotechnology-based systems, function in state-of-art applications in the fields of therapeutic delivery and disease diagnosis, and translation hurdles.

Biocompatible polymers for nanotechnology applications

Natural polymers

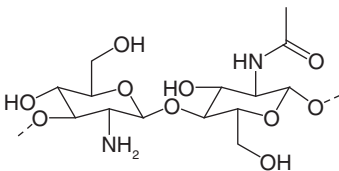
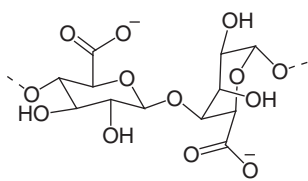
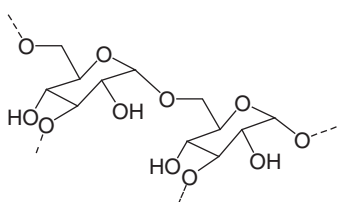
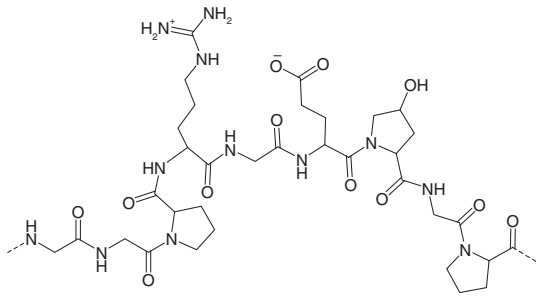
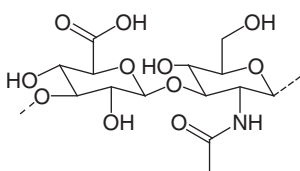
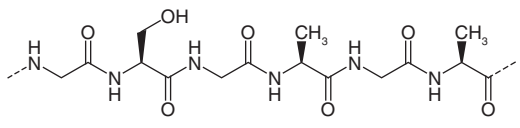
Chitosan

CHI is a natural polysaccharide of cationic nature obtained from chitin, an important component of arthropod exoskeleton and crustacean shells (Table 1). Extraction of chitin involves a primary acid treatment of decalcification followed by an alkali treatment for deproteinization. Deacetylation by boiling in NaOH results in the production of CHI, a linear polymer comprising $\beta(1-4)$ linked d-glucosamine residues with randomly distributed *N*-acetyl-D-glucosamine groups [7]. CHI can be degraded by several mechanisms, including surface erosion or enzymatic degradation through chitosanase and lysozyme [8]. In vertebrates, CHI is thought to be degraded by lysozyme and by bacterial enzymes in the colon through enzymatic hydrolytic cleavage. The large number of accessible hydroxyl and amine groups in CHI provide numerous possibilities of chemical functionalization, which may be performed in order to control properties such as hydrophobicity and electrostatic charge, or even to induce hydrogel formation through chemical cross-linking with bifunctional ligands [9–11]. CHI is widely regarded as a nontoxic polymer, being approved for dietary applications in Japan, Italy and Finland, and US FDA approved for use in wound dressings.

Alginate

ALG is a naturally occurring anionic and hydrophilic polysaccharide (Table 1). It is one of the most abundant biosynthesized materials, being typically found in brown algae, termed *Phaeophyceae* [12]. Commercially available ALGs are extracted from marine organisms by a chemical-based protocol, where the first step consists on the removal of the chelated counterions that sustain its structure in the marine organism through acidification with a mineral acid solution. The resultant insoluble alginic acid is then solubilized by using alkali such as sodium

Table 1. Chemical structure of the detailed biocompatible natural polymers.

Polymer	Chemical structure
Chitosan	 D-glucosamine/N-acetyl-D-glucosamine
Alginate	 D-manuronic acid/L-glucuronic acid
Dextran	 D-glucose/D-glucose
Gelatin	 Ala-Gly-Pro-Arg-Gly-Glu-Hyp-Gly-Pro
Hyaluronic acid	 D-gulcuronic acid/N-D-acetylglucosamine
Silk fibroin	 Gly-Ser-Gly-Ala-Gly-Ala

hydroxide or sodium carbonate in order to allow the removal of particulate matter through filtration. Finally, ALG is precipitated with ethanol, calcium salts or by using a mineral acid [13]. This polymer is composed by monomeric units of 1–4 linked β -D-mannuronic acid (M) and C-5 epimer α -L-gluronic acid (G), being organized in sequences of M (M-blocks) and G (G-blocks) residues, interspersed with M and G sequences (MG-blocks) [13]. Binding to alkali ions such as Na^+ provides the polymer with water solubility, whereas coordination to divalent ions such as Ca^{2+} , Ba^{2+} or Sr^{2+} results in hydrogel formation. The degradation kinetics of ALG can be accelerated by chemical modification of the polymeric chain. The periodate oxidation of ALG cleaves the carbon–carbon bond of the *cis*-diol group in the uronate residue and alters the chain conformation, promoting the hydrolysis of ALG in aqueous solutions [14]. This polymer may also be functionalized in order to modulate its interaction with cells. For instance, to promote cell-matrix adhesion or matrix degradation, peptides containing the arginine-glycine-aspartate (RGD) or proline-valine-glycine-leucine-isoleucine-glycine (PVGLIG) sequences, respectively, can be grafted to the polymer through carbodiimide chemistry on the carboxylic functionality, or by reductive amination on the aldehyde groups resultant from ALG oxidation [15,16].

Dextran

DEX is an extracellular bacterial homopolysaccharide synthesized from sucrose by dextransucrase enzymes (Table 1). The structure of DEX consists of linear $(1\rightarrow6)$ - α -D-glucose, with branches extending mainly from $(1\rightarrow3)$ and occasionally from $(1\rightarrow4)$ or $(1\rightarrow2)$ positions accounting for a 5% degree of branching [17]. The exact structure of each type of DEX depends on the degree of branching, which in turn depends on the specific microbial strain and, hence, on the specificity of dextransucrases involved [18]. The biodegradation of DEX involves natural enzymatic cleavage of saccharide bonds by dextran-1,6-glucosidase found in several organs such as spleen, liver, lungs, kidneys, brain and muscle tissue, as well as by dextranases expressed by bacteria in the colon [17]. The presence of a high number of hydroxyl groups in its structure makes DEX very attractive for chemical functionalization.

Gelatin

GEL is a water-soluble protein derived from collagen. Collagen is a fibrous insoluble protein widely found in nature, being the major component of skin, bone and connective tissue (Table 1). GEL is obtained by heat dissolution of collagen at alkaline (GEL type A) or acidic (GEL type B) conditions [19]. The chemical structure of GEL is represented by $(\text{Glycine-X-Proline})_n$, where X is attributed to other amino acid residues, usually lysine, arginine, methionine and valine [20]. These amino acid sequences are responsible for the organization of GEL in a triple helical structure, where helical regions form protein chains capable of retaining water, resulting in the formation of a hydrogel. The carboxylic and amine groups derived from the amino acids that compose GEL structure are attractive groups for functionalization through different chemical routes. These have been used to graft photopolymerizable moieties either on the amine by nucleophilic substitution or on the carboxylic acid functionality through carbodiimide chemistry, followed by esterification reaction, and to promote internal cross-linking. It is well accepted that proteolytic hydrolysis should be the major pathway for GEL biodegradation [21].

Hyaluronic acid

HA is produced at large scale by the *Streptococcus zooepidemicus* and *Streptococcus equi* bacteria with high yield and purity (Table 1). Apart from bacterial production, HA can be also obtained in large quantity from animal tissues [22]. HA is structurally characterized by being a large, linear glycosaminoglycan polymer composed by repeating units of D-gulucuronic acid and *N*-acetyl-D-glucosamine, linked by alternating β -(1,4) and β -(1,3) glycosidic bonds. Attempts to improve stability of this polymer often rely on chemical functionalization. The most common chemistries for HA modification include esterification on the hydroxyl functionality in the C-6 position of *N*-acetylglucosamine or C-2 position of D-gulucuronic acid, as well as esterification and carbodiimide coupling reactions on the available carboxylic groups [23]. Functionalization of the carboxylic functionality may be a viable strategy to decrease biodegradation, since it has been identified as the recognition site of the Hyal-2 enzymes. Biologically, HA plays an important role in organization and maintenance of the structural integrity of extracellular and pericellular matrices, behaving also as a signaling molecule by interacting with a variety of cell surface receptors and HA-binding proteins, to activate intracellular events that mediate several cell functions [24].

Silk fibroin

Silks are defined as fibrous proteins containing highly repetitive sequences of amino acids, which are stored in the animal as a liquid and assemble into fibers when sheared or spun during secretion (Table 1) [25]. The most extensively produced silk is the one spun from the silkworm, larva or caterpillar, of the domesticated *B. mori* moth, a member of the *Bombycidae* family [26]. SF can be defined as a macromolecular protein, which is coated by hydrophilic sericins of around 20–310 kDa, consisting mainly of glycine (44%), alanine (29%) and serine (11%). Although SF is classified by US Pharmacopeia as a nondegradable material, due to its negligible tensile strength loss *in vivo*, this macromolecular protein is susceptible to biological degradation by proteolytic enzymes such as proteinase XIV, α -chymotrypsin and collagenase IA [27,28]. The final products resulting from SF degradation are the corresponding amino acids, which are easily absorbed *in vivo*. Although SF is composed predominantly by the nonreactive amino acids glycine and alanine, functionalization can be achieved on the residues of serine, tyrosine, threonine, lysine, glutamic acid and aspartic acid present in its structure [26]. The most common methodology for SF modification is based on the reactivity of the hydroxyl group from serine residues, which can undergo esterification reactions. Tyrosine and lysine residues have also been used for attachment of oligosaccharides or synthetic polymers using a diazonium coupling strategy [29–31]. The excellent biocompatibility of SF supported its approval by FDA as a biomaterial for the fabrication of medical devices [32].

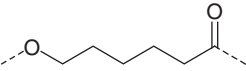
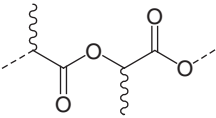
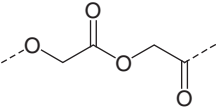
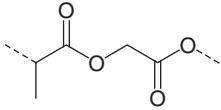
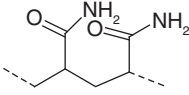
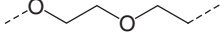
Synthetic polymers

Poly(caprolactone)

PCL is an aliphatic polyester, readily synthesized using a ring-opening polymerization of the ϵ -caprolactone (ϵ -CL) cyclic monomer (Table 2). PCL possesses a relatively low melting point, between 55 and 60°C, and a glass transition temperature of around -60°C. Thus, under physiological conditions, PCL presents a rubbery elastic state [33,34]. Its semicrystalline degree may reach values close to 70%, which decrease with increasing molecular weights (MWs) of the polymer [35]. PCL biodegradation occurs through hydrolysis of surface aliphatic ester linkages, and this is a slow degradation mechanism that permits PCL-based materials to remain physiologically active for extended periods of time (> 12 months) [36]. Therefore, the biological effects of acidic by-products resulting from PCL degradation are negligible [37]. This polymer received FDA approval for human clinical use [38]. However, PCL demonstrates high hydrophobicity and poor wettability, which avert its application for cell attachment purposes [39]. Since PCL is deficient in functional groups that can be readily modified, it is usually copolymerized or blended with other materials in order to obtain variants possessing improved biofunctionality [40]. The extended half-life of PCL networks turns it an ideal candidate for the manufacture of long-term systems in the drug delivery field [41].

Poly(lactic acid)

PLA belongs to the family of aliphatic polyesters and it is composed of lactic acid monomers, which could be obtained from renewable agricultural sources (Table 2). PLA is usually manufactured via ring-opening polymerization or direct polycondensation, either through solution or melt strategies [42]. The lactic acid monomer coexists in optically active D- or L-enantiomers. Depending on their proportion, PLA of variable properties can be obtained – poly(D-lactic acid) as a regularly structured crystalline material, poly(L-lactic acid) in the regularly structured hemicrystalline form, poly(D,L-lactic acid) as an amorphous material and meso-PLA resulting from the polymerization of meso-lactide [43]. The poly(L-lactic acid) homopolymer, often termed only PLA, has been the most widely used, possessing a crystallinity degree of around 37% and a glass transition and melting temperature of approximately 65 and 180°C, respectively [44]. Therefore, it presents a glassy elastic structure under physiological temperature, exhibiting a relatively rigid chain structure. The biodegradable properties of PLA result from the scission of its ester bonds and culminate in the generation of lactic acid and low MW oligomers, which are afterward forwarded to metabolic pathways [45]. The particularly poor absorption of water and high hydrophobicity of PLA are responsible for the prevention of cell surface adhesion and slow hydrolytic degradation of this polymer compared with its composite analogs, as PLGA [46]. PLA is deficient in modifiable functional groups, which is typical of polyesters. Nevertheless, PLA has been able to be combined with a variety of additional components such as biomimetic apatite, extracellular proteins or RGD peptides, rendering PLA amenable to more favorable biological performances [47]. FDA have accepted this polymer for human clinical applications [48].

Table 2. Chemical structure of the detailed biocompatible synthetic polymers.	
Polymer	Chemical structure
Poly(caprolactone)	 Ring-opened caprolactone
Poly(lactic acid)	 Lactic acid/Lactic acid
Poly(glycolic acid)	 Glycolic acid/Glycolic acid
Poly(lactic-co-glycolic acid)	 Lactic acid/Glycolic acid
Poly(acrylamide)	 Acrylamide/Acrylamide
Poly(ethylene glycol)	 Ethylene glycol/Ethylene glycol

Poly(glycolic acid)

PGA consists on the simplest linear aliphatic polyester, and it is composed of arrangements of different glycolic acid-based building blocks, derived from sugar cane (Table 2) [49]. Similarly to PLA, this polymer is usually obtained via monomer polymerization or condensation [50,51]. PGA presents a glass transition temperature of approximately 35°C, and a melting point of around 210°C, hence behaving as a glassy material under physiological temperature [51]. The simple structure and stereoregularity of PGA results in a high level of crystallinity (around 55%). PGA faces a bulk-type biodegradation via nonspecific scission of ester linkages, and this process can start occurring in less than 1 month. The degradation by-product, glycolic acid, is then transitioned to metabolic pathways and reacts to generate glycine [52]. PGA was one of the first biocompatible and biodegradable synthetic polymers explored in the biomedical field, as well as receiving FDA approval for human clinical applications [53]. However, the clinical translation of this material was hindered by its restricted solubility and high rate of degradation, consequently yielding considerable acidic by-products [54]. Moreover, PGA presents a hydrophobic nature and chemically inert surface, contributing to limited biological applications. This has been circumvented by strategies envisaging the conjugation of PGA with biologically active molecules and other polymers [55].

Poly(lactic-co-glycolic acid)

PLGA is an aliphatic polyester copolymer of lactic acid and glycolic acid (Table 2). The synthesis of PLGA is usually conducted via polycondensation or ring-opening polymerization of the monomers [56]. Different variants of this polymer can be obtained depending on the proportion of lactic and glycolic acid, although the most common is the 50:50 ratio one [57]. The average glass transition temperature of PLGA is 50°C, indicating its glassy elastic state

under physiologic temperature [58]. Since L-lactic acid and D,L-lactic acid originate semicrystalline and amorphous structures, respectively, and glycolic acid loses its high crystallinity level after copolymerization, PLGA is preferably amorphous [59]. The bulk biodegradation of PLGA in aqueous environments occurs through ester hydrolysis [60]. The rate of biodegradation is proportional to the content of glycolic acid as this monomer is more hydrophilic than the lactic acid one, which permits higher penetration of water into the copolymer structure [61]. Low MW values are also associated with a faster degradation, such that PLGA copolymers of 15 and 100 kDa degrade in time periods varying from weeks to months, respectively [62]. After biodegradation of PLGA, the lactic and glycolic acid by-products are forwarded to natural metabolic pathways for biorecycling [63]. PLGA belongs to the family of FDA-approved polymers for basic research and clinical use. Similarly to the previously mentioned synthetic polyesters, the hydrophobicity of PLGA becomes a disadvantage during cell loading. Furthermore, the insufficient number of modifiable functional groups per chain may pose a limitation when there is a demand for multifunctional polymers [64]. Thus, several authors have dedicated their work on this subject, successfully developing new strategies to circumvent these drawbacks [56]. The unique versatility of PLGA, which allows to formulate this polymer in multiple scales and designs, turned it into one of the most well-studied biocompatible polymers in the biomedical field.

Poly(acrylamide)

PAM is a free radical polymerized system of acrylamide monomers (Table 2). PAM alone forms linear cross-linked chains, therefore, to enhance surface area, it is often copolymerized or cross-linked with *N,N'*-methylenebisacrylamide [65]. Free radical polymerization of the monomers is initiated through the addition of redox pair catalysts ammonium persulfate and *N,N,N',N'*-tetramethyl ethylenediamine [66]. Cross-linked PAM form hydrogels and, by adjusting the total acrylamide concentration and ratio of *N,N'*-methylenebisacrylamide, PAM gels with a wide range of pore size can be produced [65]. This tunable property can be used as an excellent exchange system to either allow the permeation of biomolecules or entrap chemical elements for delayed therapeutic delivery or biological measurement. Dehydrated PAMs glass transition temperature is between 420 and 460°C, and its melting point is approximately 80°C, such that it is glassy under physiological temperatures [67]. In general, PAM is amorphous, but its crystallinity increases with the degree of polymerization [68]. PAM is extremely versatile by introducing primary amine-based monomers to the PAM matrix prior to polymerization, such as *N*-(3-aminopropyl)methacrylamide hydrochloride. Moreover, PAM can be functionalized with an array of molecules that contain succinimidyl chemistry, pre- or postmanufacture [69]. Acrylamide monomers are thought to be harmful, as they readily cross-link with nucleic acid, however, as cross-linked PAM they present biocompatibility [70].

Poly(ethylene glycol)

PEG is a linear polyether that is usually synthesized via ring-opening polymerization of ethylene oxide, triggered by nucleophilic attack of the epoxide ring (Table 2) [71]. PEG of low MW (<700 Da) appears to be a viscous and colorless liquid. Whereas high MW polymers (>1000 Da) are white crystalline solids whose melting point may increase up to around 70°C [72]. The glass transition temperature of PEG is approximately -60°C, independently of the polymer MW, such that PEG is a rubbery elastic material under physiologic temperatures [73]. Although PEG is not biodegradable, its metabolites are rapidly excreted from the physiological system via liver or kidneys [74]. The hydrophilicity of PEG results in the lowest level of protein or cell adhesion among biocompatible polymers. Thus, it is well known for its absence of immunogenicity or antigenicity, and has received FDA approval for biomedical applications [75]. The well-known 'PEGylation' arose from the stealth properties that PEG chains possess, which was explored by colloidal science as an efficient antifouling strategy to escape the immune system and increase the blood circulation time of biotechnology products [71]. Despite several PEG-based works simply depend on adsorption of the polymer, it may be engineered with functionalized architectures in order to facilitate the chemical coupling with other molecules [75].

Polymeric nanosystems

Nanoparticles

Polymeric nanoparticles are solid colloidal systems classified into nanocapsules or nanospheres. Nanocapsules have a vesicular-type structure in which the cargo is confined to a cavity surrounded by a polymer membrane, whereas nanospheres consist of structures in which the cargo is physically and homogeneously dispersed in a matrix [76]. Polymeric nanoparticles have been focus of great interest by the research community, as they possess a

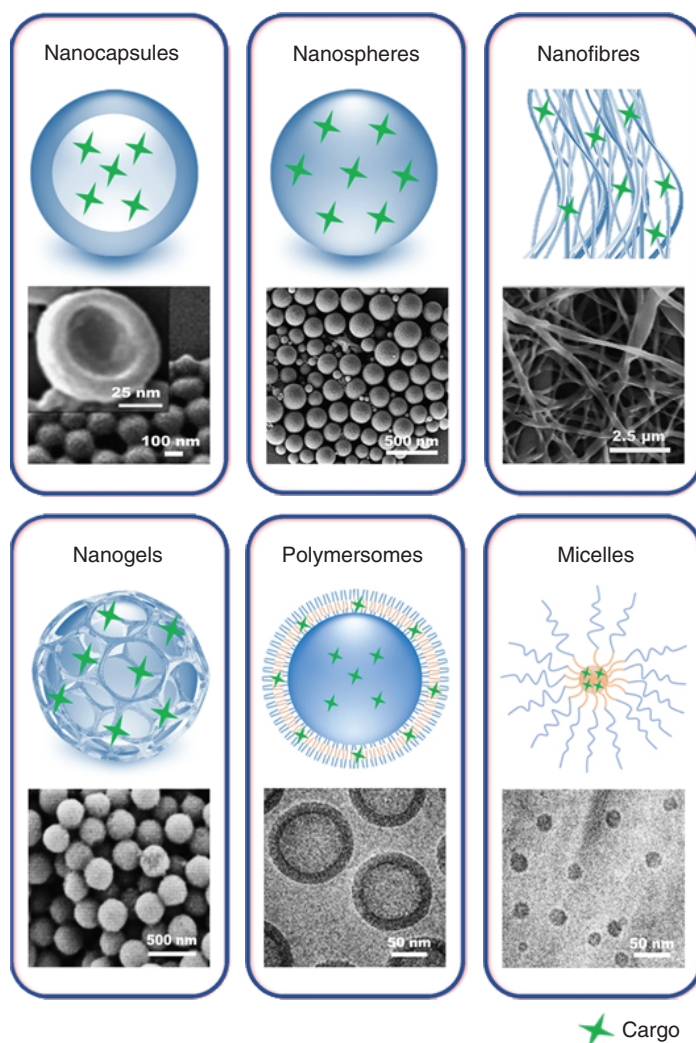


Figure 1. Diagrams and electron microscopy images showing the structure and morphology of different polymeric nanosystems.

Electron microscopy (EM) images of nanocapsules: Reproduced with permission from [114], © Royal Society of Chemistry (2015).

EM image of nanospheres: Reproduced with permission from [115], licensed with CC BY 2.0.

EM image of nanofibers: Reproduced with permission from [116], © The Korean Society of Industrial and Engineering Chemistry (2015).

EM image of nanogels: Reproduced with permission from [117], © American Chemical Society (2011).

EM image of polymersomes: Reproduced with permission from [118], © Royal Society of Chemistry (2013).

EM image of micelles: Reproduced with permission from [119], © Royal Society of Chemistry (2015).

controlled release profile of the cargo, high stability and proved efficacy regarding the encapsulation or adsorption of both hydrophilic and hydrophobic molecules of diverse MWs [77]. Polymeric nanoparticles, either nanocapsules or nanospheres, usually present spherical-shaped morphology, alongside with smooth surface, as represented in Figure 1.

Nanofibers

Nanofibers are classically defined as 1D nanomaterials, which have a diameter in the range 1–1000 nm and length of the order of 1000 nm and above [78]. In recent years, incorporation of functional bioactive materials [79], drug molecules [80,81] and nano-sized particles [79–83] into nanofibers have attracted the interest of researches due to their synergistic and hybrid characteristics. The incorporation of such systems into the nanofibers can provide better performance due to the larger surface and capability to imitate complicated structures, while maintaining the incorporated material properties and enhanced composite physical characteristics [84]. Nanofibers can be fabricated in various morphologies depending on the intended application purposes. Examples of this include cylindrical (Figure 1) [85,86], hollow [87], flat [88], beads on a string [89], ribbon [88], grooved [90], porous [91], multichannel [92], core–shell [93] and helical [94,95] structures.

Nanogels

Nanogels are defined as nanoscale dispersions of hydrogel particles, composed of swollen networks of hydrophilic or amphiphilic polyionic polymers [96]. Oppositely to other polymeric nanosystems such as nanospheres, which present a densely packed core, nanogels are characterized by softness properties and may comprise around 90%

of water content upon swelling [97]. These materials are mostly used and ideal for association with hydrophilic cargo molecules, although the literature data also report their re-engineering to incorporate hydrophobic compounds [98,99]. Some of the key characteristics of nanogels include high cargo loading capacity and stability in aqueous media, while keeping the buoyant density at reasonably low levels [100]. The release of the cargo from the nanogel may occur through swelling or shrinking phenomena in response to surrounding stimuli that trigger alterations in the network or gel-phase transitions of the nanosystem [101]. The morphology of polymeric nanogels is usually round-shaped, as represented in [Figure 1](#).

Polymersomes

Polymersomes are artificial vesicle-like structures composed of amphiphilic block copolymers, and analogs of their lipid counterparts known as liposomes [102]. Polymersomes present an aqueous lumen surrounded by a bi-layer membrane, which results from hydrophilic coronas located at both sides of the hydrophobic ring [103]. The polymersomes structure arrangement possess regions of variable hydrophilicity and may be used to entrap both hydrophobic and hydrophilic cargos [104]. The release of the cargo may occur immediately upon disassembly of the nanosystem triggered by a chemical or physical stimulus, whereas the controlled release may be achieved by tuning the permeability of the polymersome membrane according to its polymeric composition [105]. Polymersomes are known to demonstrate enhanced colloidal stability, higher mechanical strength, as well as lower chemical permeability compared with liposomes. They are also more robust and easily functionalized, and most of them demonstrate intrinsic stealth properties [106,107]. Polymersomes exhibit a hollow sphere morphology typical of vesicles, as represented in [Figure 1](#).

Micelles

Micelles are nanomaterials that spontaneously self-assemble from amphiphilic copolymers to form core-shell architectures [108]. The most common type of micelles described for biomedical applications are composed of a hydrophilic exterior and hydrophilic interior [109]. The hydrophilic portion of the copolymer assumes a steric protective shell enveloping the core, which in turn derives from the hydrophobic moiety and is sheltered from the surrounding aqueous environment [110]. Most of the key characteristics of micelles are related to the shell since it is able to prevent protein adsorption and opsonization [111]. Moreover, the corona of polymeric hydrophilic chains of the shell stabilizes the core, which is widely used as a reservoir of hydrophobic cargos [112]. Generally, the size of the hydrophilic tail of the copolymer is bigger than the one of the hydrophobic region, resulting in spherical-shaped micelles, as represented in [Figure 1](#). When the hydrophobic region is larger than the hydrophilic, diverse other morphologies including rod- or lamellae-like structures are formed [113].

Polymeric nanosystems for therapeutic delivery & disease diagnosis

Therapeutic delivery

Cancer

Several authors have dedicated their efforts to investigate novel polymeric nanotechnology therapeutic approaches for cancer malignancies. Coencapsulation has become the new fashion since it allows to synchronize tumor multidelivery, taking advantage of the synergistic effect of cargos. Deng *et al.* approached the coencapsulation of the tumor-suppressive molecule miR-34a and doxorubicin into nanoparticles of HA and CHI [120]. They verified a considerable enhancement on the antitumor effect of doxorubicin against breast cancer cells, mainly due to the suppression of expression of the antiapoptosis proto-oncogene Bcl-2 by miR-34a. Other authors explored the arrangement of polymersomes of PCL and PEG to encapsulate paclitaxel and doxorubicin on their hydrophobic ring and surrounding hydrophilic corona, respectively ([Figure 2](#)) [121]. Further functionalization of polymersomes with folate ligand to target folic acid receptor on hepatoma cells resulted in a significantly higher *in vivo* tumor growth inhibition (80%), compared with the free drug cocktail (54%) or nontargeted control (68%).

In another study, implantable electrospun PLA nanofibers were used as a multidelivery nanosystem of oxaliplatin and fluorouracil for colorectal cancer in order to avoid local recurrence after surgery [122]. The authors verified suppression of tumor recurrence and an ability to prolong the mice survival time more efficiently compared with the intravenous control treatment. In regard to monoencapsulation therapies, Morán *et al.* recently reported versatile GEL nanoparticles for encapsulation of fluorouracil [123]. This system benefited from the naturally occurring RGD motifs of GEL to target tumor cells, as well as the isoelectric point of the polymer, between 4.8 and 5.2, to minimize the trafficking of the nanoparticles from endosomes to lysosomes. Since GEL is negatively charged at physiological

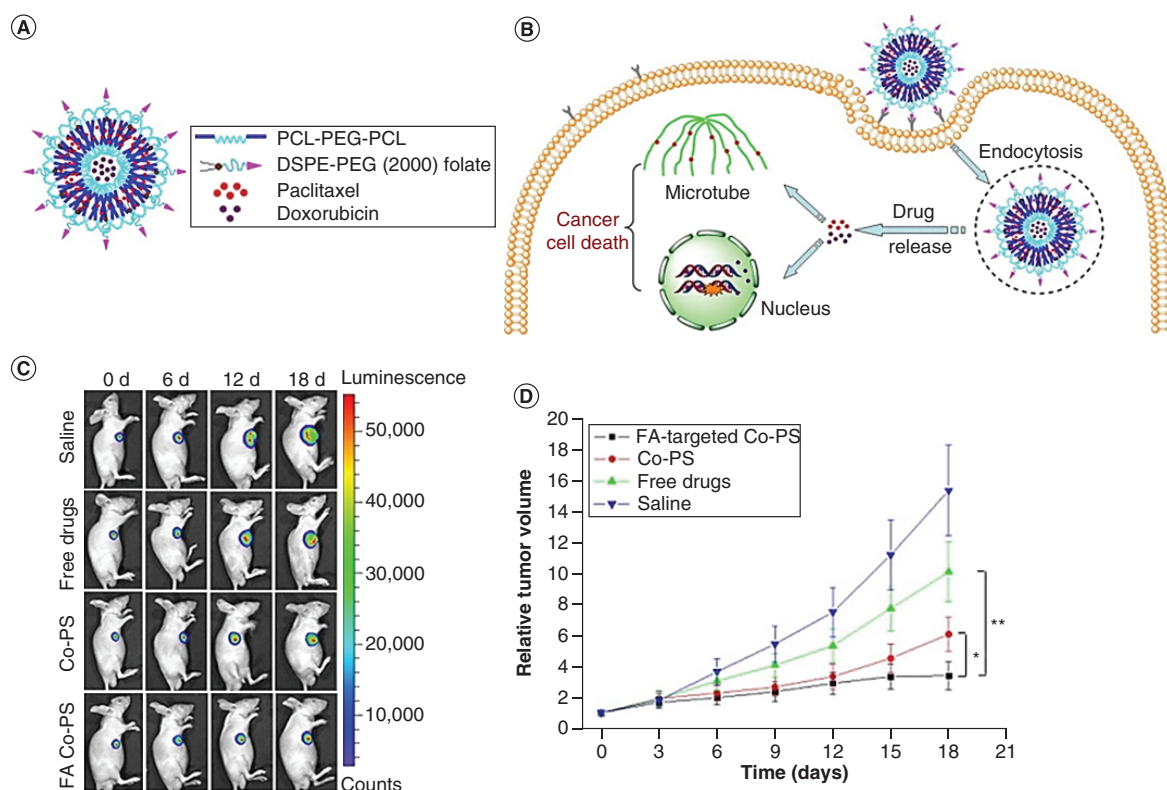


Figure 2. Therapeutic delivery of paclitaxel and doxorubicin using folate-functionalized polymersomes of poly(ϵ -caprolactone) and poly(ethylene glycol) for chemotherapy of hepatocellular carcinoma. (A) Structure of the nanosystem, in which a PEGylated distearoylphosphatidylethanolamine chain (DSPE-PEG[2000]) was used for the functionalization strategy. (B) Mechanism proposed for the endocytosis of the nanosystem in folate receptor-overexpressing BEL-7404 cells. (C) Representative *in vivo* bioluminescence images after treatment of mice with the control (saline), free drugs, nontargeted loaded system (Co-PS) and targeted loaded system (FA Co-PS). (D) Relative tumor volume after treatment of mice with the control (saline), free drugs, nontargeted loaded system (Co-PS) and targeted loaded system (FA Co-PS).

* $p < 0.05$; ** $p < 0.01$.

Co-P: Co-polymersome; DSPE: 1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine; FA: Folic acid; PCL: Polycaprolactone; PEG: Polyethylene glycol.

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pH, it can interact with the oppositely charged cargo to facilitate the entrapment process. However, once the nanosystem faces the acidic pH of endosomes, GEL becomes positively charged and immediately repulses and releases the entrapped fluorouracil intracellularly. Mottaghiab *et al.* explored the use of SF in the manufacture of gemcitabine nanoparticles for the treatment of lung cancer (Figure 3) [124]. The nanosystem targeting was achieved through attachment of the SP5-52 peptide, which recognizes tumor antiangiogenic agents of lung cancer cells. After intravenous administration, the targeted gemcitabine nanoparticles achieved enhanced survival rate and lung tissue recover compared with the free drug and nontargeted control, which could be attributed to the ability of the SP5-52 peptide to trigger the binding of targeted nanoparticles to the Flt-1 receptor, overexpressed on the surface of lung cancer cells [124].

Infectious diseases

With respect to infectious diseases, polymeric nanosystems have demonstrated considerable potential in the treatment of tuberculosis. Rawal *et al.* explored CHI nanoparticles to deliver the frontline antitubercular drug rifampicin, demonstrating their *in vivo* ability to keep higher levels of drug concentration for a longer period of time, compared with the nonencapsulated therapeutic [125]. Other authors used ALG polymer as matrix of nanosystems for coencapsulation of rifampicin and another antitubercular drug, isoniazid [126]. *In vivo* mice studies revealed that drugs loaded into ALG nanoparticles exhibited higher extent and rate of uptake by lungs in comparison with the

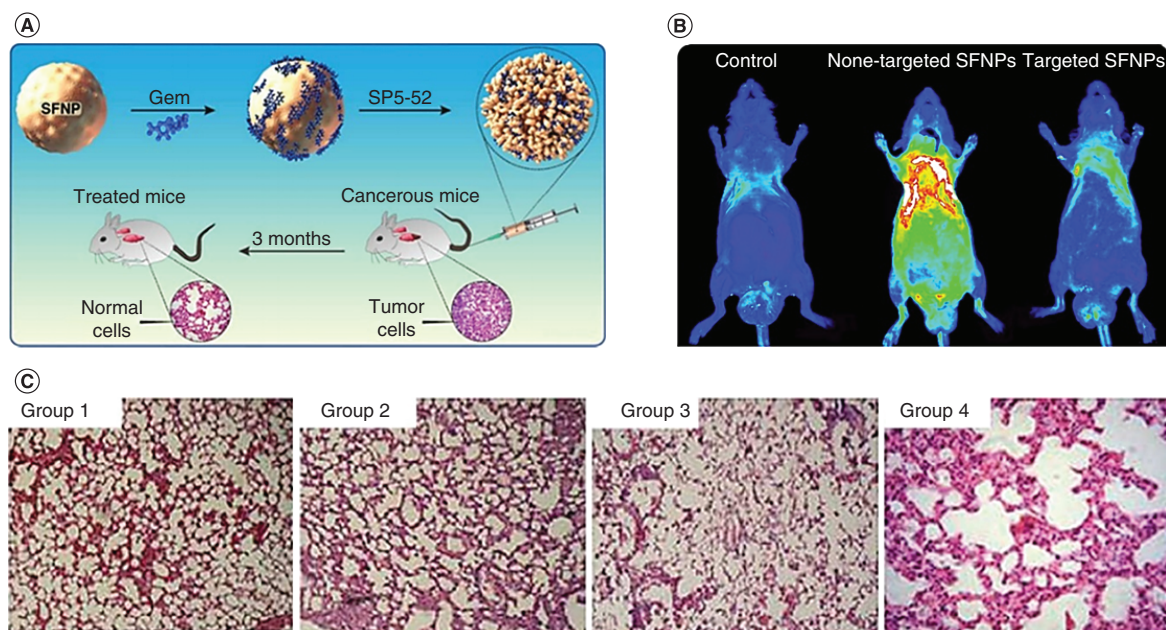


Figure 3. Nanosystem of silk fibroin nanoparticles functionalized with the SP5-52 peptide for delivery of gemcitabine to treat lung tumors. (A) Delivery nanosystem of SF nanoparticles loaded with Gem. **(B)** *In vivo* mice imaging after injection of targeted and nontargeted nanoparticles, compared with the noninjected group (control). **(C)** Histopathological assessment of lung tissue after treatment, in which Groups 1, 2, 3 and 4 correspond to animals injected with only phosphate-buffered saline (control), free drug, nontargeted and targeted nanoparticles, respectively.

Gem: Gemcitabine; NP: Nanoparticle; SF: Silk fibroin.

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nonencapsulated therapeutics, which was explained by the more prolonged retention of the nanoparticles at the target site, resulting in higher level of phagocytosis by alveolar macrophages. Upadhyay *et al.* also approached a strategy of combination therapy using micelles of PMA and PLA for delivery of rifampicin and isoniazid [127]. The effectiveness of these loaded micelles was tested *in vitro* against susceptible and resistant *Mycobacterium tuberculosis* strains, with considerably lower values of minimal inhibitory concentration obtained for the nanosystem compared with the nonencapsulated drugs. Nanosystems of biocompatible polymers have also driven the development of novel therapy approaches to fight HIV infection. Gong *et al.* proposed the encapsulation of the antiretroviral elvitegravir in a PLGA nanosystem that was able to enhance the intracellular concentration of the drug in HIV-infected monocytic cells, as well as the viral suppression ability in HIV-infected primary macrophages [128]. In another study, a PLGA nanosystem was also developed to formulate tenofovir disoproxil fumarate, in an attempt to enhance the oral absorption of this drug [129]. A relative fold increase of 6.8 and 1.7 was verified for the oral bioavailability and mean residence time of the tenofovir disoproxil fumarate-loaded nanosystem, respectively, compared with the free drug. Efavirenz is an example of another antiretroviral that has been delivered to HIV by means of biocompatible polymeric nanosystems [130,131].

Metabolic disorders

The application of polymeric nanosystems in the treatment of metabolic disorders has turned the efforts toward diabetes mellitus. Chou *et al.* proposed a polymeric nanogel of CHI as an injectable delivery system for insulin, in order to achieve a long-term management of the basal levels of this peptide hormone (Figure 4) [132]. Lysozyme was strategically included in the system, envisaging gel disassembly and consequent insulin release due to the digestion of glycosidic bonds of CHI by the enzyme. The authors verified that a single injection of the nanogel formulation in a diabetic mouse model was able to keep the blood glucose at considerably lower values compared with the administration of free insulin, in a time period of 10 days [132]. Alibolandi *et al.* investigated another type of nanosystem to deliver insulin based on polymersomes of DEX and PLGA [133]. Studies conducted in diabetic rats evidenced that the drug bioavailability increased from 0.62 to 9.77% for the same orally administered dosage

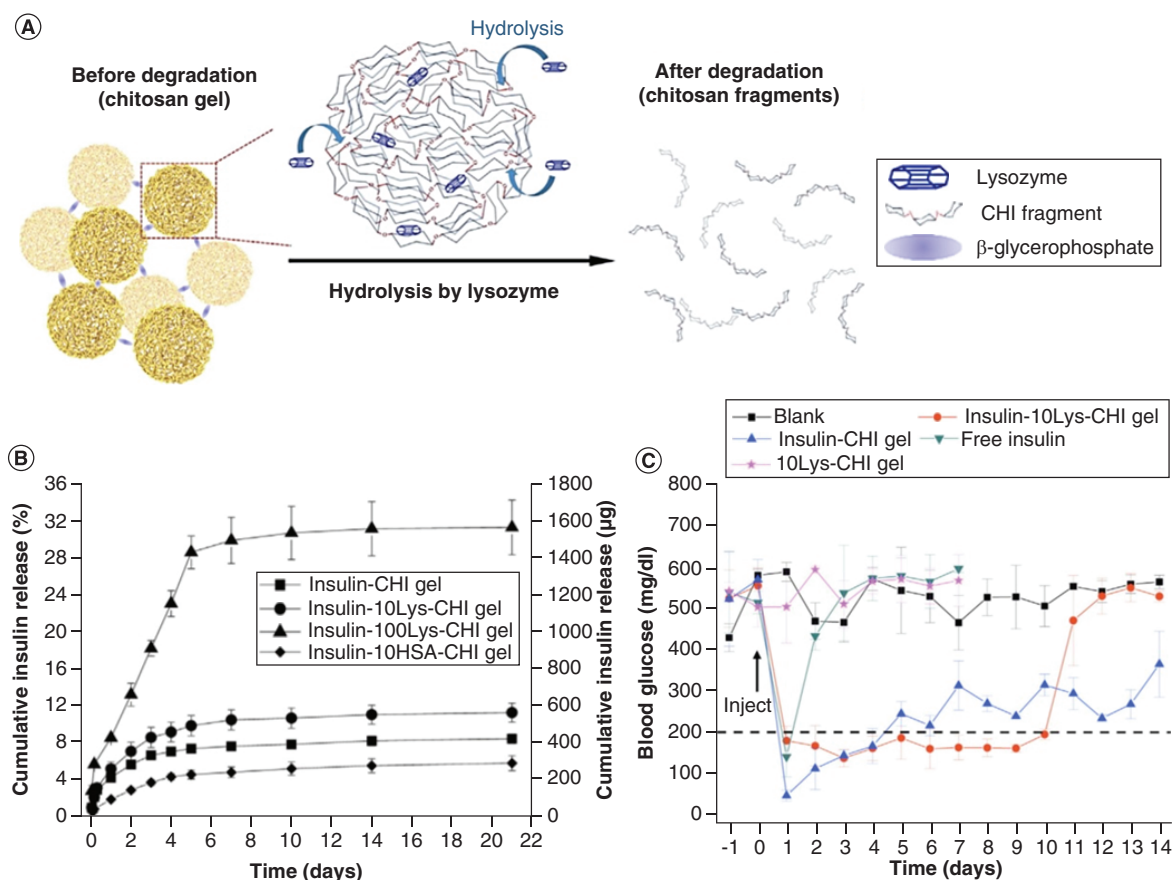


Figure 4. Polymeric nanogel of chitosan and lysozyme to deliver insulin in the context of diabetes mellitus. (A) Illustration of the developed nanosystem. **(B)** Release of insulin from CHI gels without lysozyme (insulin-CHI-gel), with 10 μ g/ml lysozyme (insulin-10Lys-CHI-gel), with 100 μ g/ml lysozyme (insulin-100Lys-CHI gel) or with human serum albumin control at 10 μ g/ml. **(C)** Blood glucose levels of diabetic mice treated with phosphate-buffered saline solution control (blank), insulin-loaded CHI gel with 10 μ g/ml lysozyme (insulin-10Lys-CHI gel), insulin-loaded CHI gel without lysozyme (insulin-CHI gel), free insulin and unloaded CHI gel with 10 μ g/ml lysozyme (10Lys-CHI gel). CHI: Chitosan.

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of free insulin and insulin-loaded polymersomes, respectively. A plethora of other alternatives have emerged in the field of diabetes therapy, with novel nanosystems proposed for the encapsulation of antidiabetic therapeutics as exenatide [134–136], metformin [137] or glucagon-like peptide-1 [138]. Osteoporosis has also been focus of attention in recent literature [139]. Dong *et al.* suggested a nanosystem of polymeric nanoparticles of CHI and HA to codeliver alendronate and curcumin as a bone antiresorptive therapeutic and density enhancer, respectively [140]. *In vitro* results confirmed the potential of the loaded nanosystem to promote proliferation, differentiation and mineralization in the extracellular matrix of the preosteoblastic model of MC3T3-E1 cells, thus triggering active bone formation. Other authors explored the use of intravenous PLGA nanoparticles to deliver RANK siRNA, which is a gene silencer of the RANK receptor, also known as receptor activator of NF- κ B, and related to the differentiation and activation of osteoclasts [141]. Significant *in vivo* reductions in the order of 50% were observed in the levels of RANK mRNA, differentiation of osteoclast precursors to mature osteoclasts, and osteoclastic activity. In another study, Narayanan *et al.* approached the encapsulation of the human parathyroid hormone 1–34 in CHI and PEG nanoparticles [142]. Following oral administration in rats, the nanosystem provided a bioavailability of around 100 pg/ml, whereas the free hormone was only able to reach a maximum of 30 pg/ml. These findings stressed out the ability of the chosen nanoparticles to trigger intestinal uptake of the therapeutic from the digestive tract of the animals.

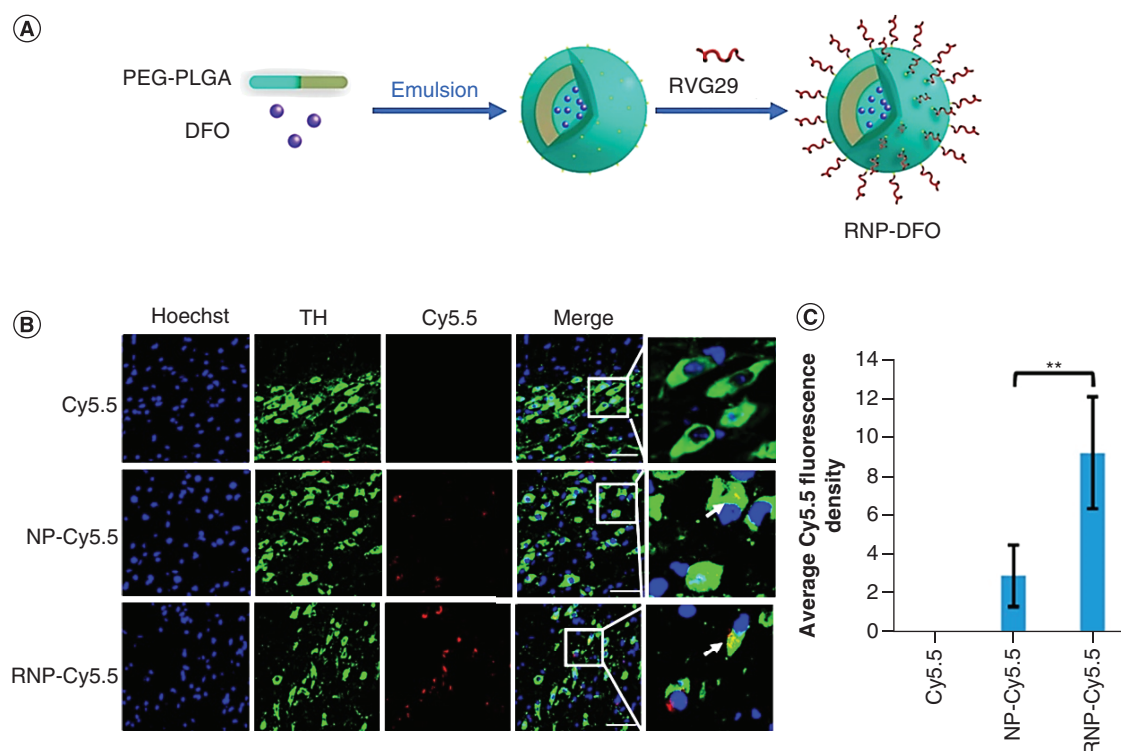


Figure 5. Targeted nanosystem of poly(lactic-co-glycolic acid) and poly(ethylene glycol) to deliver deferoxamine to the brain and trigger reversibility of functional deficits in Parkinsonian mice. (A) Manufacture of PLGA and PEG nanoparticles loaded with DFO and functionalized with a peptide derived from the rabies virus glycoprotein (RVG29), which binds the nicotinic acetylcholine receptor of the blood–brain barrier. RNP-DFO represents the final nanosystem. **(B)** Representative confocal images of the substantia *nigra* of treated mice, in which the Cy5.5 fluorescent dye was encapsulated in the nanoparticles to mimic the drug. The animals were treated with free Cy5.5, nontargeted and targeted Cy5.5 nanoparticles (NP-Cy5.5 and RNP-Cy5.5, respectively). Hoechst and TH were used to stain the nuclei and neurons, respectively. **(C)** Quantitative measurement of Cy5.5 fluorescence related with the confocal analysis. ** $p < 0.01$.

DFO: Deferoxamine; PEG: Poly(ethylene glycol); PLGA: Poly(lactic-co-glycolic acid); TH: Tyrosine hydroxylase.

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CNS diseases

Among the reasons for the inadequacies in current therapies, the crossing of the blood–brain barrier (BBB) is one of the biggest challenges in what concerns therapeutic delivery to the brain, even under disease conditions [143]. Sun *et al.* presented a polymeric nanosystem of polysorbate 80-coated PLGA nanoparticles to deliver the neuroprotective puerarin to the brain, suitable for ischemic cerebrovascular disease [144]. After intravenous administration, the nanosystem exhibited a maximum concentration in the brain of treated rats of around 150 ng/ml, compared with a value of approximately 75 ng/ml for the free therapeutic. Coating with polysorbate-80 promotes the adsorption of apolipoproteins B and E on the surface of nanoparticles, hence triggering their transport to the brain through interaction with the low-density lipoprotein receptor of the BBB [145]. The same effect may be achieved by a coating with poloxamer 188 [146], which was used by Khalin *et al.* in the development of an intravenous PLGA nanosystem to deliver the neurotrophic factor to traumatic brain injury [147]. The authors verified a significant increase in the levels of the neurotrophic factor in both ipsilateral and contralateral hemispheres of the injured brain of mice, compared with the free therapeutic. Other authors focused their research on the development of intravenous nanoparticles of PLGA and PEG to deliver deferoxamine, in order to prevent oxidative stress occurring from the excess of iron deposition in the brain under Parkinson's disease (Figure 5) [148]. The nanoparticles were targeted to the brain via functionalization with a peptide derived from the rabies virus glycoprotein, which binds the nicotinic acetylcholine receptor of the BBB. The investigation of oxidative stress indicators, as reactive oxygen species, demonstrated that their levels in the brain of parkinsonian mice were significantly reduced by the loaded nanoparticles, compared with the free deferoxamine [148].

Zhang *et al.* envisaged an intravenous PLA and PEG nanosystem to deliver the β -sheet breaker peptide H10, dual-functionalized with the TGN shuttle and QSH peptides to target the BBB and brain A β 42, respectively, for Alzheimer treatment [149]. The nanosystem promoted considerable enhancements on the spatial learning and memory of diseased mice, while the free therapeutic reached only a certain degree of improvement. Qi *et al.* focused on the therapy of acute spinal cord injuries, proposing intravenous nanoparticles of DEX modified with ibuprofen to deliver methylprednisolone, with further evaluation of treated rats in regard to the hindlimb motor function [150]. The authors demonstrated significant improvements in tilt angles and Tarlov scores of rats treated with the loaded nanoparticles, in comparison with animals treated with free methylprednisolone.

Disease diagnosis

Polymeric nanosystems can be used to encapsulate contrast agents such as barium, iodine and gold, or labeled with radioisotopes for medical imaging applications. The application of contrast agent-loaded nanoparticles reduces the rapid distribution and elimination of the contrast species, which minimize adverse reactions such as renal toxicity [151]. Imaging modalities benefiting from polymeric nanoparticles used as contrast agents include positron emission tomography (PET), MRI and x-ray imaging. PET is an imaging modality used to detect intravenously injected nanoparticles labeled with a positron-emitting radioisotope, such as ^{18}F and ^{64}Cu . DEX nanoparticles labeled with ^{64}Cu were previously employed to monitor inflammation in atherosclerotic plaque [152], whereas polystyrene latex nanoparticles coated with the same radioisotope were used to image the uptake of therapeutics by lung tissue in patients with acute and chronic respiratory diseases [153]. Iron oxides have been encapsulated into polymeric nanoparticles as novel MRI contrast agents, which is the case of intravenously injected PAM nanoparticles containing iron oxide crystals that were used for MRI of brain tumors in rats [154]. Surface modification of the nanoparticles with PEG chains has been reported to improve the nanosystem plasma half-life, which lead to longer lasting MRI imaging [154]. Regarding x-ray, core-shell nanoparticles of poly(2-methacryloyloxyethyl[2,3,5-triiodobenzoate])/g- Fe_2O_3 and iodine-loaded nanosystems were described as a tool to image several physiologic architectures such as liver, kidney, spleen and lymph nodes in animal models [155].

Fluorescent nanosensors are optical-based sensors typically used for assessment of subcellular biochemical parameters [156]. Nanosensors that entrap sensing elements based on the matrix pore size generally utilize reduction in pore dimension, through alteration of the composition of the polymeric matrix, in order to physically limit fluorophore escape. Entrapping fluorophores in this manner is ideally suited to nanoparticle matrices, which maintain a rigid structure and do not swell or deform when in circulation for extended periods [157]. Almdal *et al.* have reported that PAM-based nanoparticles, prepared by embedding fluorophores within the nanosensors matrix, can leach 50% of their fluorophores within 20 h [158]. Another approach consists of covalently link fluorophores directly to the nanoparticle matrix, to eliminate uncertainties of fluorophore loss [159]. Ow *et al.* have shown that conjugation of fluorescent dyes to a core-shell nanoparticle can enhance brightness of a nanosensor by up to 20-times greater than the constituent fluorophore [160]. This has been attributed to the exclusion of solvent-based interferences. Due to the versatility of the nanosensor matrix, and its ability to shield sensing elements, arrays of sophisticated fluorescence sensing techniques are being developed. Doussineau *et al.* reviewed the range of sensing elements that have and are being developed for fluorescent nanosensors, which include reversible analyte binders, dynamic collisional quenchers and receptor-mediated elements [161]. Fluorescent nanosensors have been reported for the detection of: key biochemical molecules such as glucose [162], calcium [163] or adenosine triphosphate [164]; metals such as iron [165] or mercury [166]; and macromolecules such as proteins [157,167] or nucleic acids [168].

Translation into clinics: current challenges

Despite the diversity of therapeutic and/or diagnostic applications that have been described for polymer nanotechnology systems, the translation into clinics is still behind expectations. One of the eminent challenges in regard to this matter is the poor control over design features of the nanosystems, in which the so-called protein corona plays a key role. As extensively reviewed elsewhere [169–171], polymer nanotechnology systems are expected to be promptly coated by a dynamic and multicomposition layer of endogenous proteins after administration, which seems to be both patient- and nanosystem-specific. This layer has the capacity to alter the surface properties of the designed nanosystem and change its predicted biological fate in a dramatic manner, turning the preclinical studies insufficient to give precise data about the concrete potential of the nanosystem [172]. It is therefore of uttermost importance to unveil the mechanisms behind protein corona formation and exact composition for each specific type of nanosystem, which is further expected to give insights on how to design novel nanosystems accounting

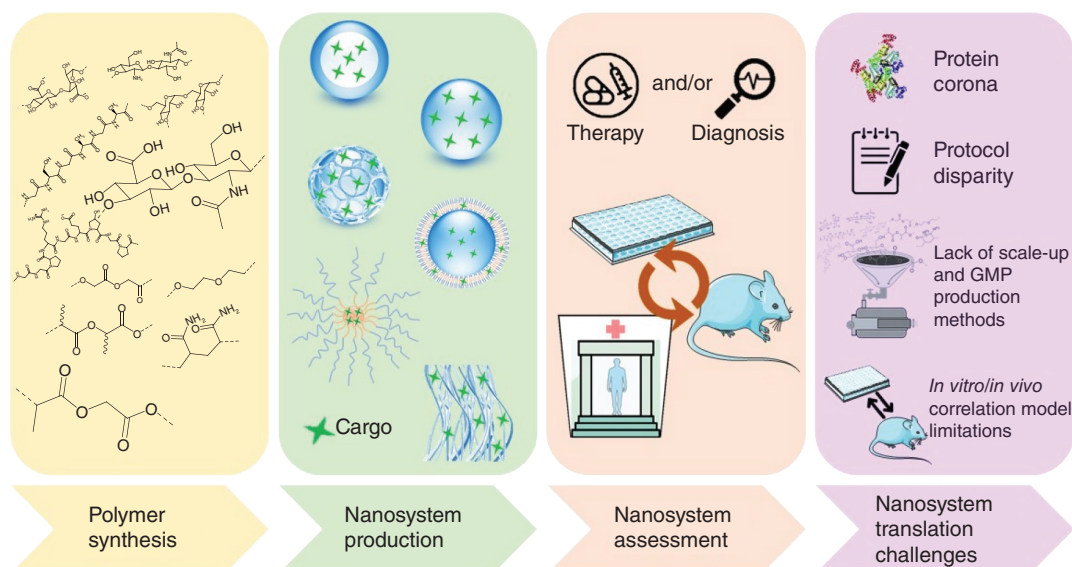


Figure 6. Polymer nanotechnology workflow from polymer synthesis to nanosystem translation challenges.

for the impact of the protein corona *a priori*. Despite great efforts, testing models mimicking protein corona formation *in vivo* to challenge the developed nanosystems are still missing. On the other hand, the research field would benefit from the standardization of protocols to fully develop and characterize each novel nanosystem in the preclinical phase, hence avoiding disparity of experimental conditions and data among different studies. Steps toward this have been recently initiated with the proposal of the Minimum Information Reporting in Bio–Nano Experimental Literature reporting standards, termed MIRIBEL [173]. These reporting standards are based on a checklist that allows better reproducibility and quantitative research, presenting minimal requirements for a good publication practice in the bio–nano field and divided in: material characterization, biological characterization and details of experimental protocols. For instance, in the case of *in vivo* experiments, the age and gender of animals may have a significant impact on the therapeutic and/or diagnostic outcomes, which, if not accounted for, make comparisons difficult. Besides this, laboratory methods for polymer nanotechnology production are often associated with limited material scaling and purity degree, hence causing major quality change from batch-to-batch and low-throughput [174]. Thus, other aspects that pose a challenge to the translation of polymer nanotechnology systems are the lack of scale-up and GMP manufacturing methods. Finally, bridging the gap between polymer nanotechnology development and clinics would involve to ameliorate the *in vitro/in vivo* correlation [175,176]. *In vitro* data are usually associated with poor prediction of the nanosystem fate *in vivo*, and this elucidates the need to exploit more advanced human-mimicking or human-derived *in vitro* models with capacity to recapitulate disease features such as cell heterogeneity, cell–cell/–extracellular matrix interactions and tissue architecture in a more realistic manner.

Figure 6 summarizes the different steps of polymer nanotechnology development from polymer synthesis to nanosystem translation challenges.

Conclusion & future perspective

In summary, natural and synthetic polymers have demonstrated great potential for the manufacture of diverse nanomaterials and the consequent development of future translative therapies and diagnostics. Due to the chemical versatility, size tunability and assembly into a variety of structures, polymers have augmented the development of multipurpose nanomaterials for key health burdens such as cancer, infectious, metabolic and nervous system diseases. Recent literature evidences multiple biomedical advantages of these nanomaterials, which result in: effective delivery of therapeutic cargos to the targeted disease, subcellular diagnosis of key biological parameters and avenues open to a combined theranostic potential. However, the translation of polymeric nanotechnology into clinics is still poor when considering the vast number of applications that have been published in the recent years. Translation drawbacks often include poor understanding of the protein corona-nanosystem interaction, lack of

protocol standardization, limited scale-up and GMP-grade production methods, as well as misleading preclinical assessment due to the use of poorly realistic *in vitro* models. Despite the promising potential of biocompatible polymers to develop novel nanosystems, research efforts should now focus on ways to move the nanosystems that have been developed from the bench to the bedside.

Executive summary

Biocompatible polymers for nanotechnology applications

- There is currently a range of possibilities of biocompatible polymers, either natural or synthetic, to construct therapeutic delivery and diagnostic nanosystems.
- Among the different possibilities, the criteria to choose the polymer are based on the desired biological application of the nanosystem and take into consideration the properties of the material such as availability of chemical groups in the prospect of surface functionalization or type of biodegradation.

Polymeric nanosystems

- The progress in the nanotechnology field has allowed the development of nanosystems, ranging from fiber- to gel-like structures, which may be selected according to the properties of the loaded cargo such as spatial volume, hydrophilicity and type of release.

Polymeric nanosystems for therapeutic delivery & disease diagnosis

- Polymeric nanosystems have benefited the area of therapeutic delivery by providing more effective therapeutic solutions for key health burdens due to precise/targeted drug delivery and reduction of side effects, enhanced pharmacokinetic and pharmacodynamic drug properties, possibility of drug sustained release and drug protection against clearance.
- Advancements have also been accomplished in the field of disease diagnosis, since polymeric nanosystems allow a controlled and/or less toxic body distribution of loaded contrast agents/radioisotopes, as well as the development of fluorescent nanosensors that access intracellular compartments and evaluate fundamental subcellular biochemical parameters.
- The translation of these nanosystems into the clinics still requires great efforts, mainly focused on adopting suitable scale-up/GMP manufacturing protocols and using more realistic human-like preclinical models.

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References

Papers of special note have been highlighted as: ●● of considerable interest

1. Molina CP, Badyalak SF. Translational smart materials in tissue engineering. In: *Smart Materials for Tissue Engineering: Applications*. Wang Q (Ed.). The Royal Society of Chemistry, London, UK, 39–66 (2017).
2. Damodaran VB, Bhatnagar D, Murthy NS. *Biomedical Polymers: Synthesis and Processing*. Springer, Cham, Switzerland (2016).

3. Williams DF. On the mechanisms of biocompatibility. *Biomaterials* 29(20), 2941–2953 (2008).
4. Steffens D, Braghirolli DI, Maurmann N, Pranke P. Update on the main use of biomaterials and techniques associated with tissue engineering. *Drug Discov. Today* 23(8), 1474–1488 (2018).
5. Von Roemeling C, Jiang W, Chan CK, Weissman IL, Kim BYS. Breaking down the barriers to precision cancer nanomedicine. *Trends Biotechnol.* 35(2), 159–171 (2017).
6. Sur S, Rathore A, Dave V, Reddy KR, Chouhan RS, Sadhu V. Recent developments in functionalized polymer nanoparticles for efficient drug delivery system. *Nano-Structures & Nano-Objects* 20, 100397 (2019).
7. Puvvada Ys VS, Sukhvasi S. Extraction of chitin from chitosan from exoskeleton of shrimp for application in pharmaceutical industry. *Int. J. Curr. Pharm. Res.* 1(9), 258–263 (2012).
8. Kharkar PM, Kiick KL, Kloxin AM. Designing degradable hydrogels for orthogonal control of cell microenvironments. *Chem. Soc. Rev.* 42(17), 7335–7372 (2013).
9. Du Y-Z, Wang L, Yuan H, Wei X-H, Hu F-Q. Preparation and characteristics of linoleic acid-grafted chitosan oligosaccharide micelles as a carrier for doxorubicin. *Colloids Surf. B* 69(2), 257–263 (2009).
10. Hu FQ, Meng P, Dai YQ *et al.* PEGylated chitosan-based polymer micelle as an intracellular delivery carrier for anti-tumor targeting therapy. *Eur. J. Pharm. Biopharm.* 70(3), 749–757 (2008).
11. Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. *Adv. Drug Deliv. Rev.* 62(1), 83–99 (2010).
12. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. *Prog. Polym. Sci.* 37(1), 106–126 (2012).
13. Pawar SN, Edgar KJ. Alginate derivatization: a review of chemistry, properties and applications. *Biomaterials* 33(11), 3279–3305 (2012).
14. Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidized alginate and its potential application for tissue engineering. *Biotechnol. Prog.* 17(5), 945–950 (2001).
15. Bouhadir KH, Hausman DS, Mooney DJ. Synthesis of cross-linked poly(aldehyde guluronate) hydrogels. *Polymer* 40(12), 3575–3584 (1999).
16. Fonseca KB, Bidarra SJ, Oliveira MJ, Granja PL, Barrias CC. Molecularly designed alginate hydrogels susceptible to local proteolysis as three-dimensional cellular microenvironments. *Acta Biomater.* 7(4), 1674–1682 (2011).
17. Baldwin AD, Kiick KL. Polysaccharide-modified synthetic polymeric biomaterials. *Biopolymers* 94(1), 128–140 (2010).
18. Vettori MHPB, Franchetti SMM, Contiero J. Structural characterization of a new dextran with a low degree of branching produced by *Leuconostoc mesenteroides* FT045B dextranase. *Carbohydr. Polym.* 88(4), 1440–1444 (2012).
19. Djagny VB, Wang Z, Xu S. Gelatin: a valuable protein for food and pharmaceutical industries: review. *Crit. Rev. Food Sci. Nutr.* 41(6), 481–492 (2001).
20. Ha TLB, Quan TM, Vu DN, Si DM. Naturally derived biomaterials: preparation and application. In: *Regenerative Medicine and Tissue Engineering* Andrades JA (Ed.). IntechOpen, Rijeka, Croatia, 247–274 (2013).
21. Dalev P, Staromanova E, Dalev D *et al.* Biodegradation of chemically modified gelatin films in a simulated natural environment. *Biotechnol. Biotechnol. Equip.* 15(2), 116–123 (2001).
22. Schanté C, Zuber G, Herlin C, Vandamme TF. Synthesis of N-alanyl-hyaluronamide with high degree of substitution for enhanced resistance to hyaluronidase-mediated digestion. *Carbohydr. Polym.* 86(2), 747–752 (2011).
23. Rinaudo M. Main properties and current applications of some polysaccharides as biomaterials. *Polym. Int.* 57(3), 397–430 (2008).
24. Dicker KT, Gurski LA, Pradhan-Bhatt S, Witt RL, Farach-Carson MC, Jia X. Hyaluronan: a simple polysaccharide with diverse biological functions. *Acta Biomater.* 10(4), 1558–1570 (2014).
25. Craig CL. Evolution of arthropod silks. *Annu. Rev. Entomol.* 42, 231–267 (1997).
26. Pereira RFP, Silva MM, De Zea Bermudez V. *Bombyx mori* silk fibers: an outstanding family of materials. *Macromol. Mater. Eng.* 300(12), 1171–1198 (2015).
27. Cao Y, Wang B. Biodegradation of silk biomaterials. *Int. J. Mol. Sci.* 10(4), 1514–1524 (2009).
28. Melke J, Midha S, Ghosh S, Ito K, Hofmann S. Silk fibroin as biomaterial for bone tissue engineering. *Acta Biomater.* 31, 1–16 (2016).
29. Liu W, Zhou Z, Zhang S *et al.* Precise protein photolithography (P3): high performance biopatterning using silk fibroin light chain as the resist. *Adv. Sci.* 4(9), 1700191 (2017).
30. Gotoh Y, Niimi S, Hayakawa T, Miyashita T. Preparation of lactose-silk fibroin conjugates and their application as a scaffold for hepatocyte attachment. *Biomaterials* 25(6), 1131–1140 (2004).
31. Sampaio S, Miranda TMR, Santos JG, Soares GMB. Preparation of silk fibroin–poly(ethylene glycol) conjugate films through click chemistry. *Polym. Int.* 60(12), 1737–1744 (2011).
32. Wang Y, Kim HJ, Vunjak-Novakovic G, Kaplan DL. Stem cell-based tissue engineering with silk biomaterials. *Biomaterials* 27(36), 6064–6082 (2006).

33. Fanovich MA, Jaeger P. Sorption and diffusion of compressed carbon dioxide in polycaprolactone for the development of porous scaffolds. *Mater. Sci. Eng. C* 32(4), 961–968 (2012).
34. Vieira AC, Vieira JC, Ferra JM, Magalhães FD, Guedes RM, Marques AT. Mechanical study of PLA–PCL fibers during *in vitro* degradation. *J. Mech. Behav. Biomed.* 4(3), 451–460 (2011).
35. Jenkins MJ, Harrison KL. The effect of molecular weight on the crystallization kinetics of polycaprolactone. *Polym. Adv. Technol.* 17(6), 474–478 (2006).
36. Lin CH, Su JM, Hsu SH. Evaluation of type II collagen scaffolds reinforced by poly(epsilon-caprolactone) as tissue-engineered trachea. *Tissue Eng. Part C Methods* 14(1), 69–77 (2008).
37. Woodruff MA, Hutmacher DW. The return of a forgotten polymer – polycaprolactone in the 21st century. *Prog. Polym. Sci.* 35(10), 1217–1256 (2010).
38. Sharma S, Mohanty S, Gupta D, Jassal M, Agrawal AK, Tandon R. Cellular response of limbal epithelial cells on electrospun poly-ε-caprolactone nanofibrous scaffolds for ocular surface bioengineering: a preliminary *in vitro* study. *Mol. Vis.* 17, 2898–2910 (2011).
39. Zhu Y, Gao C, Shen J. Surface modification of polycaprolactone with poly(methacrylic acid) and gelatin covalent immobilization for promoting its cytocompatibility. *Biomaterials* 23(24), 4889–4895 (2002).
40. Chen T, Cai T, Jin Q, Ji J. Design and fabrication of functional polycaprolactone. *E-Polymers* 15, 3–13 (2015).
41. Mondal D, Griffith M, Venkatraman SS. Polycaprolactone-based biomaterials for tissue engineering and drug delivery: current scenario and challenges. *Int. J. Polym. Mater.* 65(5), 255–265 (2016).
42. Pang X, Zhuang X, Tang Z, Chen X. Polylactic acid (PLA): research, development and industrialization. *Biotechnol. J.* 5(11), 1125–1136 (2010).
43. Lim L-T, Auras R, Rubino M. Processing technologies for poly(lactic acid). *Prog. Polym. Sci.* 33(8), 820–852 (2008).
44. KM Nampoothiri, Nair NR, John RP. An overview of the recent developments in polylactide (PLA) research. *Bioresour. Technol.* 101(22), 8493–8501 (2010).
45. Qi X, Ren Y, Wang X. New advances in the biodegradation of poly(lactic acid). *Int. Biodeterior. Biodegradation* 117, 215–223 (2017).
46. Lasprilla AJ, Martinez GA, Lunelli BH, Jardini AL, Maciel Filho R. Poly-lactic acid synthesis for application in biomedical devices – a review. *Biotechnol. Adv.* 30(1), 321–328 (2012).
47. Rasal RM, Janorkar AV, Hirt DE. Poly(lactic acid) modifications. *Prog. Polym. Sci.* 35(3), 338–356 (2010).
48. Parveen S, Sahoo SK. Polymeric nanoparticles for cancer therapy. *J. Drug Target.* 16(2), 108–123 (2008).
49. Barros BS, Zaenglein AL. The use of cosmeceuticals in acne: help or hoax? *Am. J. Clin. Dermatol.* 18(2), 159–163 (2017).
50. Kaihara S, Matsumura S, Mikos AG, Fisher JP. Synthesis of poly(L-lactide) and polyglycolide by ring-opening polymerization. *Nat. Protoc.* 2(11), 2767–2771 (2007).
51. Mehta R, Kumar V, Bhunia H, Upadhyay SN. Synthesis of poly(lactic acid): a review. *J. Macromol. Sci. C* 45(4), 325–349 (2005).
52. Middleton JC, Tipton AJ. Synthetic biodegradable polymers as orthopedic devices. *Biomaterials* 21(23), 2335–2346 (2000).
53. Shibata A, Yada S, Terakawa M. Biodegradability of poly(lactic-co-glycolic acid) after femtosecond laser irradiation. *Sci. Rep.* 6, 27884 (2016).
54. Vroman I, Tighzert L. Biodegradable polymers. *Materials* 2(2), 307–344 (2009).
55. Lee K-B, Yoon KR, Woo SI, Choi IS. Surface modification of poly(glycolic acid) (PGA) for biomedical applications. *J. Pharm. Sci.* 92(5), 933–937 (2003).
56. Martins C, Sousa F, Araújo F, Sarmento B. Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications. *Adv. Healthc. Mater.* 7(1), 1701035 (2017).
57. Bala I, Hariharan S, Kumar MNVR. PLGA nanoparticles in drug delivery: the state of the art. *Crit. Rev. Ther. Drug Carrier Syst.* 21(5), 387–422 (2004).
58. Hines DJ, Kaplan DL. Poly(lactic-co-glycolic acid) controlled release systems: experimental and modeling insights. *Crit. Rev. Ther. Drug Carrier Syst.* 30(3), 257–276 (2013).
59. Kapoor DN, Bhatia A, Kaur R, Sharma R, Kaur G, Dhawan S. PLGA: a unique polymer for drug delivery. *Ther. Deliv.* 6(1), 41–58 (2015).
60. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers* 3(3), 1377–1397 (2011).
61. Luderer F, Löbner M, Rohm HW *et al.* Biodegradable sirolimus-loaded poly(lactide) nanoparticles as drug delivery system for the prevention of in-stent restenosis in coronary stent application. *J. Biomater. Appl.* 25(8), 851–875 (2011).
62. Gentile P, Chiono V, Carmagnola I, Hatton PV. An overview of poly(lactic-co-glycolic acid) (PLGA)-based biomaterials for bone tissue engineering. *Int. J. Mol. Sc.* 15(3), 3640–3659 (2014).

63. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. *J. Control. Rel.* 161(2), 505–522 (2012).
64. Yu Y, Chen C-K, Law W-C, Sun H, Prasad PN, Cheng C. A degradable brush polymer–drug conjugate for pH-responsive release of doxorubicin. *Polym. Chem.* 6(6), 953–961 (2015).
65. Denisin AK, Pruitt BL. Tuning the range of polyacrylamide gel stiffness for mechanobiology applications. *ACS Appl. Mater. Interfaces* 8(34), 21893–21902 (2016).
66. Desai AS, Chauhan VM, Johnston APR, Esler T, Aylott JW. Fluorescent nanosensors for intracellular measurements: synthesis, characterization, calibration, and measurement. *Front. Physiol.* 4, (2014).
67. Lin DC, Yurke B, Langrana NA. Mechanical properties of a reversible, DNA-crosslinked polyacrylamide hydrogel. *J. Biomech. Eng.* 126(1), 104–110 (2004).
68. Yan F, Zheng C, Zhai X, Zhao D. Preparation and characterization of polyacrylamide in cationic microemulsion. *J. Appl. Polym. Sci.* 67(4), 747–754 (1998).
69. Chauhan VM, Orsi G, Brown A, Pritchard DI, Aylott JW. Mapping the pharyngeal and intestinal pH of *Caenorhabditis elegans* and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. *ACS Nano* 7(6), 5577–5587 (2013).
70. Aras D, Cakar Z, Ozkavukcu S, Can A, Cinar O. *In vivo* acrylamide exposure may cause severe toxicity to mouse oocytes through its metabolite glycidamide. *PLoS One* 12(2), e0172026 (2017).
71. D'souza AA, Shegokar R. Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications. *Expert Opin. Drug Deliv.* 13(9), 1257–1275 (2016).
72. Thomas A, Muller SS, Frey H. Beyond poly(ethylene glycol): linear polyglycerol as a multifunctional polyether for biomedical and pharmaceutical applications. *Biomacromolecules* 15(6), 1935–1954 (2014).
73. Törmälä P. Determination of glass transition temperature of poly(ethylene glycol) by spin probe technique. *Eur. Polym. J.* 10(6), 519–521 (1974).
74. Efthimiadou E, Tapeinos C, Bilalis P, Kordas G. New approach in synthesis, characterization and release study of pH-sensitive polymeric micelles, based on PLA-Lys-b-PEGm, conjugated with doxorubicin. *J. Nanopart. Res.* 13(12), 6725–6736 (2011).
75. Ryan SM, Mantovani G, Wang X, Haddleton DM, Brayden DJ. Advances in PEGylation of important biotech molecules: delivery aspects. *Expert Opin. Drug Deliv.* 5(4), 371–383 (2008).
76. Pardridge WM. Blood–brain barrier delivery. *Drug Discov. Today* 12(1), 54–61 (2007).
77. Cheng CJ, Tietjen GT, Saucier-Sawyer JK, Saltzman WM. A holistic approach to targeting disease with polymer nanoparticles. *Nat. Rev. Drug. Discov.* 14(4), 239–247 (2015).
78. Horrocks AR, Anand SC. *Handbook of Technical Textiles: Technical Textile Applications*. Woodhead Publishing, Sawston, UK, (2016).
79. Dong B, Smith ME, Wnek GE. Encapsulation of multiple biological compounds within a single electrospun fiber. *Small* 5(13), 1508–1512 (2009).
80. Kenawy E-R, Bowlin GL, Mansfield K *et al.* Release of tetracycline hydrochloride from electrospun poly(ethylene-co-vinylacetate), poly(lactic acid), and a blend. *J. Control. Rel.* 81(1), 57–64 (2002).
81. Jiang H, Fang D, Hsiao B, Chu B, Chen W. Preparation and characterization of ibuprofen-loaded poly(lactide-co-glycolide)/poly(ethylene glycol)-g-chitosan electrospun membranes. *J. Biomater. Sci. Polym. Ed.* 15(3), 279–296 (2004).
82. Liwen J, Carl S, Saad AK, Xiangwu Z. Preparation and characterization of silica nanoparticulate–polyacrylonitrile composite and porous nanofibers. *Nanotechnology* 19(8), 085605 (2008).
83. Ionescu LC, Lee GC, Sennett BJ, Burdick JA, Mauck RL. An anisotropic nanofiber/microsphere composite with controlled release of biomolecules for fibrous tissue engineering. *Biomaterials* 31(14), 4113–4120 (2010).
84. Zhang C-L, Yu S-H. Nanoparticles meet electrospinning: recent advances and future prospects. *Chem. Soc. Rev.* 43(13), 4423–4448 (2014).
85. Niu H, Zhang J, Xie Z, Wang X, Lin T. Preparation, structure and supercapacitance of bonded carbon nanofiber electrode materials. *Carbon* 49(7), 2380–2388 (2011).
86. Zhou Z, Lai C, Zhang L *et al.* Development of carbon nanofibers from aligned electrospun polyacrylonitrile nanofiber bundles and characterization of their microstructural, electrical, and mechanical properties. *Polymer* 50(13), 2999–3006 (2009).
87. Li D, Xia Y. Direct fabrication of composite and ceramic hollow nanofibers by electrospinning. *Nano Lett.* 4(5), 933–938 (2004).
88. Koombhongse S, Liu W, Reneker DH. Flat polymer ribbons and other shapes by electrospinning. *J. Polym. Sci. B* 39(21), 2598–2606 (2001).
89. Fong H, Chun I, Reneker DH. Beaded nanofibers formed during electrospinning. *Polymer* 40(16), 4585–4592 (1999).
90. Huang C, Niu H, Wu J, Ke Q, Mo X, Lin T. Needleless electrospinning of polystyrene fibers with an oriented surface line texture. *J. Nanomater.* 2012, 7 (2012).
91. Bogwitzki M, Czado W, Frese T *et al.* Nanostructured fibers via electrospinning. *Adv. Mater.* 13(1), 70–72 (2001).

92. Zhao T, Liu Z, Nakata K *et al.* Multichannel TiO₂ hollow fibers with enhanced photocatalytic activity. *J. Mater. Chem.* 20(24), 5095–5099 (2010).
93. Sun Z, Zussman E, Yarin AL, Wendorff JH, Greiner A. Compound core–shell polymer nanofibers by co-electrospinning. *Adv. Mater.* 15(22), 1929–1932 (2003).
94. Chang G, Shen J. Helical nanoribbons fabricated by electrospinning. *Macromol. Mater. Eng.* 296(12), 1071–1074 (2011).
95. Lin T, Wang H, Wang X. Self-crimping bicomponent nanofibers electrospun from polyacrylonitrile and elastomeric polyurethane. *Adv. Mater.* 17(22), 2699–2703 (2005).
96. Ferreira SA, Gama FM, Vilanova M. Polymeric nanogels as vaccine delivery systems. *Nanomedicine* 9(2), 159–173 (2013).
97. Li Z, Barnes JC, Bosoy A, Stoddart JF, Zink JI. Mesoporous silica nanoparticles in biomedical applications. *Chem. Soc. Rev.* 41(7), 2590–2605 (2012).
98. Soni G, Yadav KS. High encapsulation efficiency of poloxamer-based injectable thermoresponsive hydrogels of etoposide. *Pharm. Dev. Technol.* 19(6), 651–661 (2014).
99. Li N, Wang J, Yang X, Li L. Novel nanogels as drug delivery systems for poorly soluble anticancer drugs. *Colloids. Surf. B Biointerfaces* 83(2), 237–244 (2011).
100. Kohli E, Han H-Y, Zeman AD, Vinogradov SV. Formulations of biodegradable nanogel carriers with 5'-triphosphates of nucleoside analogs that display a reduced cytotoxicity and enhanced drug activity. *J. Control. Rel.* 121(1), 19–27 (2007).
101. Neamtu I, Rusu AG, Diaconu A, Nita LE, Chiriac AP. Basic concepts and recent advances in nanogels as carriers for medical applications. *Drug Deliv.* 24(1), 539–557 (2017).
102. Lopresti C, Lomas H, Massignani M, Smart T, Battaglia G. Polymersomes: nature inspired nanometer sized compartments. *J. Mater. Chem.* 19(22), 3576–3590 (2009).
103. Onaca O, Enea R, Hughes DW, Meier W. Stimuli-responsive polymersomes as nanocarriers for drug and gene delivery. *Macromol. Biosci.* 9(2), 129–139 (2009).
104. Christian DA, Cai S, Bowen DM, Kim Y, Pajewski JD, Discher DE. Polymersome carriers: from self-assembly to siRNA and protein therapeutics. *Eur. J. Pharm. Biopharm.* 71(3), 463–474 (2009).
105. Messenger L, Gaitzsch J, Chierico L, Battaglia G. Novel aspects of encapsulation and delivery using polymersomes. *Curr. Opin. Pharmacol.* 18, 104–111 (2014).
106. Meng F, Zhong Z. Polymersomes spanning from nano- to microscale: advanced vehicles for controlled drug delivery and robust vesicles for virus and cell mimicking. *J. Phys. Chem. Lett.* 2(13), 1533–1539 (2011).
107. Kita-Tokarczyk K, Grumelard J, Haeefe T, Meier W. Block copolymer vesicles – using concepts from polymer chemistry to mimic biomembranes. *Polymer* 46(11), 3540–3563 (2005).
108. Connot J, Silva JM, Fernandes JG *et al.* Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking. *Front. Chem.* 2, 105 (2014).
109. Gothwal A, Khan I, Gupta U. Polymeric micelles: recent advancements in the delivery of anticancer drugs. *Pharm. Res.* 33(1), 18–39 (2016).
110. Aliabadi HM, Lavasanifar A. Polymeric micelles for drug delivery. *Expert Opin. Drug Deliv.* 3(1), 139–162 (2006).
111. Deshmukh AS, Chauhan PN, Noolvi MN *et al.* Polymeric micelles: basic research to clinical practice. *Int. J. Pharm.* 532(1), 249–268 (2017).
112. Torchilin VP. Micellar nanocarriers: pharmaceutical perspectives. *Pharm. Res.* 24(1), 1–16 (2006).
113. Kang N, Perron M-È, Prud'homme RE, Zhang Y, Gaucher G, Leroux J-C. Stereocomplex block copolymer micelles: core–shell nanostructures with enhanced stability. *Nano Lett.* 5(2), 315–319 (2005).
114. Carenza E, Jordan O, Martínez-San Segundo P *et al.* Encapsulation of VEGF165 into magnetic PLGA nanocapsules for potential local delivery and bioactivity in human brain endothelial cells. *J. Mater. Chem. B* 3(12), 2538–2544 (2015).
115. Ghasemian E, Vatanara A, Rouholamini Najafabadi A, Rouini MR, Gilani K, Darabi M. Preparation, characterization and optimization of sildenafil citrate loaded PLGA nanoparticles by statistical factorial design. *Daru* 21(1), 68 (2013).
116. Son HY, Kim I, Nam YS. On-surface synthesis of metal nanostructures on solid and hydrated polymer nanofibers coated with polydopamine. *J. Ind. Eng. Chem.* 30, 220–224 (2015).
117. Schutz CA, Juillerat-Jeanneret L, Kauper P, Wandrey C. Cell response to the exposure to chitosan-TPP//alginate nanogels. *Biomacromolecules* 12(11), 4153–4161 (2011).
118. Meeuwissen SA, Bruckers SMC, Chen Y, Pochan DJ, Van Hest JCM. Spontaneous shape changes in polymersomes via polymer/polymer segregation. *Polym. Chem.* 5(2), 489–501 (2014).
119. Shang X, Fan X, Yang S, Xie Z, Guo Y, Hu Z. Synthesis, characterization and self-assembly behavior of zwitterionic amphiphilic triblock copolymers bearing pendant amino acid residues. *RSC Adv.* 5(116), 96181–96188 (2015).

120. Deng X, Cao M, Zhang J *et al.* Hyaluronic acid-chitosan nanoparticles for co-delivery of MiR-34a and doxorubicin in therapy against triple negative breast cancer. *Biomaterials* 35(14), 4333–4344 (2014).
121. Zhu D, Wu S, Hu C *et al.* Folate-targeted polymersomes loaded with both paclitaxel and doxorubicin for the combination chemotherapy of hepatocellular carcinoma. *Acta Biomater.* 58, 399–412 (2017).
122. Zhang J, Wang X, Liu T, Liu S, Jing X. Antitumor activity of electrospun polylactide nanofibers loaded with 5-fluorouracil and oxaliplatin against colorectal cancer. *Drug Deliv.* 23(3), 794–800 (2016).
123. Moran MC, Carazo J, Busquets MA. Dual responsive gelatin-based nanoparticles for enhanced 5-fluorouracil efficiency. *Colloids. Surf. B Biointerfaces* 172, 646–654 (2018).
124. Mottaghitalab F, Kiani M, Farokhi M *et al.* Targeted delivery system based on gemcitabine-loaded silk fibroin nanoparticles for lung cancer therapy. *ACS Appl. Mater. Interfaces* 9(37), 31600–31611 (2017).
125. Rawal T, Parmar R, Tyagi RK, Butani S. Rifampicin loaded chitosan nanoparticle dry powder presents an improved therapeutic approach for alveolar tuberculosis. *Colloids. Surf. B* 154, 321–330 (2017).
126. Garg T, Goyal AK, Rath G, Murthy RS. Spray-dried particles as pulmonary delivery system of anti-tubercular drugs: design, optimization, *in vitro* and *in vivo* evaluation. *Pharm. Dev. Technol.* 21(8), 951–960 (2016).
127. Upadhyay S, Khan I, Gothwal A *et al.* Conjugated and entrapped HEMA-PLA nano-polymeric micelles based dual delivery of first line anti TB drugs: improved and safe drug delivery against sensitive and resistant Mycobacterium tuberculosis. *Pharm. Res.* 34(9), 1944–1955 (2017).
128. Gong Y, Chowdhury P, Midde NM, Rahman MA, Yallapu MM, Kumar S. Novel elvitegravir nanoformulation approach to suppress the viral load in HIV-infected macrophages. *Biochem. Biophys. Rep.* 12, 214–219 (2017).
129. Shailender J, Ravi PR, Saha P, Dalvi A, Myneni S. Tenofovir disoproxil fumarate loaded PLGA nanoparticles for enhanced oral absorption: effect of experimental variables and *in vitro*, *ex vivo* and *in vivo* evaluation. *Colloids. Surf. B* 158, 610–619 (2017).
130. Nunes R, Araújo F, Barreiros L *et al.* Noncovalent PEG coating of nanoparticle drug carriers improves the local pharmacokinetics of rectal anti-HIV microbicides. *ACS Appl. Mater. Interfaces* 10(41), 34942–34953 (2018).
- **The authors demonstrated the therapeutic potential of a poly(lactic-co-glycolic acid) and poly(ethylene glycol) nanosystem to deliver efavirenz as an anti-HIV rectal microbicide.**
131. Martins C, Araújo F, Gomes MJ *et al.* Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy. *Eur. J. Pharm. Biopharm.* 138, 111–124 (2018).
132. Chou H-S, Larsson M, Hsiao M-H *et al.* Injectable insulin-lysozyme-loaded nanogels with enzymatically-controlled degradation and release for basal insulin treatment: *in vitro* characterization and *in vivo* observation. *J. Control. Rel.* 224, 33–42 (2016).
133. Alibolandi M, Alabdollah F, Sadeghi F *et al.* Dextran-b-poly(lactide-co-glycolide) polymersome for oral delivery of insulin: *in vitro* and *in vivo* evaluation. *J. Control. Rel.* 227, 58–70 (2016).
134. Li X, Wang C, Liang R *et al.* The glucose-lowering potential of exenatide delivered orally via goblet cell-targeting nanoparticles. *Pharm. Res.* 32(3), 1017–1027 (2015).
135. Nguyen H-N, Wey S-P, Juang J-H *et al.* The glucose-lowering potential of exendin-4 orally delivered via a pH-sensitive nanoparticle vehicle and effects on subsequent insulin secretion *in vivo*. *Biomaterials* 32(10), 2673–2682 (2011).
136. Shi Y, Sun X, Zhang L *et al.* Fc-modified exenatide-loaded nanoparticles for oral delivery to improve hypoglycemic effects in mice. *Sci. Rep.* 8(1), 726 (2018).
137. Kumar S, Bhanjana G, Verma RK, Dhingra D, Dilbaghi N, Kim K-H. Metformin-loaded alginate nanoparticles as an effective antidiabetic agent for controlled drug release. *J. Pharm. Pharmacol.* 69(2), 143–150 (2016).
138. Araújo F, Shrestha N, Shahbazi M-A *et al.* The impact of nanoparticles on the mucosal translocation and transport of GLP-1 across the intestinal epithelium. *Biomaterials* 35(33), 9199–9207 (2014).
139. Barry M, Pearce H, Cross L, Tatullo M, Gaharwar AK. Advances in nanotechnology for the treatment of osteoporosis. *Curr. Osteoporos. Rep.* 14(3), 87–94 (2016).
140. Dong J, Tao L, Abourehab MaS, Hussain Z. Design and development of novel hyaluronate-modified nanoparticles for combo-delivery of curcumin and alendronate: fabrication, characterization, and cellular and molecular evidences of enhanced bone regeneration. *Int. J. Biol. Macromol.* 116, 1268–1281 (2018).
141. Sezlev Bilecen D, Uludag H, Hasirci V. Development of PEI-RANK siRNA complexloaded PLGA nanocapsules for the treatment of osteoporosis. *Tissue Eng. Part A* 25, 34–43 (2018).
142. Narayanan D, Anitha A, Jayakumar R, Chennazhi KP. *In vitro* and *in vivo* evaluation of osteoporosis therapeutic peptide PTH 1–34 loaded pegylated chitosan nanoparticles. *Mol. Pharm.* 10(11), 4159–4167 (2013).
143. Saraiva C, Praça C, Ferreira R, Santos T, Ferreira L, Bernardino L. Nanoparticle-mediated brain drug delivery: overcoming blood–brain barrier to treat neurodegenerative diseases. *J. Control. Rel.* 235, 34–47 (2016).
144. Sun D, Xue A, Zhang B, Lou H, Shi H, Zhang X. Polysorbate 80-coated PLGA nanoparticles improve the permeability of acetylpuerarin and enhance its brain-protective effects in rats. *J. Pharm. Pharmacol.* 67(12), 1650–1662 (2015).

145. Kreuter J, Shamenkov D, Petrov V *et al.* Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood—brain barrier. *J. Drug Target.* 10(4), 317–325 (2002).
146. Petri B, Bootz A, Khalansky A *et al.* Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated poly(butyl cyanoacrylate) nanoparticles: revisiting the role of surfactants. *J. Control. Rel.* 117(1), 51–58 (2007).
147. Khalin I, Alyautdin R, Wong TW, Gnanou J, Kocherga G, Kreuter J. Brain-derived neurotrophic factor delivered to the brain using poly(lactide-co-glycolide) nanoparticles improves neurological and cognitive outcome in mice with traumatic brain injury. *Drug Deliv.* 23(9), 3520–3528 (2016).
148. You L, Wang J, Liu T *et al.* Targeted brain delivery of rabies virus glycoprotein 29-modified deferoxamine-loaded nanoparticles reverses functional deficits in parkinsonian mice. *ACS Nano* 12(5), 4123–4139 (2018).
- **The authors reported the therapeutic potential of a poly(lactic-co-glycolic acid) and poly(ethylene glycol) nanosystem to deliver deferoxamine in the context of Parkinson's disease.**
149. Zhang C, Zheng X, Wan X *et al.* The potential use of H102 peptide-loaded dual-functional nanoparticles in the treatment of Alzheimer's disease. *J. Control. Rel.* 192, 317–324 (2014).
150. Qi L, Jiang H, Cui X *et al.* Synthesis of methylprednisolone loaded ibuprofen modified dextran based nanoparticles and their application for drug delivery in acute spinal cord injury. *Oncotarget* 8(59), 99666–99680 (2017).
151. Vera DR, Mattrey RF. A molecular CT blood pool contrast agent. *Acad. Radiol.* 9(7), 784–792 (2002).
152. Nahrendorf M, Zhang H, Hembrador S *et al.* Nanoparticle PET-CT imaging of macrophages in inflammatory atherosclerosis. *Circulation* 117(3), 379–387 (2008).
153. Rossin R, Muro S, Welch MJ, VR Muzykantov, DP Schuster. *In vivo* imaging of ⁶⁴Cu-labeled polymer nanoparticles targeted to the lung endothelium. *J. Nucl. Med.* 49(1), 103–111 (2008).
154. Moffat BA, Reddy GR, Mcconville P *et al.* A novel polyacrylamide magnetic nanoparticle contrast agent for molecular imaging using MRI. *Mol. Imaging* 2(4), 15353500200303163 (2003).
155. Galperin A, Margel D, Baniel J, Dank G, Biton H, Margel S. Radiopaque iodinated polymeric nanoparticles for x-ray imaging applications. *Biomaterials* 28(30), 4461–4468 (2007).
156. Benjaminsen RV, Sun H, Henriksen JR, Christensen NM, Almdal K, Andresen TL. Evaluating nanoparticle sensor design for intracellular pH measurements. *ACS Nano* 5(7), 5864–5873 (2011).
157. Welser K, Adsley R, Moore BM, Chan WC, Aylott JW. Protease sensing with nanoparticle based platforms. *Analyst* 136(1), 29–41 (2011).
158. Almdal K, Sun H, Poulsen AK *et al.* Fluorescent gel particles in the nanometer range for detection of metabolites in living cells. *Polym. Adv. Technol.* 17(9–10), 790–793 (2006).
159. Schulz A, Wotschadlo J, Heinze T, Mohr GJ. Fluorescent nanoparticles for ratiometric pH-monitoring in the neutral range. *J. Mater. Chem.* 20(8), 1475–1482 (2010).
- **The authors elucidated the diagnostic potential of a poly(acrylamide) nanosystem for ratiometric pH monitorization.**
160. Ow H, Larson DR, Srivastava M, Baird BA, Webb WW, Wiesner U. Bright and stable core-shell fluorescent silica nanoparticles. *Nano Lett.* 5(1), 113–117 (2005).
161. Doussineau T, Schulz A, Lapresta-Fernandez A *et al.* On the design of fluorescent ratiometric nanosensors. *Chem. Eur. J.* 16(34), 10290–10299 (2010).
162. Fehr M, Lalonde S, Lager I, Wolff MW, Frommer WB. *In vivo* imaging of the dynamics of glucose uptake in the cytosol of COS-7 cells by fluorescent nanosensors. *J. Biol. Chem.* 278(21), 19127–19133 (2003).
163. Schulz A, Woolley R, Tabarin T, Mcdonagh C. Dextran-coated silica nanoparticles for calcium-sensing. *Analyst* 136(8), 1722–1727 (2011).
164. Nielsen LJ, Olsen LF, Ozalp VC. Aptamers embedded in polyacrylamide nanoparticles: a tool for *in vivo* metabolite sensing. *ACS Nano* 4(8), 4361–4370 (2010).
165. Sumner JP, Kopelman R. Alexa Fluor 488 as an iron sensing molecule and its application in PEBBLE nanosensors. *Analyst* 130(4), 528–533 (2005).
166. Li M, Wang Q, Shi X, Hornak LA, Wu N. Detection of Mercury(II) by quantum Dot/DNA/gold nanoparticle ensemble based nanosensor via nanometal surface energy transfer. *Anal. Chem.* 83(18), 7061–7065 (2011).
167. Welser K, Perera MDA, Aylott JW, Chan WC. A facile method to clickable sensing polymeric nanoparticles. *Chem. Commun.* doi:10.1039/b914358k(43), 6601–6603 (2009).
168. Wang RE, Zhang Y, Cai J, Cai W, Gao T. Aptamer-based fluorescent biosensors. *Curr. Med. Chem.* 18(27), 4175–4184 (2011).
169. Hadjidemetriou M, Kostarelos K. Evolution of the nanoparticle corona. *Nat. Nanotechnol.* 12(4), 288–290 (2017).
170. Schöttler S, Landfester K, Mailänder V. Controlling the stealth effect of nanocarriers through understanding the protein corona. *Angew. Chem Int. Ed.* 55(31), 8806–8815 (2016).

171. Nguyen VH, Lee B-J. Protein corona: a new approach for nanomedicine design. *Int. J. Nanomed.* 12, 3137–3151 (2017).
172. Corbo C, Molinaro R, Parodi A, Toledano Furman NE, Salvatore F, Tasciotti E. The impact of nanoparticle protein corona on cytotoxicity, immunotoxicity and target drug delivery. *Nanomedicine* 11(1), 81–100 (2015).
173. Faria M, Björnmalm M, Thurecht KJ *et al.* Minimum information reporting in bio-nano experimental literature. *Nat. Nanotechnol.* 13(9), 777–785 (2018).
- **Reporting standards that allow better reproducibility and quantitative research, presenting minimal requirements for a good publication practice in the bio–nano field.**
174. Agrahari V, Agrahari V. Facilitating the translation of nanomedicines to a clinical product: challenges and opportunities. *Drug Discov. Today* 23(5), 974–991 (2018).
175. Han X, Corson N, Wade-Mercer P *et al.* Assessing the relevance of *in vitro* studies in nanotoxicology by examining correlations between *in vitro* and *in vivo* data. *Toxicology* 297(1), 1–9 (2012).
176. Henriksen-Lacey M, Carregal-Romero S, Liz-Marzán LM. Current challenges toward *in vitro* cellular validation of inorganic nanoparticles. *Bioconjug. Chem.* 28(1), 212–221 (2017).



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Modelling protein therapeutic co-formulation and co-delivery with PLGA nanoparticles continuously manufactured by microfluidics

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Formulating protein therapeutics into nanoparticles (NPs) of poly(lactic-co-glycolic acid) (PLGA) provides key features such as protection against clearance, sustained release and less side effects by possible attachment of targeting ligands. These NPs also offer the potential for protein combination therapy, which is expected to exploit synergetic bioresponses, avoid multiple dosage regimens and consequent mis-dosing. Since the conventional manufacture of protein-loaded PLGA NPs is still associated with low-throughput, new continuous manufacturing methods such as microfluidics have been established. Herein, PLGA NPs continuously manufactured through microfluidics are proposed for co-formulation and -delivery of two model proteins consisting of bovine serum albumin (BSA) conjugated to either fluorescein (FITC) or tetramethylrhodamine (TRITC) isothiocyanates. Protein co-formulated NPs of 100 nm, monodispersed and with 70% of association efficiency were obtained. The microfluidic setup allowed a production rate of around 7 g of particles per day and demonstrated scale-up capacity. Model proteins were released in a controlled manner and without significant changes in their secondary structure. Studies in macrophage-like cells proved that protein co-formulated PLGA NPs did not impair metabolic activity (>70%). The cellular association of the proteins was around 2-times higher when co-formulated into PLGA NPs, compared to the free protein controls. Moreover, the cellular association of the co-formulated proteins was 4-times higher than the physical mixture of NPs individually loaded with each protein type. This work has demonstrated the effectiveness of continuously manufactured PLGA NPs for co-formulating and enhancing the cellular association of co-delivered model proteins, providing a proof-of-concept foundation for future protein combination nanotherapies.

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1. Introduction

The protein therapeutic market is an important segment of the pharmaceutical industry and is anticipated to be worth \$230 billion by 2021.¹ The exponential growth of this market is correlated with the superior therapeutic performance over small-molecule drugs, which is due to higher bioactivity and

specificity.² Protein-based therapeutics have therefore been widely investigated for the treatment of various diseases, with more than 200 Food and Drug Administration (FDA)-approvals for clinical use since 1980.³

Although proteins hold great potential as therapeutics, their delivery poses a significant challenge. Protein therapeutics are highly affected by physiological conditions, which may compromise their stability and trigger their proteolytic degradation, opsonization, as well as complexation with biologic products leading to conformational changes and consequent loss of function.⁴ Moreover, lipophilic cell membranes restrict the passage of proteins with intracellular targets, which are subject to challenges in interacting with cells and accessing sub-cellular compartments.⁵ As a result, protein therapeutics can be diverted away from their target, resulting in side effects emerging from off-site accumulation and the need for repeated administrations. Polymer-based nanoparticles (NPs) have been identified as a promising strategy to circumvent these drawbacks by offering a protective vehicle for the formulation of protein therapeutics. This is

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because, they have been found to prevent the rapid clearance of the protein therapeutics whilst providing their controlled release over time.^{6,7} The FDA-approved poly(lactic-co-glycolic acid) (PLGA) polymer is the most widely utilized polymer for protein therapeutic-loaded NPs with biodegradable and biocompatible properties.⁸ PLGA is a surface-functionalizable polymer that allows further attachment of a variety of targeting ligands, which are key to inducing higher accumulation of the NPs at the target site, therefore reducing potential side effects.⁹ However, conventional bulk processes for synthesis of protein therapeutic-loaded PLGA NPs are often related to slow- and low-throughput, unsuccessful association efficiency and lack of batch-to-batch consistency.^{10,11} These pitfalls are being overcome by the rapid development of continuous manufacturing methods such as microfluidics that offer the possibility of precisely control the particle-formation environment and enable the production of PLGA NPs with tunable physicochemical characteristics and higher drug loading capacity, as well as monodisperse batches.^{12–14} Microfluidics also allows a reproducible, time-saving up-scale manufacturing of NPs, which is crucial to reach industrial and clinical batch sizes.¹⁵

Another challenge with manufacturing protein therapeutic-loaded PLGA NPs is the harsh environment encountered when employing the double emulsion systems commonly used in the formulation process. The shear stress inherent to the immiscibility of the phases involved in these systems leads to the interfacial adsorption of proteins, which is the major cause of their denaturation.^{16,17} Typical nanoprecipitation-based processes would be more appropriate due to the absence of oily-aqueous interfaces, although they are mostly designed for the formulation of highly hydrophobic cargos.¹⁸ Some authors have introduced modifications in the nanoprecipitation process to accomplish the formulation of hydrophilic compounds into hydrophobic polymeric matrices, however, these modifications generally require more complex co-solvent systems.^{19–24}

The development of PLGA NPs for protein delivery has made notable advances, while their potential to co-formulate protein therapeutics and perform combination therapy is yet to be fully investigated. Co-formulation of multiple therapeutics into polymeric NPs is expected to simplify delivery to patients by reducing multiple dosage regimens, achieve synergistic bioresponses by tuning the ratio of the loaded cargos, as well as to provide a simultaneous targeting to multiple therapeutic active sites.²⁵ Combination therapy may be particularly useful for multi-cytokine treatments, since immune responses trigger several biochemical cascades in which many molecular networks need to be targeted simultaneously.²⁶

Bovine serum albumin (BSA) is an ideal protein candidate for preliminary model studies due to its relatively high molecular weight (around 66.5 kDa) and economical price point.²⁷ The conjugation of fluorochromes as either fluorescein (FITC) or tetramethylrhodamine (TRITC) isocyanates with BSA is well known to significantly change its physicochemical properties, which produces fluorochrome-

dependent protein profiles.²⁸ For example, protein solubility can be considerably altered by the labelling fluorochrome, due to a net-increase in the total number of attractive sites in the protein-fluorochrome system, as reported by Quinn *et al.*²⁹ Therefore, BSA-FITC and BSA-TRITC can be distinguished from each other and from the unlabelled BSA with respect to their physiochemical characteristics and used as two different model proteins, to study continuous manufacturing and encapsulation into polymeric NPs.

This study reports PLGA NPs for all-in-one co-formulation and -delivery of two models of protein therapeutics consisting of BSA-FITC and BSA-TRITC. The study addresses continuous manufacturing by investigating a microfluidic modified-nanoprecipitation technique for the production of protein co-formulated PLGA NPs, with further assessment of their impact in the association of the cargos with macrophage-like cells.

2. Experimental

2.1 Materials

The X-junction microfluidic device (Quartz Droplet Junction Chip, 190 μm etch depth) was acquired from Dolomite (Royston, UK). BSA-FITC, BSA-TRITC, Tween®-80, sodium chloride, phosphate buffered saline (PBS), phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, USA); acetone from Thermo Fisher Scientific (Waltham, MA, USA); high glucose with ultraglutamine Dulbecco's modified Eagle medium (DMEM) from Lonza (Basel, Switzerland); Triton X-100 from Spi-Chem (West Chester, PA, USA); and fetal bovine serum (FBS) and penicillin-streptomycin from Gibco (Waltham, MA, USA). Acid-terminated PLGA polymer (50 : 50 lactic to glycolic ratio, molecular weight of 10 kDa) was kindly provided by Dr Mohammad Al-Natour and its synthesis was described elsewhere.³⁰

The human monocytic leukaemia THP-1 cell line was purchased from ATCC (Manassas, VA, USA).

2.2 Methods

2.2.1 Nanoparticles manufacturing and process optimization. The organic phase used for the microfluidic manufacturing of the NPs consisted of a mixture of PLGA in acetone, for which the concentration was optimized between 0.25% and 1% w/v. The aqueous phase, in turn, was kept as a 2% w/v solution of Tween®-80, and both BSA-FITC and BSA-TRITC were added to this phase to obtain protein-loaded PLGA NPs. For the BSA-FITC and BSA-TRITC individual formulation, a concentration of 33 $\mu\text{g mL}^{-1}$ and 67 $\mu\text{g mL}^{-1}$ of BSA-FITC and BSA-TRITC in the 2% w/v solution of Tween®-80, respectively, was used. Whereas, for the BSA-FITC and BSA-TRITC co-formulation, a concentration of 25 $\mu\text{g mL}^{-1}$ and 8 $\mu\text{g mL}^{-1}$ of BSA-FITC and BA-TRITC in the 2% w/v solution of Tween®-80, respectively, was used.

The microfluidic chip displayed a three-inlets X-layout, in which the organic and aqueous phases were injected through the central and the two outer inlets, respectively (Fig. 1). The

total flow rate was varied from 25:75 to 500:1500 $\mu\text{L min}^{-1}$ regarding the organic:aqueous phases. Each experiment was conducted until 10 mg of PLGA NPs were produced (equivalent to 2 min run time using the final flow rate conditions). NPs were washed three times with ultrapure water and recovered by ultrafiltration using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Billerica, MA, USA) with a molecular weight cut-off (MWCO) of 100 kDa.

Scale-up experiment. A scale-up experiment was performed in relation to the mass of PLGA in the final batch of unloaded NPs. The batch size was scaled 5- and 25-times, corresponding to a final mass of PLGA of 50 mg and 250 mg, respectively.

2.2.2 Characterization of nanoparticles

Mean particle size, size distribution and surface charge. NPs were characterized for their average size (Z-average) and polydispersity index (PDI) by dynamic light scattering (DLS), and zeta-potential (ζ -potential) through laser Doppler anemometry (LDA), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). For these measurements, samples were diluted (1:100) in an ionic solution of 10 mM sodium chloride.

Association efficiency. The amount of loaded BSA-FITC and BSA-TRITC was directly quantified in order to determine the association efficiency (AE) of the developed nanosystems. The calculation of AE followed eqn (1).³¹

$$AE_{\text{BSA}}(\%) = \frac{\text{Quantified loaded mass of BSA}}{\text{Initial mass of BSA}} \times 100 \quad (1)$$

The amount of loaded BSA-FITC and BSA-TRITC was spectrophotometrically quantified after the extraction of the proteins from the matrix of the NPs according to a protocol

adapted from.³² Briefly, the protein-loaded NPs were treated with a solution of 3 M NaOH for 24 h at 100 rpm in an orbital shaker (Thermo Scientific, Madison, WI, USA), to promote the disaggregation of the polymeric matrix and release of the associated BSA-FITC and BSA-TRITC. The suspension was then sonicated for 1 h and centrifuged at 14 000 rpm for 15 min (Hermle Z 300, Hermle Labortechnik GMBH, Wehingen, DE). The supernatant was filtered through a 0.22 μm syringe filter (Millex® GP, Millipore, Bedford, MA, USA). The absorbance of the filtrate was read in a Varian Cary® 50 Bio UV-VIS spectrophotometer (Agilent Technologies, Stockport, UK) at 488 nm and 543 nm for the detection of BSA-FITC and BSA-TRITC, respectively.

Morphology. The morphological features of the NPs were analyzed by transmission electron microscopy (TEM) using a Philips Tecnai BioTwin-12 microscope (FEI, Eindhoven, NL) at an accelerating voltage of 120 kV. Images were recorded using a Gatan SIS Megaview IV digital camera (Gatan Inc., Warrendale, PA). Samples were prepared by dropping 20 μL of the suspension of the NPs onto a 300-mesh copper grid and negatively-stained with 20 μL of 1% w/v uranyl acetate.

2.2.3 In vitro release study. To investigate the *in vitro* protein release profile, PLGA NPs formulated with BSA-FITC (55 mg) and BSA-TRITC (55 mg) or co-formulated with BSA-FITC and BSA-TRITC (140 mg) were dispersed in PBS, pH 7.4, to reach the total volume of 2 mL. The samples were then stirred at 37 °C and 100 rpm in an orbital shaker incubator (Incu-Shake MINI, SciQuip, Staffordshire, UK). Aliquots of 1 mL were collected at specific time points (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5 and 24 h) during the assay, and the withdrawn volume was replaced with pre-heated PBS, pH 7.4, to maintain sink conditions. All the collected aliquots were centrifuged at 14 000 rpm for 15 min, and the

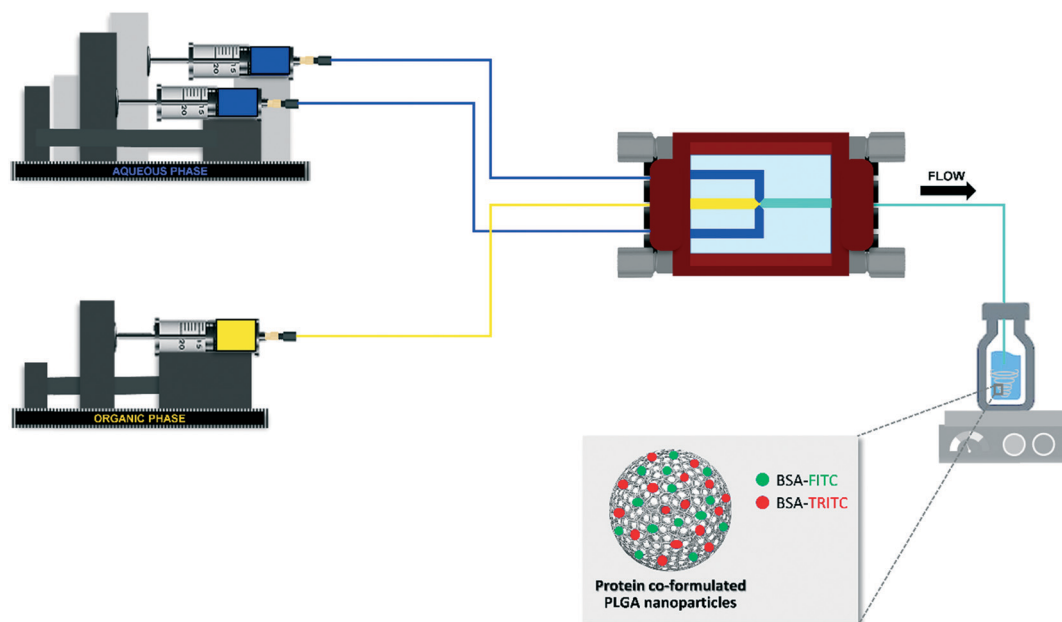


Fig. 1 Schematic representation of the microfluidic setup.

spectrophotometric absorbance of the supernatant was read at 488 nm and 543 nm for the detection of BSA-FITC and BSA-TRITC, respectively.

2.2.4 Structural stability of the released proteins. Alterations in the secondary structure of the total protein content released from PLGA NPs were assessed by circular dichroism (CD) spectroscopy. PLGA NPs individually formulated with BSA-FITC (55 mg) and BSA-TRITC (55 mg) or co-formulated with BSA-FITC and BSA-TRITC (140 mg) were dispersed in PBS (pH 7.4, 2 mL). The samples were then stirred at 37 °C and 100 rpm in an orbital shaker incubator (Incu-Shake MINI, SciQuip, Staffordshire, UK) for 24 h. After centrifugation at 14 000 rpm for 15 min, the supernatant was concentrated to 240 µg mL⁻¹ of protein by ultrafiltration using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Billerica, MA, USA) with a MWCO of 10 kDa. The spectral profile of each sample was compared with the respective native and denatured (90 °C, 1 h) controls. The concentration of native and denatured controls was defined as 1 mg mL⁻¹ and 200 µg mL⁻¹, respectively. The ellipticity value of proteins was scanned over 200–260 nm wavelength using an Aviv Model 400 spectrometer (Aviv Biomedical Inc, Lakewood, New Jersey, USA). A quartz cell was used, and the bandwidth and time-per-point were 1 nm and 1 s, respectively. The mean residue ellipticity (MRE) was calculated according to eqn (2).³³

$$\text{MRE} = \frac{(\theta_{\text{sample}} - \theta_{\text{media}}) \times \text{MW}}{(N - 1) \times 1 \times C} \quad (2)$$

where θ is the raw ellipticity value (mdeg), MW is the molecular weight of BSA (66.5 kDa), N is the number of residues of BSA (583), 1 is the cuvette path length (1 mm), and C is the protein concentration in mg mL⁻¹. The α -helix percentage was also calculated according to eqn (3).³⁴

$$\alpha\text{-helix (\%)} = \frac{-\text{MRE}_{208\text{nm}} - 4000}{33\,000 - 4000} \quad (3)$$

2.2.5 Cell culturing. THP-1 cells were grown in suspension in tissue culture flasks (passage 14–25). The cells were maintained in DMEM medium containing FBS (10%, v/v) and penicillin–streptomycin (1%, v/v). Cultures were kept in an incubator (CellCulture CO₂ incubator, ESCO GB Ltd., Downton, UK) at 37 °C with 5% CO₂, in a water saturated atmosphere. After reaching confluency, cells were centrifuged and split into new tissue culture flasks.

2.2.6 Cellular metabolic activity assay. The influence of samples on metabolic activity was assessed in macrophage-like cells differentiated from the THP-1 cell line using the MTT assay. THP-1 cells were seeded in a 96-well plate (1 × 10⁵ cells per well) in 200 µL complete DMEM medium at a PMA concentration of 10 ng mL⁻¹ in order to induce their differentiation into macrophage-like cells.³⁵ After 24 h of incubation, medium was removed and cells were washed twice with 200 µL PBS, pH 7.4. Then, cells were incubated with the samples in concentrations of 0.03 µM, 0.003 µM and 0.0003 µM (in

medium) determined in relation to the proteins, for 24 h. Afterwards, solutions were discarded, cells were washed with 200 µL PBS, pH 7.4, and treated with 200 µL of MTT solution (0.5 mg mL⁻¹, in medium), during 4 h in the dark. Formazan crystals, resulting from the reduction of MTT by viable cells, were solubilized with 100 µL of DMSO, with of 20 min gentle shaking at 100 rpm at room temperature. The absorbance was measured at 590 nm and 630 nm. A negative control (NC), consisting of cells incubated with 1% of Triton X-100 in medium solution (0% of metabolic activity), and a positive control (PC), consisting of cells incubated only with medium (100% of metabolic activity), were also prepared and treated similarly to the sample wells. Metabolic activity was expressed as a percentage compared to the controls according to eqn (4).

$$\text{Metabolic activity (\%)} = \frac{\text{Experimental value} - \text{NC}}{\text{PC} - \text{NC}} \times 100 \quad (4)$$

2.2.7 Cell-nanoparticle interaction. The influence of proteins in their free form or formulated into PLGA NPs on cell association was investigated through flow cytometry analysis in macrophage-like cells differentiated from the THP-1 cell line following a protocol previously developed by Costa *et al.*³⁶ THP-1 cells were seeded in a 24-well plate (5 × 10⁵ cells per well) in 1 mL complete DMEM medium at a PMA concentration of 10 ng mL⁻¹ in order to induce their differentiation into macrophage-like cells.³⁵ After 24 h, cells were washed twice with pre-warmed 1 mL PBS, pH 7.4, and incubated with samples (0.03 µM, determined in relation to the proteins) in 500 µL DMEM for 3 h at 37 °C. After incubation, the cells were washed twice with 1 mL PBS, pH 7.4, and detached with 90 µL trypsin–EDTA. The cells were then washed with 1 mL complete medium and PBS, pH 7.4, through centrifugation at 1300 rpm during 5 min, and fixed with 4% w/v paraformaldehyde (PFA) in PBS, pH 7.4, for 10 min. PFA was removed through centrifugation washes. Finally, cells were resuspended in PBS, pH 7.4, and placed in cytometer tubes. The quantification of proteins associated with macrophage-like cells was done using a FACSAria I cytometer (BD Biosciences, San Jose, CA, USA). The results were analyzed using the FlowJo vX.0.7 software.

2.2.8 Statistical analysis. The results were represented as mean ± standard deviation from a minimum of three independent experiments. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a *post hoc* test (Tukey's honestly significant difference). Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$. The statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

In this work, PLGA NPs were co-formulated with BSA-FITC and BSA-TRITC *via* a microfluidic modified-nanoprecipitation technique. The manufacturing of these NPs was performed

using a X-junction microfluidic chip. An acetone organic phase consisting of PLGA polymer and an aqueous solution of Tween®-80 were used. The standard nanoprecipitation methodology was first described by Fessi *et al.*³⁷ who detailed how materials for delivery could be solubilized in the organic phase. In this article, it is shown that the BSA-FITC and BSA-TRITC model proteins can also be included in the aqueous Tween®-80 solution.

The continuous manufacturing of polymeric NPs using microfluidics is a highly versatile technique in which many parameters must be optimized in order to produce the desired particulate system. Therefore, to clarify the manufacturing method, the influence of PLGA concentration in the organic phase and total flow rate of the system was investigated, whilst maintaining the ratio of organic:aqueous solvent phase and aqueous phase concentration of Tween®-80.

An increase in the PLGA concentration of the organic phase from 0.25% w/v to 1% w/v produced a significant increase ($*p < 0.05$) in the Z-average of NPs from around 110 nm to 140 nm, respectively (Fig. 2A). This increase in size can be explained by the rise in the number and consequent fusion of PLGA chains within the vicinity of nucleation sites during particle formation.³⁸ PDI values lower than 0.2 across the same range of concentrations (Fig. 2B) demonstrated size distribution uniformity of all NP populations, which is a typical characteristic of microfluidic-produced particles. This is because, the manufacturing device allows the controlled separation of the nucleation and growth stages through the distance away from the location where the initial mixing of the organic and aqueous phase occurs.^{11,39} The ζ -potential, in turn, significantly dropped ($**p < 0.01$) from around -9 mV to -17 mV with increasing concentrations of PLGA (Fig. 2C) as a result of the higher density of polymer carboxyl end-groups exposed on the surface of the NPs.⁴⁰ Increasing concentrations of PLGA also resulted in a considerable increase

in the production rate of NPs (Fig. 2D) from around 0.2 g per day to 0.7 g per day. The production rate is usually an undervalued parameter in most bulk and even continuous manufacturing methods used for PLGA NPs production. However, this parameter has attracted a greater focus in recent years. This is because, the production rate directly correlates with the capability of the manufacturing method to address the throughput required for the application in industrial and clinical settings.^{41,42}

These physicochemical properties of NPs are ideal for systemic administration and have been previously shown to permit transit through and escape from the spleen and liver.^{43,44} Furthermore, these properties also permit the application of these particles for cancer nanomedicine. This is because, their Z-average enables enhanced permeability and retention (EPR) effect by migrating through the vascular fenestrations of tumours.⁴⁵ More particularly, the size dimension of the developed NPs could be also useful in the field of brain drug delivery since it is generally accepted that it facilitates the transport of particles through the vascular endothelial cells of the blood-brain barrier.^{46,47} PLGA at 1% w/v in acetone solvent was defined as the organic phase to proceed with further studies, as it provided the highest possible polymer concentration and consequent NPs production rate, whilst maintaining homogeneous monodistributed NPs.⁴⁸

The optimization of the manufacturing process involved an increase in the total flow rate from 100 $\mu\text{L min}^{-1}$ to 2000 $\mu\text{L min}^{-1}$. These flow rates corresponded to an increase in the organic phase flow rate from 25 $\mu\text{L min}^{-1}$ to 75 $\mu\text{L min}^{-1}$ and aqueous flow rate 500 $\mu\text{L min}^{-1}$ to 1500 $\mu\text{L min}^{-1}$. Higher total flow rates led to a significant decrease in the Z-average of the PLGA NPs ($**p < 0.01$, Fig. 3A), which is in accordance with previously published literature by Zhang *et al.*⁴⁹ They have explained that the faster diffusion of the organic phase into the aqueous phase enabled higher nucleation rates and

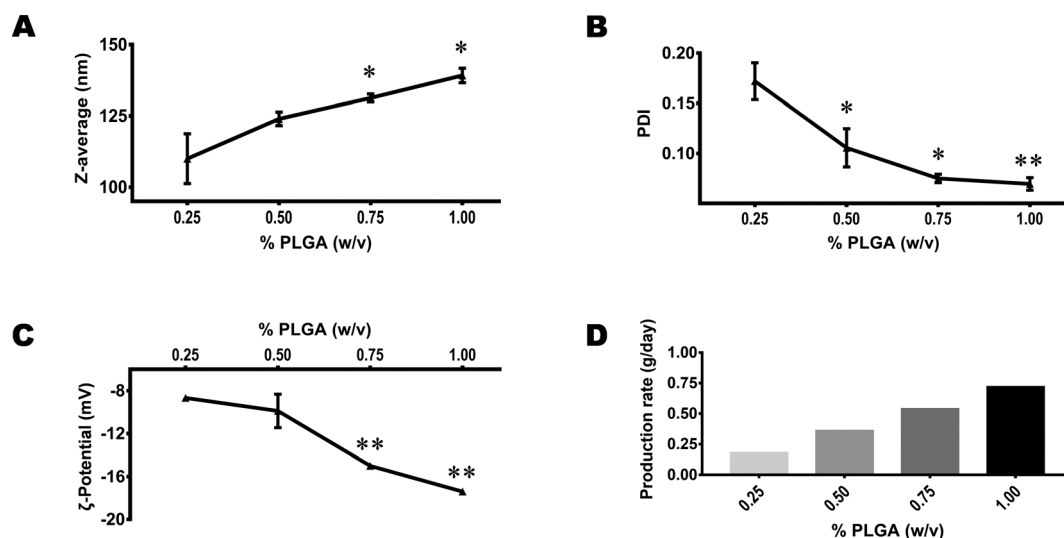


Fig. 2 Optimization of PLGA concentration for the manufacturing of unloaded NPs, using an organic:aqueous flow rate of 50:150 $\mu\text{L min}^{-1}$. NP properties of (A) Z-average, (B) PDI and (C) ζ -potential were investigated, as well as the (D) manufacturing productivity. Final conditions were defined as 1% w/v PLGA. Error bars represent the mean $\pm$ SD ($n = 3$) and the significance levels were assigned at $*p < 0.05$ and $**p < 0.01$.

the formation of multiple, smaller spatial supersaturation zones. This places the nucleation stage in a privileged position compared to the growth one, since more nuclei are formed and only limited amount of polymer is left to grow them. Therefore, lower diameter NPs can be obtained. The PDI (Fig. 3B) and ζ -potential (Fig. 3C) did not change significantly across the same range of total flow rate, keeping values less than 0.2 and around -20 mV, respectively. Increasing total flow rates resulted in a greater increase in the production rate of the PLGA NPs (Fig. 3D) from around 0.4 g per day to 7 g per day. It is important to highlight that the obtained maximum production rate of 7 g per day demonstrated superior yields to previously reported literature data for polymeric nanosystems, that were manufactured using a single microfluidic device, e.g. 0.0072–0.0144 g per day,⁵⁰ 0.012–0.079 g per day,⁵¹ 0.156 g per day⁵² and 0.252 g per day.⁵³ Based on the data presented in Fig. 3, the final total flow rate was defined as 2000 $\mu\text{L min}^{-1}$, which corresponded to an organic:aqueous flow rate of 500:1500 $\mu\text{L min}^{-1}$. These greater flow rates provided the highest productivity, while maintaining homogeneous monodisperse PLGA NPs.

To ensure the controlled, reproducible continuous manufacturing of PLGA NPs using microfluidics, the scale of the manufacturing method was investigated. Specifically, a scale-up of the final yield by $\times 5$ (50 mg) and $\times 25$ (250 mg) was conducted. The Z-average, PDI and ζ -potential properties did not change significantly and demonstrated to be independent on the scale-up (Table 1), which indicated the strength of the herein proposed continuous microfluidic manufacturing process. This successful up-production in a continuous-flow setup offers potential to speed up the translation of the developed nanomedicines from laboratory to industry and clinical scales.⁵⁴

PLGA NPs were manufactured unloaded, individually formulated with BSA-FITC or BSA-TRITC, as well as co-formulated with BSA-FITC and BSA-TRITC. The Z-average (around 120 nm), PDI (around 0.1) and ζ -potential (around 15 mV) of all protein-loaded formulations did not exhibit significant changes compared to unloaded NPs (Table 2). PLGA nanosystems individually formulated with either BSA-FITC or BSA-TRITC presented an AE of around 80%. Although there is limited information in the literature highlighting the loading of BSA-TRITC into PLGA NPs, the obtained BSA-FITC AE value was far higher than the ones reported by other authors, approximately around 30–45%.^{32,34,55} Moreover, the co-formulated nanosystem also presented high AE for both BSA-FITC and BSA-TRITC, corresponding to values of around 70% and 80%, respectively. It was hypothesized that the selected concentration of Tween®-80 endowed the protein-containing aqueous phase with an optimal viscosity to promote its diffusion through the organic phase. This provides enhanced dispersion of BSA-FITC and/or BSA-TRITC molecules through the PLGA matrix. It was furthermore envisaged that any residual Tween®-80 coating the surface of the NPs could act as an entrapment layer for BSA-FITC and/or BSA-TRITC molecules residing at the polymer-surfactant interface.

TEM imaging was used as a tool to provide more insight into the size and morphology of the developed NPs. Monodisperse particles with a homogenous surface and spherical morphology were observed (Fig. 4). All formulations presented a size of NPs of around 100 nm, which is in accordance with DLS measurements.

Protein release was evaluated *in vitro* in buffered saline (pH 7.4), to represent an intravenous environment. PLGA NPs individually loaded with BSA-FITC were characterized by an initial rapid burst effect, with around 80% of the protein

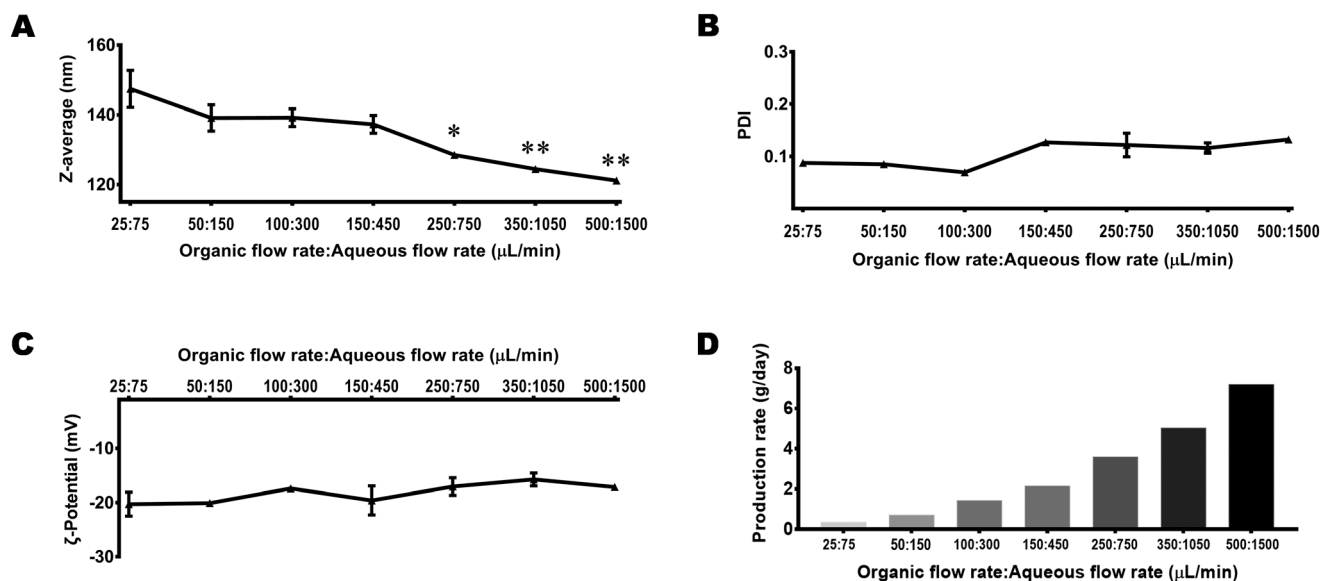


Fig. 3 Optimization of the total flow rate when manufacturing unloaded NPs. NP properties of (A) Z-average, (B) PDI and (C) ζ -potential were investigated, as well as the (D) manufacturing productivity. Final conditions were defined as an organic:aqueous flow rate of 500:1500 $\mu\text{L min}^{-1}$. Error bars represent the mean $\pm$ SD ($n = 3$) and the significance levels were assigned as * $p < 0.05$ and ** $p < 0.01$.

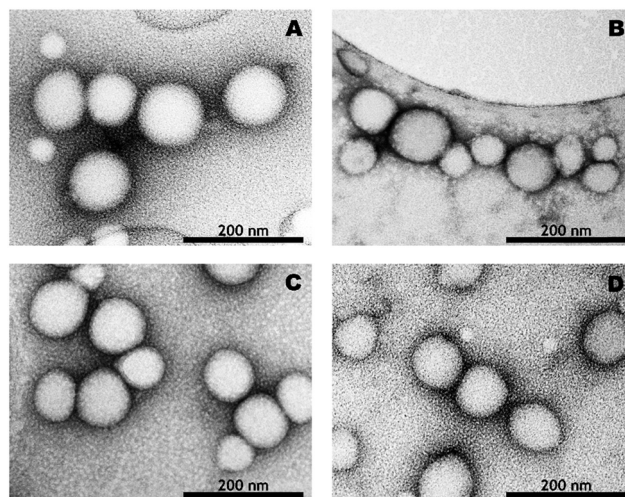
Table 1 Evaluation of the impact of a scale-up manufacturing on the physicochemical properties of NPs of Z-average, PDI and ζ -potential

Scaling	Z-Average (nm)	PDI	ζ -Potential (mV)
Standard scale	116.9 $\pm$ 1.7	0.105 $\pm$ 0.006	-17.2 $\pm$ 1.1
5-times scale-up	121.9 $\pm$ 3.8	0.109 $\pm$ 0.015	-16.6 $\pm$ 2.8
25-times scale-up	120.9 $\pm$ 2.3	0.107 $\pm$ 0.013	-16.5 $\pm$ 3.6

being recovered in the first 2 h (Fig. 5A). In comparison, PLGA NPs individually loaded with BSA-TRITC, significantly less of the protein was recovered (around 50%) over the same time period and the overall release was more sustained (Fig. 5B). This results suggested that BSA-FITC may have a tendency to be located close or on the surface of the NPs, whereas BSA-TRITC could be dispersed into the polymeric PLGA matrix.⁵⁶ Since the TRITC fluorochrome is more hydrophobic than FITC,⁵⁷ it also rendered BSA-TRITC protein more hydrophobicity than BSA-FITC. The PLGA polymer is known to present a hydrophobic backbone and, thus, it is prone to retain hydrophobic and hydrophilic cargos into its matrix and close to its surface, respectively, which can explain the previous release results.⁵⁶

Co-formulated BSA-FITC and BSA-TRITC PLGA NPs exhibited a release profile of BSA-TRITC comparable to the BSA-TRITC individual formulation, with also around 50% of the protein recovered over the first 2 h (Fig. 5C). However, the release profile of BSA-FITC underwent significant alterations compared to the individual formulation, with only around 20% of the protein recovered over 2 h (Fig. 5C). It was hypothesized that this difference was related to the opposite net charge values of FITC and TRITC fluorochromes. FITC is negatively charged under neutral pH and presents a net value of -1.02, whereas TRITC possesses a net positive charge of +0.99.⁵⁸ BSA conjugated with the positively charged TRITC, which is hypothesised to be distributed through the polymeric matrix of PLGA NPs, may exert electrostatic interactions with BSA conjugated with the negatively charged FITC, which would be expected to be located closer to the particles' surface. This electrostatic attraction could therefore be responsible for retaining the BSA-FITC for prolonged time periods, sustaining its release, in comparison to the BSA-FITC individual formulation.

Protein structure is important since alterations in the secondary structure of proteins are likely to be related to their denaturation, which ultimately culminates in aggregation and loss of biologic function.^{34,59} One of the major disadvan-

**Fig. 4** TEM images for (A) unloaded NPs, individually loaded (B) BSA-FITC and (C) BSA-TRITC NPs, and (D) BSA-FITC and BSA-TRITC co-formulated NPs.

tages of the loading of protein therapeutics into polymeric NPs is their denaturation after the manufacturing process, mainly due to the deposition of these molecules in the organic-aqueous phase of emulsions and/or the contact of the organic solvents with their nonpolar amino acids.⁶⁰ Therefore, maintenance of the secondary structure of proteins after the release from PLGA NPs was studied through CD, where controls of each protein type in its native and denatured form were used for comparison purposes.

All CD spectra presented double minima at around 220 nm and 210 nm, which is characteristic of the α -helical structure of BSA^{61,62} (Fig. 6). The spectra of BSA-FITC and BSA-TRITC released from the individually and co-formulated PLGA NPs were comparable to the native forms of the proteins. They were also distinguishable from the denatured control, which produced diminished absorbance bands. The α -helix content was calculated in order to confirm the structural similarities between the released samples and native controls. The calculated values were found in the interval from 50–60% as literature data reports for BSA,^{34,63} with variations in the α -helix content relative to the control of only around 0%, 4% and 3% for individually loaded BSA-FITC NPs (Fig. 6A) and BSA-TRITC PLGA NPs (Fig. 6B), and co-formulated BSA-FITC and BSA-TRITC PLGA NPs (Fig. 6C). The CD results confirmed that the proposed continuous microfluidic manufacturing method for production of

Table 2 Evaluation of the physicochemical properties of NPs of Z-average, PDI, ζ -potential and AE after individual formulation with BSA-FITC or BSA-TRITC, and co-formulation with BSA-FITC and BSA-TRITC

NPs	Z-Average (nm)	PDI	ζ -Potential (mV)	AE (%)	
				BSA-FITC	BSA-TRITC
Unloaded	116.9 $\pm$ 1.7	0.105 $\pm$ 0.006	-17.2 $\pm$ 1.1	—	—
BSA-FITC	117.4 $\pm$ 2.1	0.102 $\pm$ 0.012	-14.1 $\pm$ 2.3	77.7 $\pm$ 4.0	—
BSA-TRITC	121.3 $\pm$ 2.6	0.104 $\pm$ 0.002	-15.7 $\pm$ 2.1	—	79.9 $\pm$ 3.2
BSA-FITC and BSA-TRITC	118.7 $\pm$ 4.1	0.106 $\pm$ 0.002	-14.6 $\pm$ 2.7	66.3 $\pm$ 4.5	77.5 $\pm$ 5.7

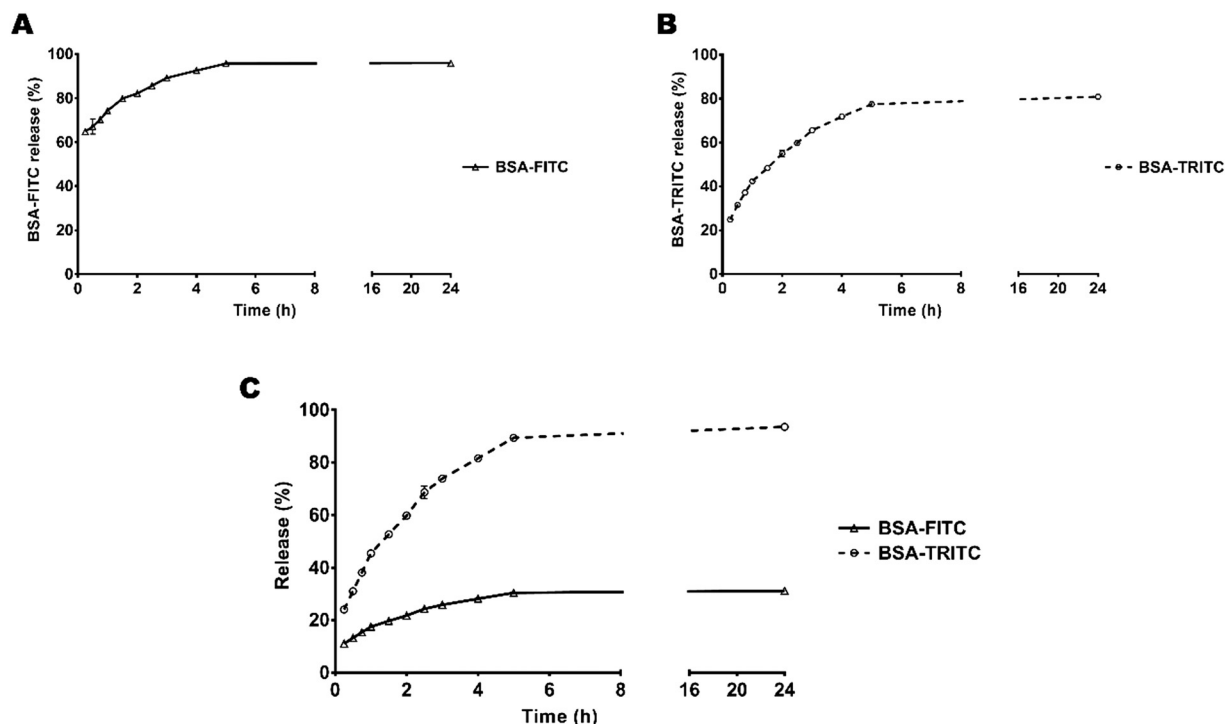


Fig. 5 *In vitro* release profile of individually loaded (A) BSA-FITC and (B) BSA-TRITC NPs, and (C) BSA-FITC and BSA-TRITC co-formulated NPs. Error bars represent the mean $\pm$ SD ($n = 3$).

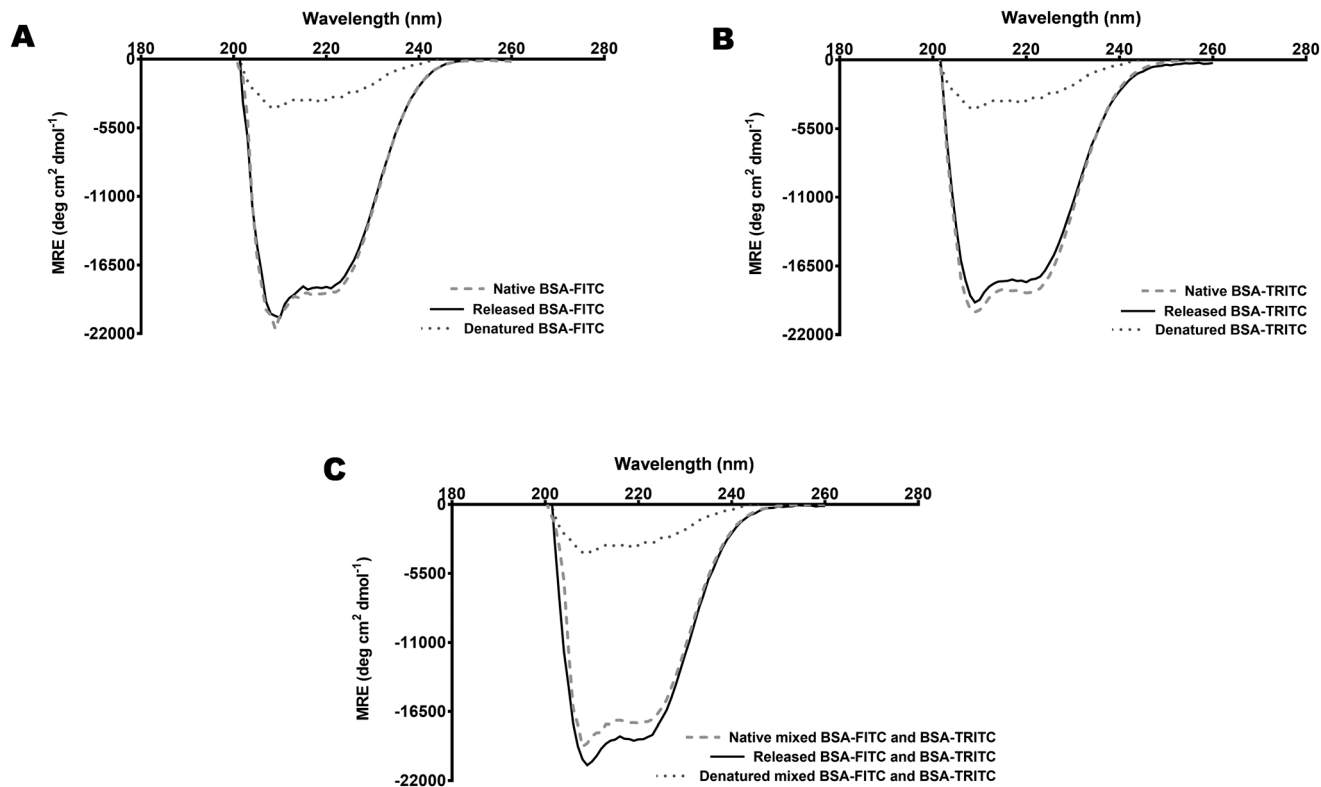


Fig. 6 CD spectral profile of the total released protein from individually formulated (A) BSA-FITC NPs and (B) BSA-TRITC NPs, and (C) BSA-FITC and BSA-TRITC co-formulated NPs. Spectral profile of the respective native and thermally denatured total protein is also represented. Results are presented as mean ($n = 3$).

polymeric PLGA NPs was successfully able to encapsulate model proteins, either individually or as a co-formulation, in the absence of deviations in the secondary structure of the released proteins.

The influence of the developed nanosystems on metabolic activity was accessed through a MTT assay in macrophage-like cells differentiated from the THP-1 cell line (Fig. 7). This cell line was selected only as a model due to its well described, easy and relatively inexpensive culture. Protein concentrations ranging from 0.0003–0.03 μM were investigated for their effects on metabolic activity. The metabolic activity of macrophage-like cells was found to be always above 70% when in contact with the free BSA-FITC, BSA-TRITC, and mixed BSA-FITC and BSA-TRITC at all evaluated concentrations. This demonstrated the absence of cytotoxicity associated with the free proteins and was expected since the major molarity contribution from the protein-fluorochrome complex originates from the BSA, for which the normal physiologic concentration is much higher than the maximum one herein tested (around 0.76 M in blood serum).^{64,65} Unloaded NPs also exhibited cell metabolic activity greater than 70% for all tested concentrations, as well as NPs individually loaded with BSA-FITC or BSA-TRITC and co-formulated with BSA-FITC and BSA-TRITC. The polymeric matrix of NPs did not induce cytotoxicity and did not compromise cell viability. This observation was also true for the physical mixture of NPs individually loaded with BSA-FITC or BSA-TRITC in amounts equivalent to the co-formulated NPs. Therefore, it is important to note, according to the 10993-5 norm from the International Organization for Standardization (ISO) for *in vitro* pre-clinical evaluation of the cytotoxicity of medical devices, these PLGA NP formulations may be considered non-toxic and potentially safe.⁶⁶

It is well reported that the hydrophilicity of proteins is one of the main characteristics responsible for their poor interaction with the hydrophobic lipid bilayer of cells.⁶⁷ This may be especially critical for protein-based drugs that require an efficient intracellular transport and delivery to exert a certain therapeutic effect.⁶⁸ Therefore, the potential of the developed NPs to improve cell–protein interaction was evaluated

in macrophage-like cells differentiated from the THP-1 cell line (Fig. 8).

The loading of BSA-FITC into PLGA NPs did not appear to improve cell interaction, with no significant differences observed in comparison to the free BSA-FITC (Fig. 8A). This correlated to the release studies (Fig. 5A) that suggested that BSA-FITC may be preferably located on the surface of the NPs which, as a result, may adopt hydrophilic properties that may hinder cell association. Whereas, the loading of BSA-TRITC into PLGA NPs significantly improved ($***p < 0.001$) cell interaction compared to the free BSA-TRITC (Fig. 8B). This again confirmed observations from the release studies (Fig. 5B) and suggested that the more hydrophobic BSA-TRITC protein may be preferably dispersed through the polymeric core of PLGA NPs, rather than on their surface. The hydrophilic properties of BSA-TRITC were therefore camouflaged by the hydrophobic ones of the PLGA NPs, hence favouring cell interaction. These findings were in accordance with research published by Miklavžin *et al.*, who showed that the complexation of erythropoietin with polyelectrolytes to render a more hydrophobic complex was able to enhance the interaction and consequent permeability of the protein through Caco-2 cell monolayers.⁶⁹ Apart from the hydrophilicity, it was hypothesized that the developed PLGA NPs might profit from residual Tween®-80 molecules on their surface to bind the scavenger receptor of macrophage-like cells, hence promoting an enhanced cell–protein interaction. Tween®-80 has the capacity to adsorb apolipoprotein E, which is a known ligand of this type of cell receptor and thus mediates cell-NP binding.^{70,71} Co-formulated PLGA NPs significantly increased the cell association of co-formulated BSA-FITC and BSA-TRITC ($**p < 0.01$), compared to the mix of the free form of the proteins (Fig. 8C). This finding highlights an important advance in this proof-of-concept study due to the usefulness of developing a delivery system capable of enhancing protein co-delivery. The co-formulated PLGA NPs demonstrated a significantly higher cell association ($***p < 0.001$) compared to the physical mixture of NPs individually loaded with BSA-FITC and BSA-TRITC (Fig. 8C), which reinforced the superior biologic performance of the all-in-one nanosystem.

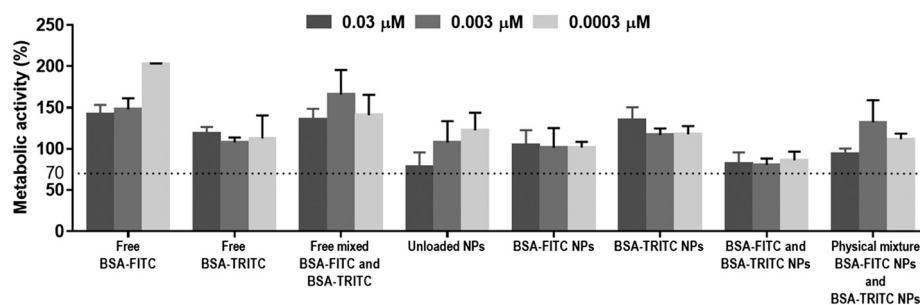


Fig. 7 Metabolic activity of macrophage-like THP-1 cells after 3 h exposure to the free and nanoformulated proteins. Sample data were normalized to the positive (buffer, 100%) and negative (lysis buffer, 0%) controls. Error bars represent the mean $\pm$ SD ($n = 3$).

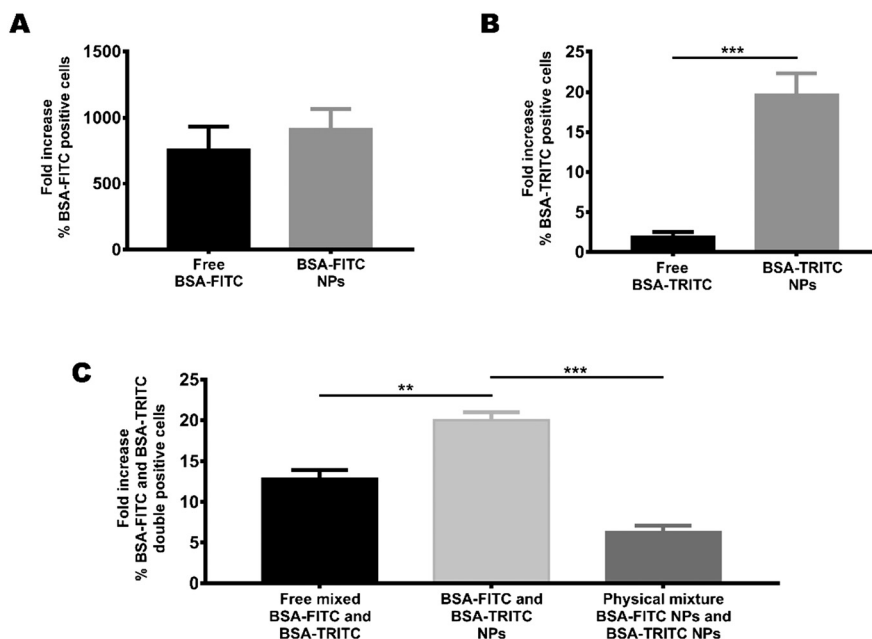


Fig. 8 Flow cytometric analysis of the interaction of macrophage-like THP-1 cells with (A) free and NP formulated BSA-FITC, (B) free and NP formulated BSA-TRITC and (C) free and NP co-formulated BSA-FITC and BSA-TRITC, after a 3 h exposure assay. The fold increase was calculated relative to untreated cells. Error bars represent the mean $\pm$ SD ($n = 3$) and significance levels were assigned as ** $p < 0.01$ and *** $p < 0.001$.

4. Conclusion

A continuous microfluidic manufacturing method that enabled the engineering of monodisperse BSA-FITC and BSA-TRITC co-formulated PLGA NPs with a high association efficiency is reported herein. The enhanced production rate and scale-up potential of the demonstrated microfluidic method indicated the capacity to manufacture monodisperse PLGA NPs in industrial and clinical batch sizes. The microfluidic manufacture process preserved the protein secondary structure and did not appear to induce denaturation upon release. Co-formulated BSA-FITC and BSA-TRITC PLGA NPs did not present cytotoxicity and significantly enhanced the association of BSA-FITC and BSA-TRITC with macrophage-like cells compared to the free mixed form of the proteins and even the physical mixture of NPs individually loaded with each protein type. Therefore, it is anticipated that this model study will pave the way towards novel combination therapies based on PLGA nanosystems that require the co-delivery of protein therapeutics to a range of pathologies. Perspectives for future work include the co-formulation of other proteins and investigation of the preservation of bioactivity post-release, as well as studies of the efficacy of the nanosystem *in vivo*.

Conflicts of interest

There are no conflicts to declare.

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References

- 1 S. S. Dewan, *Global Markets for Bioengineered Protein Drugs*, BCC Research, 2017.
- 2 W. Gao and M. Sun, *Theranostics*, 2018, **8**, 3872–3873.
- 3 S. S. Usmani, G. Bedi, J. S. Samuel, S. Singh, S. Kalra, P. Kumar, A. A. Ahuja, M. Sharma, A. Gautam and G. P. S. Raghava, *PLoS One*, 2017, **12**, e0181748.
- 4 L. R. Brown, *Expert Opin. Drug Delivery*, 2005, **2**, 29–42.
- 5 V. Torchilin, *Drug Discovery Today: Technol.*, 2008, **5**, e95–e103.
- 6 M. S. Akash, K. Rehman and S. Chen, *Pharm. Dev. Technol.*, 2016, **21**, 367–378.
- 7 S. Mohammadi-Samani and B. Taghipour, *Pharm. Dev. Technol.*, 2015, **20**, 385–393.
- 8 J. Hajavi, M. Ebrahimian, M. Sankian, M. R. Khakzad and M. Hashemi, *J. Biomed. Mater. Res., Part A*, 2018, **106**, 2540–2551.
- 9 C. Martins, F. Sousa, F. Araújo and B. Sarmiento, *Adv. Healthcare Mater.*, 2017, **7**, 1701035.
- 10 R. Juliano, *Nat. Rev. Drug Discovery*, 2013, **12**, 171–172.
- 11 J. Ma, S. M.-Y. Lee, C. Yi and C.-W. Li, *Lab Chip*, 2017, **17**, 209–226.
- 12 S. Rezvantalab and M. Keshavarz Moraveji, *RSC Adv.*, 2019, **9**, 2055–2072.
- 13 X. Li and X. Jiang, *Adv. Drug Delivery Rev.*, 2018, **128**, 101–114.
- 14 C. Martins, F. Araújo, M. J. Gomes, C. Fernandes, R. Nunes, W. Li, H. A. Santos, F. Borges and B. Sarmiento, *Eur. J. Pharm. Biopharm.*, 2018, **138**, 111–124.
- 15 A. Gdowski, K. Johnson, S. Shah, I. Gryczynski, J. Vishwanatha and A. Ranjan, *J. Nanobiotechnol.*, 2018, **16**, 12.
- 16 M. Witting, K. Obst, W. Friess and S. Hedtrich, *Biotechnol. Adv.*, 2015, **33**, 1355–1369.
- 17 M. M. Pakulska, S. Miersch and M. S. Shoichet, *Science*, 2016, **351**, aac4750.
- 18 D. Duchêne, G. Ponchel and D. Wouessidjewe, *Adv. Drug Delivery Rev.*, 1999, **36**, 29–40.
- 19 K. S. Yadav and K. K. Sawant, *AAPS PharmSciTech*, 2010, **11**, 1456–1465.
- 20 U. Bilati, E. Allémann and E. Doelker, *AAPS PharmSciTech*, 2005, **6**, E594–E604.
- 21 L. Peltonen, J. Aitta, S. Hyvonen, M. Karjalainen and J. Hirvonen, *AAPS PharmSciTech*, 2004, **5**, E16.
- 22 J. Xu, S. Zhang, A. Machado, S. Lecommandoux, O. Sandre, F. Gu and A. Colin, *Sci. Rep.*, 2017, **7**, 4794.
- 23 U. Bilati, E. Allémann and E. Doelker, *Eur. J. Pharm. Sci.*, 2005, **24**, 67–75.
- 24 L. Peltonen, P. Koistinen, M. Karjalainen, A. Hakkinen and J. Hirvonen, *AAPS PharmSciTech*, 2002, **3**, E32.
- 25 L. Wu, D. Leng, D. Cun, C. Foged and M. Yang, *J. Controlled Release*, 2017, **260**, 78–91.
- 26 E. Garaci, F. Pica, P. Sinibaldi-Vallebona, P. Pierimarchi, A. Mastino, C. Matteucci and G. Rasi, *Int. Immunopharmacol.*, 2003, **3**, 1145–1150.
- 27 P. Li, L.-W. Qi, E. H. Liu, J.-L. Zhou and X.-D. Wen, *TrAC, Trends Anal. Chem.*, 2008, **27**, 66–77.
- 28 S. Bingaman, V. H. Huxley and R. E. Rumbaut, *Microcirculation*, 2003, **10**, 221–231.
- 29 M. K. Quinn, N. Gnan, S. James, A. Ninarello, F. Sciortino, E. Zaccarelli and J. J. McManus, *Phys. Chem. Chem. Phys.*, 2015, **17**, 31177–31187.
- 30 M. Al-Natour, *PhD Thesis*, University of Nottingham, 2019.
- 31 J. das Neves and B. Sarmiento, *Acta Biomater.*, 2015, **18**, 77–87.
- 32 A. S. Guedj, A. J. Kell, M. Barnes, S. Stals, D. Goncalves, D. Girard and C. Lavigne, *Int. J. Nanomed.*, 2015, **10**, 5965–5979.
- 33 M. E. Fealey, B. P. Binder, V. N. Uversky, A. Hinderliter and D. D. Thomas, *Biophys. J.*, 2018, **114**, 550–561.
- 34 S. Y. Lee and H. J. Cho, *J. Colloid Interface Sci.*, 2017, **490**, 391–400.
- 35 P. B. Aldo, V. Craveiro, S. Guller and G. Mor, *Am. J. Reprod. Immunol.*, 2013, **70**, 80–86.
- 36 A. Costa, B. Sarmiento and V. Seabra, *Eur. J. Pharm. Sci.*, 2018, **114**, 103–113.
- 37 H. Fessi, F. Puisieux, J. P. Devissaguet, N. Ammoury and S. Benita, *Int. J. Pharm.*, 1989, **55**, R1–R4.
- 38 J. Aubry, F. Ganachaud, J. P. Cohen Addad and B. Cabane, *Langmuir*, 2009, **25**, 1970–1979.
- 39 Q. Guo, Q. Zhu, T. Miao, J. Tao, X. Ju, Z. Sun, H. Li, G. Xu, H. Chen and L. Han, *J. Controlled Release*, 2019, **303**, 117–129.
- 40 R. Nicolette, D. F. d. Santos and L. H. Faccioli, *Int. Immunopharmacol.*, 2011, **11**, 1557–1563.
- 41 I. U. Khan, C. A. Serra, N. Anton and T. F. Vandamme, *Expert Opin. Drug Delivery*, 2015, **12**, 547–562.
- 42 S. Yadavali, H.-H. Jeong, D. Lee and D. Issadore, *Nat. Commun.*, 2018, **9**, 1222.
- 43 D. H. Jo, J. H. Kim, T. G. Lee and J. H. Kim, *Nanomedicine*, 2015, **11**, 1603–1611.
- 44 N. Hoshyar, S. Gray, H. Han and G. Bao, *Nanomedicine*, 2016, **11**, 673–692.
- 45 E. Blanco, H. Shen and M. Ferrari, *Nat. Biotechnol.*, 2015, **33**, 941–951.
- 46 R. P. Moura, C. Martins, S. Pinto, F. Sousa and B. Sarmiento, *Expert Opin. Drug Delivery*, 2019, **16**, 271–285.
- 47 T. T. Zhang, W. Li, G. Meng, P. Wang and W. Liao, *Biomater. Sci.*, 2016, **4**, 219–229.
- 48 R. H. Fang, K. N. H. Chen, S. Aryal, C.-M. J. Hu, K. Zhang and L. Zhang, *Langmuir*, 2012, **28**, 13824–13829.
- 49 H.-X. Zhang, J.-X. Wang, L. Shao and J.-F. Chen, *Ind. Eng. Chem. Res.*, 2010, **49**, 4156–4161.
- 50 P. M. Valencia, E. M. Pridgen, M. Rhee, R. Langer, O. C. Farokhzad and R. Karnik, *ACS Nano*, 2013, **7**, 10671–10680.
- 51 R. Othman, G. T. Vladislavjević, H. C. Hemaka Bandulasena and Z. K. Nagy, *Chem. Eng. J.*, 2015, **280**, 316–329.
- 52 J. Sun, L. Zhang, J. Wang, Q. Feng, D. Liu, Q. Yin, D. Xu, Y. Wei, B. Ding, X. Shi and X. Jiang, *Adv. Mater.*, 2015, **27**, 1402–1407.
- 53 J.-M. Lim, N. Bertrand, P. M. Valencia, M. Rhee, R. Langer, S. Jon, O. C. Farokhzad and R. Karnik, *Nanomedicine*, 2014, **10**, 401–409.

- 54 T. Baby, Y. Liu, A. P. J. Middelberg and C.-X. Zhao, *Chem. Eng. Sci.*, 2017, **169**, 128–139.
- 55 N. Rescignano, L. Tarpani, R. Tiribuzi, S. Montesano, S. Martino, L. Latterini, J. M. Kenny and I. Armentano, *Macromol. Biosci.*, 2013, **13**, 1204–1212.
- 56 H. S. Yoo and T. G. Park, *J. Controlled Release*, 2001, **70**, 63–70.
- 57 J. W. Goding, in *Monoclonal Antibodies*, ed. J. W. Goding, Academic Press, London, 3rd edn, 1996, pp. 352–399, DOI: 10.1016/B978-012287023-1/50060-2.
- 58 L. Yin, W. Wang, S. Wang, F. Zhang, S. Zhang and N. Tao, *Biosens. Bioelectron.*, 2015, **66**, 412–416.
- 59 H. Sah, *J. Controlled Release*, 1999, **58**, 143–151.
- 60 R. Gaudana, V. Khurana, A. Parenky and A. K. Mitra, *J. Drug Delivery*, 2011, **2011**, 458128.
- 61 N. K. Kunda, I. M. Alfagih, S. R. Dennison, H. M. Tawfeek, S. Somavarapu, G. A. Hutcheon and I. Y. Saleem, *Pharm. Res.*, 2015, **32**, 1341–1353.
- 62 S. Vakilian, S. Mashayekhan, I. Shabani, M. Khorashadizadeh, A. Fallah and M. Soleimani, *Int. J. Biol. Macromol.*, 2015, **75**, 248–257.
- 63 S. Rawat, C. Raman Suri and D. K. Sahoo, *Biochem. Biophys. Res. Commun.*, 2010, **392**, 561–566.
- 64 R. E. Wang, L. Tian and Y.-H. Chang, *J. Pharm. Biomed. Anal.*, 2012, **63**, 165–169.
- 65 S. Choi, E. Y. Choi, D. J. Kim, J. H. Kim, T. S. Kim and S. W. Oh, *Clin. Chim. Acta*, 2004, **339**, 147–156.
- 66 K. Gellynck, V. Kodeck, E. Van De Walle, K. Kersemans, F. De Vos, H. Declercq, P. Dubruel, L. Vlaminck and M. Cornelissen, *Exp. Biol. Med.*, 2015, **240**, 446–457.
- 67 R. Zhang, X. Qin, F. Kong, P. Chen and G. Pan, *Drug Delivery*, 2019, **26**, 328–342.
- 68 R. Solaro, F. Chiellini and A. Battisti, *Materials*, 2010, **3**, 1928–1980.
- 69 A. Miklavžin, M. Cegnar, J. Kerč and J. Kristl, *Acta Pharm.*, 2018, **68**, 275–293.
- 70 A. S. Joshi, A. Gahane and A. K. Thakur, *RSC Adv.*, 2016, **6**, 108545–108557.
- 71 C. Neyen, A. Plüddemann, P. Roversi, B. Thomas, L. Cai, D. R. van der Westhuyzen, R. B. Sim and S. Gordon, *Biochemistry*, 2009, **48**, 11858–11871.



Chapter 11

Microfluidic Manufacturing of Multitargeted PLGA/PEG Nanoparticles for Delivery of Taxane Chemotherapeutics

Cláudia Martins and Bruno Sarmento

Abstract

Taxane chemotherapeutics have played a key role in the treatment of various types of cancer throughout the past years. However, the drawbacks inherent to the pharmaceutical formulation of taxanes are still a reality and mainly due to the low aqueous solubility of these medicines, as well as to the nontargeted therapy and consequent side effects. Nanoparticles (NPs) of poly(lactic-co-glycolic acid) (PLGA) and polyethylene glycol (PEG) have sparked broad interest in this field and demonstrated capacity of improving taxanes' formulation. If, in one hand, the PLGA core of these NPs is able to solubilize drugs, on the other hand, the PEG shell promotes immune escape and presents chemical end groups for the attachment of targeting ligands. Advances in the design of these nanosystems resulted in the development of multitargeted PLGA/PEG NPs achieved by dual-ligand functionalization. The multitargeting offers a promising alternative to the delivery of taxanes across successive cell types or compartments and to the synergetic exploitation of more than one transporter on the cell surface. Besides the upgrade in the design of multitargeted PLGA/PEG NPs, their manufacturing has also evolved from bulk assembly to continuous-flow, high-throughput technologies such as microfluidics. This technology relies on microchannel platforms described to enable the production of large-scale batches of NPs in a better time-saving manner, with higher drug loading, reproducibility, and lower polydispersity. Herein, a detailed microfluidic method for the preparation of multitargeted, taxane-loaded PLGA/PEG NPs is described. Focus is given to the setting up of the microfluidic system and conditions required to manufacture these NPs by using polymers of PLGA and PEG previously elsewhere functionalized with two generic targeting ligands.

Key words Cancer, Chemotherapy, Microfluidics, Multitargeting, PLGA/PEG nanoparticles, Targeting ligands, Taxanes

1 Introduction

Taxanes are chemotherapeutic drugs commonly prescribed in oncology for the treatment of various cancers. Some examples of these drugs include paclitaxel, docetaxel, and cabazitaxel, commercially known as Taxol[®], Taxotere[®], and Jevtana[®], respectively [1]. The mechanism of action of taxanes is initiated upon their binding to tubulin, which becomes stably polymerized and

interrupts the regular cell division, ultimately leading to cell cycle arrest and apoptosis [2].

Despite taxanes have been widely used in anticancer therapies either as single agents or in combination with other drugs, their formulation still poses a great deal of challenge. The structure of taxanes presents lipophilic substituents that render them very poor aqueous solubility and stability [3]. Besides the high concentration of excipients included in commercial taxane formulations to circumvent these drawbacks, current nontargeted intravenous regimens inevitably result in serious side effects [4]. These side effects are dose-limiting and are reported to include anemia, neutropenia, and neuropathy, among others [5]. To bypass these pitfalls, nanoparticulate systems have been engineered to allow the formulation and delivery of taxane chemotherapeutics [6–8]. Particularly in this field, the biocompatible, biodegradable, and Food and Drug Administration (FDA)-approved poly(lactic-co-glycolic acid) (PLGA) polymer has been widely explored [9–11]. Nanoparticles (NPs) of PLGA are described to enable the solubilization of taxanes within their hydrophobic core and further sustained release of these drugs over time [12]. This has potential to increase the maximum tolerated dose and, consequently, to make possible the administration of higher therapeutic doses [13]. The hydrophilic polyethylene glycol (PEG) polymer has been strategically used together with PLGA, forming PLGA/PEG NPs, thus providing the development of nanosystems with an outer shell of PEG around the PLGA core. This avoids the opsonin adsorption commonly associated with hydrophobic surfaces such as those derived from PLGA, consequently increasing the half-life of circulating PLGA/PEG NPs [14].

The research efforts toward improved nanoparticulate systems for drug delivery have changed the paradigm of PLGA/PEG NPs manufacturing and design over the past years. Continuous-flow manufacturing techniques such as microfluidics have emerged as a hot area and are gaining advantage over conventional methods of assembly of these NPs [15]. The controlled microenvironment of microfluidic chips enables the production of monodisperse batches and smaller NPs with remarkably higher drug-loading capacity [16, 17]. Microfluidics also offer the possibility of scale-up of the manufacturing of drug-loaded NPs in a highly reproducible and time-saving manner, thus promising an accelerated industrial and clinical translation of these nanomedicines [18]. On the other hand, surface tailoring has demonstrated to be key to modulate the responsiveness of PLGA/PEG NPs to certain biologic stimuli [19]. Different studies have attempted the functionalization of NPs with targeting ligands that recognize specific cell transporters usually overexpressed or preferentially present at the site of interest [20–22]. These ligands cover a wide spectrum of activity and may consist but are not restricted to antibodies, peptides, aptamers, and

small molecules [23]. The main advantages of ligand functionalization include a higher accumulation and/or probability of intracellular transport of drug-loaded NPs at the target site, therefore reducing side effects [24]. This functionalization process mostly relies on chemical routes by coupling end groups of the polymer matrix of NPs with reactive moieties of the ligands. The PEG fraction of PLGA/PEG NPs have been widely exploited for the purpose of this chemical conjugation since its versatile structure provides an anchor for a plethora of different end groups such as carboxylic acid, amine, maleimide, and hydrazide [25]. Several chemical mechanisms can be afterward selected to couple the PEG end group with the reactive moiety of the ligand, including the carbodiimide cross-linking and click chemistry strategies [26]. Recent works have focus on the development of therapeutic NPs simultaneously functionalized with two ligands to achieve a biologic multitargeting [27, 28]. These complex multitargeted NPs have been mostly designed to circumvent the unmet needs of a (1) successive targeting of two different cell types such as in the case of intravenous treatments of brain cancer, in which a first ligand is necessary to promote NPs' transport across the blood–brain barrier and a second one to provide accumulation at brain tumor cells [29]; (2) successive targeting of two different cell compartments such as nucleus delivery, in which a first ligand promotes cell endocytosis and a second one is responsible for further migration of the NPs to the nucleus [30]; and (3) simultaneous targeting of the same cell type through exploitation of more than one transporter such as in cancer cells overexpressing multiple receptors that may provide a synergetic intracellular movement of NPs [31]. The optimal density and ratio of the two ligands, as well as the length and rigidity of the PEG polymer, are among the variables that deserve careful consideration [32]. Since these multitargeted PLGA/PEG nanosystems should undergo multistage chemical procedures, it may be preferable to conjugate the ligands with the polymer matrix prior to the manufacturing of the NPs. Despite avoiding premature drug release, this allows a more accurate control over the reaction yield and physiochemical characterization of the final polymeric conjugate [33]. Therefore, one of the two reactive ends of the PEG fraction is firstly conjugated with the PLGA core polymer, whereas the other one is further used to attach each of the targeting ligands.

Gathering all this information, the protocol herein described proposes a microfluidic method for the manufacturing of multitargeted PLGA/PEG NPs encapsulating taxane chemotherapeutics. The multitargeting is achieved by including two different generic polymers, elsewhere functionalized prior to the manufacturing of the NPs. These generic polymers result from the conjugation of PLGA with two different PEG models (PEG1 and PEG2) and further conjugation of the last ones with two

different ligand models (Ligand1 and Ligand2). For simplicity from here onward, these functionalized polymers will be referred to as PLGA-PEG1-Ligand1 and PLGA-PEG2-Ligand2.

2 Materials

2.1 Reagents and Consumables

- PLGA (PURASORB[®] PDLG 5004A) from Corbion (Amsterdam, The Netherlands).
- Acetone and acetonitrile from Thermo Fisher Scientific (Waltham, MA, USA).
- Tween[®]-80 from Sigma-Aldrich (St. Louis, MO, USA).
- Syringe filter 0.22 μm from Membrane Solutions (Dallas, TX, USA).
- Disposable syringes from Henke-Sass Wolf GmbH (Tuttlingen, Germany).
- Female to female Luer lock, polytetrafluoroethylene tubing 1/16" $\times$ 0.5 mm, end fittings, ferrules, and T-connector from Dolomite Microfluidics (Royston, UK).

2.2 Equipments

- Glass scintillation vials from Sigma-Aldrich (St. Louis, MO, USA).
- Syringe pumps from Braintree Scientific, Inc. (Braintree, MA, USA).
- Quartz droplet X-junction microfluidic chip (190 μm etch depth), H-interface holder, linear connector 4-way, and seals for linear connector from Dolomite Microfluidics (Royston, UK).
- Microscope equipped with a digital camera from Leica (ICC50 HD, Zurich, Switzerland).
- Magnetic stirrer from IKA-Werke (GmbH & Co, Staufen, Germany).

3 Methods

3.1 Phase Preparation

3.1.1 Organic Phase

1. Dissolve the plain PLGA, PLGA-PEG1-Ligand1, and PLGA-PEG2-Ligand2 in acetone at a total polymer concentration of 1% (w/v) in a scintillation vial (*see Note 1*). This total polymer concentration includes 80%, 10%, and 10% of plain PLGA, PLGA-PEG1-Ligand1, and PLGA-PEG2-Ligand2, respectively (*see Note 2*).
2. Add the taxane drug corresponding to a theoretical drug loading of 15% to the previous solution (*see Note 3*). Skip this step

for the obtention of multitargeted, unloaded PLGA/PEG NPs.

3. Let the polymers and the drug dissolve overnight, assuring that the top of the scintillation vial is tightly sealed in order to prevent acetone evaporation (*see Note 4*). A clear and nonfilamentous solution should be visible after overnight dissolution (*see Note 5*).

3.1.2 Aqueous Phase

1. Prepare an aqueous solution of 2% (w/v) Tween[®]-80, pH = 7.4 (*see Note 6*).
2. Filter the 2% (w/v) Tween[®]-80 solution through a 0.22 μ m syringe filter in order to eliminate possible impurities that can result in the clogging of the microfluidic chip.

3.2 Microfluidic System Setup

1. Fit a female to female Luer lock on the syringes for the infusion of the organic and aqueous phases, and mount them on the respective syringe pump. The organic and aqueous phases correspond to the single inner and two outer stream inlets of the microfluidic chip, respectively (as shown in Fig. 1).
2. Use a set of end fitting and ferrule to connect the tubing with the female to female Luer lock adaptor of the syringes. This joint should be reasonably tight but without giving rise to a high-pressure spot. The length of the tubing should be the shortest possible so that the probability of flow instability in result of vibration and phase dead volume can be lowered.
3. Attach a T-connector to the tubing of the aqueous phase in order to diverge the flow and simultaneously supply the two outer stream inlets of the microfluidic chip (*see Note 7*). The tubings of the T-connector outlets should be exactly of the same length to avoid flow discrepancy in between them.
4. Place the microfluidic chip on the H-interface holder, and fix the inlet and outlet tubings on each side of the chip by using both the linear connector 4-way and its seal.
5. Mount the chip area of the microfluidic setup on a microscope equipped with a digital camera.
6. Assure that all components of the microfluidic setup are on the same horizontal plane, hence avoiding fluctuations of the flow along the *zz* axis.

3.3 Microfluidic Run

1. Flush the microfluidic system with acetonitrile solvent to get rid of any impurities or blockages (*see Note 8*).
2. Fill the syringes with the organic and aqueous phases, making sure that all air bubbles are driven out and thus will not disturb the flow (*see Note 9*).

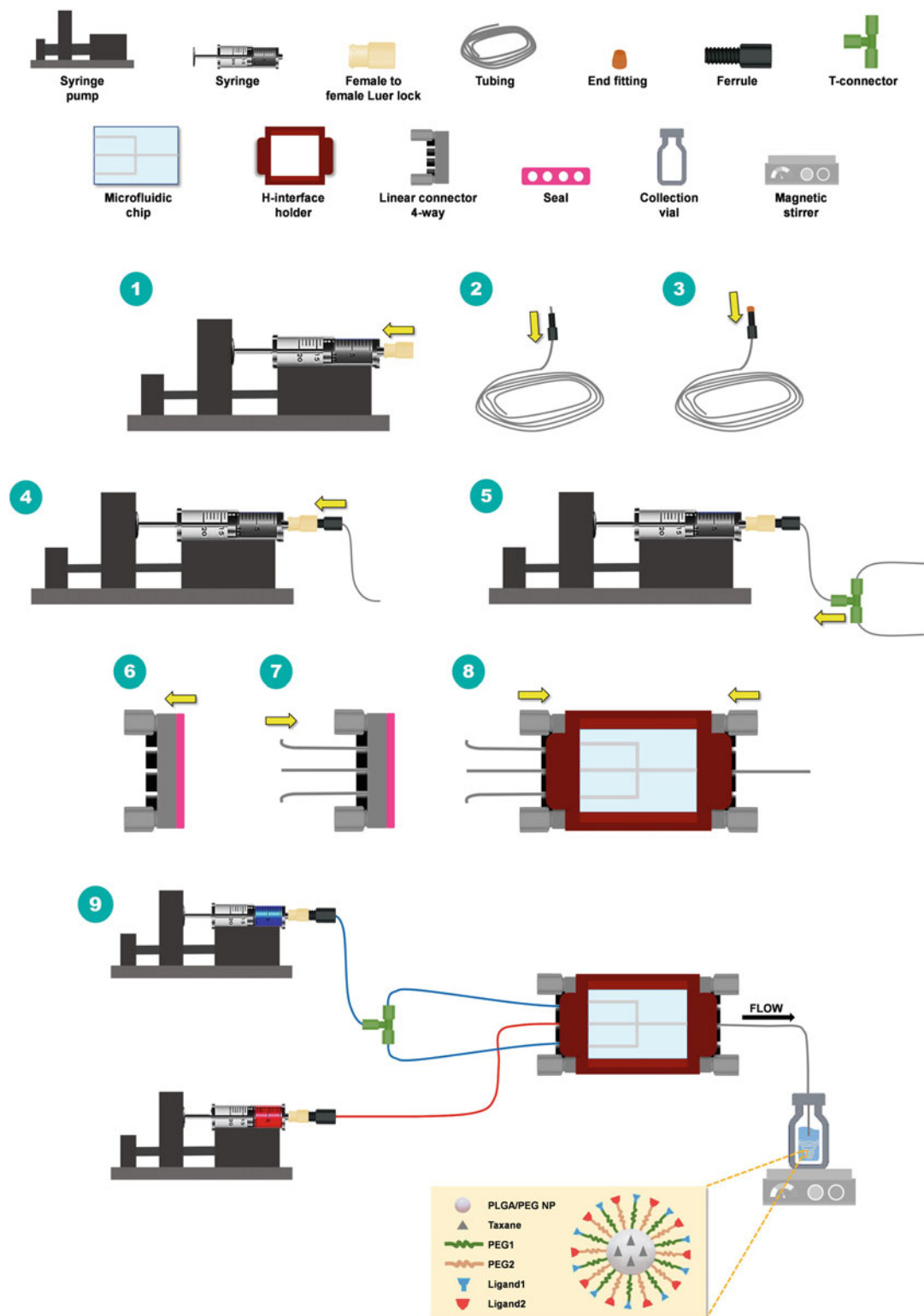


Fig. 1 Platform components and setup procedure of the microfluidic system. (1) Fit a female to female Luer lock on each syringe; attach a set of (2) ferrule and (3) end fitting to the tubing and (4) connect it with the

3. Set the syringe pumps to supply a total flow rate of the organic and aqueous phases of 500 and 1500 $\mu\text{L}/\text{min}$, respectively (*see Note 10*).
4. Turn on the aqueous flow and only after the organic one, therefore avoiding the clogging of the microfluidic system. Assure that the bench in which the microfluidic setup is placed is free of vibrational artifacts that may disturb the flow conditions (*see Note 11*).
5. Start the collection of NPs once the flow stabilizes.
6. Let the NPs suspend under mechanical stirring for 30 min at 200 rpm and room temperature to evaporate the acetone solvent.
7. Wash the microfluidic setup by flushing the microfluidic system with acetonitrile solvent to remove any blockages of polymer or drug (*see Note 8*).

4 Notes

1. It is recommended to evaluate the integrity of the ligands in the functionalized polymers after exposure to the organic solvent, especially in the case of proteins. Certain organic solvents are able to disrupt the hydration shell that typically surrounds protein molecules, hence compromising the balance of interactions responsible for maintaining their native conformation [34]. Some examples of techniques used to assess the integrity of a protein ligand after exposure to a specific solvent may include attenuated transmission reflectance Fourier transform infrared spectroscopy (ATR-FTIR) for the evaluation of alterations in the secondary structure, as well as circular dichroism (CD) and fluorescence spectroscopy for the detection of alterations in the secondary and tertiary structures, respectively [35].
2. The percentage of 80%, 10%, and 10% of plain PLGA, PLGA-PEG1-Ligand1, and PLGA-PEG2-Ligand2, respectively, in relation to the total polymer mass may vary according to the purpose of the formulation. It may be necessary to increase the percentage of the functionalized polymers in order to achieve a higher ligand density on the surface of NPs. Ligand density should be optimized to find a proper balance between high

Fig. 1 (continued) female to female Luer lock adaptor of the syringes; (5) attach a T-connector to the tubing of the aqueous phase; (6) couple each linear connector 4-way with its seal and (7) connect it with the tubing; (8) place the microfluidic chip on the H-interface holder, and fix the inlet and outlet tubings by screwing the linear connectors 4-way. (9) Fill the syringes with the organic (red) and aqueous (blue) phases, and the system is ready to be turned on

avidity to associate with the target and incite mechanisms of intracellular transport and low avidity to avoid irreversible ligand-target linkage [36].

3. The theoretical taxane loading of 15% should be adjusted a priori to meet the optimal value of drug association efficiency and practical loading. These two parameters are specific for each drug and are mainly governed by the partition coefficient of the taxane between the organic and aqueous phases selected for the manufacturing of the NPs [37, 38].
4. Polymer and drug solubilization may be accelerated through sonication or heating. However, preliminary studies are needed to investigate whether these processes are able to damage the structure of the ligands or the taxane.
5. The incomplete solubilization of the polymers and the drug in the organic solvent may lead to the clogging of the microfluidic setup or even to the manufacturing of highly polydisperse populations of NPs.
6. Careful case-by-case consideration is required to assess the impact of the chosen surfactant and its concentration in the recognition of the target by ligand-functionalized PLGA/PEG NPs. Recent data elucidates the negative role of the surfactant that remains on the surface of NPs after their manufacturing and purification, including direct effects on the ligand such as alterations in its structure, orientation, or molecular affinity, as well as a reduced availability of the ligand to bind the target due to steric hindrance phenomena [39]. Other surfactants such as poloxamers and poly(vinyl alcohol) (PVA) can be employed as Tween[®]-80 substitutes in the microfluidic method herein described.
7. Alternatively to the T-connector, two separate syringe pumps may be used to infuse each of the two aqueous stream inlets.
8. Alternative processes such as sonication in acetonitrile, flowing of compressed air, or washing with alkaline concentrates can be used to clean precipitate blockages of difficult removal.
9. The presence of air bubbles is one of the most challenging issues in setups with micro-sized channels such as microfluidic chips. Air bubbles are capable of dilating and contracting which turns the flow unstable and results in the manufacturing of highly polydisperse populations of NPs. Moreover, air bubbles present an obstacle for the fixed flow rate that is applied by the syringe pumps, ultimately leading to a significant increase in the pressure within the microchannels and possible disruption of the setup.
10. One of the main advantages of microfluidic setups in comparison with the bulk assembly of NPs is the laminar pattern of the

flow which cannot be attained at the macroscale and is mainly controlled by the chosen flow rates [40]. This pattern is governed by viscous forces and characterized by multistreamlines that always flow parallelly to the fluid direction. In opposition to the laminar pattern, the turbulent one is characterized by chaotic flow vortexes with nonparallel-shaped streamlines [17]. Turbulent flow patterns generate a gradient of NP concentration along the microchannels, hence originating highly polydisperse batches of particles and lowering the production rate [41]. Fluid mechanics may be used as a tool to predict the type of flow pattern that is possible to encounter within a microfluidic setup according to the selected flow rates. One of the most important parameters to be investigated in this field is the Reynolds number, which can be calculated according to Eq. 1 [42, 43]:

$$\text{Reynolds number} = \frac{\rho v L}{\mu} \quad (1)$$

where ρ , v , and μ represent the density (kg m^{-3}), velocity (m s^{-1}), and dynamic viscosity (Pa s) of the fluid, respectively, and L is the microchannel diameter (m). A Reynolds number lower than 1800 is usually associated with a laminar flow pattern, whereas higher than 2300 is related to a turbulent one [44]. The values of Reynolds number between 1800 and 2300 belong to the interval of transition from laminar to turbulent flow patterns. Thus, flow rates are key players in the tunability of parameters of fluid mechanics inside microfluidic setups and should be optimized to obtain populations of NPs with the desired features.

11. The immediate outcome of vibrational artifacts that disturb the flow conditions is the onset of a turbulent flow pattern and a consequent increase in the value of polydispersity of NPs.

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References

- Perez EA (2009) Microtubule inhibitors: differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. *Mol Cancer Ther* 8(8):2086–2095. <https://doi.org/10.1158/1535-7163.Mct-09-0366>
- Gligorov J, Lotz JP (2004) Preclinical pharmacology of the taxanes: implications of the differences. *Oncologist* 9(Suppl 2):3–8
- Elhissi A, Mahmood R, Parveen I, Vali A, Ahmed W, Jackson MJ (2018) Taxane formulations: from plant to clinic. In: Jackson MJ, Ahmed W (eds) *Micro and nanomanufacturing volume II*. Springer, Cham, pp 23–33. https://doi.org/10.1007/978-3-319-67132-1_2
- Yared JA, Tkaczuk KHR (2012) Update on taxane development: new analogs and new formulations. *Drug Des Devel Ther* 6:371–384. <https://doi.org/10.2147/DDDT.S28997>
- Harrison MR, Holen KD, Liu G (2009) Beyond taxanes: a review of novel agents that target mitotic tubulin and microtubules, kinases, and kinesins. *Clin Adv Hematol Oncol* 7(1):54–64
- Gaucher G, Marchessault RH, Leroux J-C (2010) Polyester-based micelles and nanoparticles for the parenteral delivery of taxanes. *J Control Release* 143(1):2–12. <https://doi.org/10.1016/j.jconrel.2009.11.012>
- Feng L, Mumper RJ (2013) A critical review of lipid-based nanoparticles for taxane delivery. *Cancer Lett* 334(2):157–175. <https://doi.org/10.1016/j.canlet.2012.07.006>
- Reul R, Renette T, Bege N, Kissel T (2011) Nanoparticles for paclitaxel delivery: a comparative study of different types of dendritic polyesters and their degradation behavior. *Int J Pharm* 407(1):190–196. <https://doi.org/10.1016/j.ijpharm.2011.01.028>
- Enlow EM, Luft JC, Napier ME, DeSimone JM (2011) Potent engineered PLGA nanoparticles by virtue of exceptionally high chemotherapeutic loadings. *Nano Lett* 11(2):808–813. <https://doi.org/10.1021/nl104117p>
- Rezvantalab S, Drude NI, Moraveji MK, Güvener N, Koons EK, Shi Y, Lammers T, Kiessling F (2018) PLGA-based nanoparticles in cancer treatment. *Front Pharmacol* 9:1260–1260. <https://doi.org/10.3389/fphar.2018.01260>
- Rafiei P, Haddadi A (2017) Pharmacokinetic consequences of PLGA nanoparticles in docetaxel drug delivery. *Pharm Nanotechnol* 5(1):3–23. <https://doi.org/10.2174/2211738505666161230110108>
- Khalil NM, TCFd N, Casa DM, Dalmolin LF, ACd M, Hoss I, Romano MA, Mainardes RM (2013) Pharmacokinetics of curcumin-loaded PLGA and PLGA-PEG blend nanoparticles after oral administration in rats. *Colloids Surf B Biointerfaces* 101:353–360. <https://doi.org/10.1016/j.colsurfb.2012.06.024>
- Kroon J, Kooijman S, Cho N-J, Storm G, van der Pluijm G (2016) Improving taxane-based chemotherapy in castration-resistant prostate cancer. *Trends Pharmacol Sci* 37(6):451–462. <https://doi.org/10.1016/j.tips.2016.03.003>
- Song Z, Feng R, Sun M, Guo C, Gao Y, Li L, Zhai G (2011) Curcumin-loaded PLGA-PEG-PLGA triblock copolymeric micelles: preparation, pharmacokinetics and distribution in vivo. *J Colloid Interface Sci* 354(1):116–123. <https://doi.org/10.1016/j.jcis.2010.10.024>
- Garg S, Heuck G, Ip S, Ramsay E (2016) Microfluidics: a transformational tool for nanomedicine development and production. *J Drug Target* 24(9):821–835. <https://doi.org/10.1080/1061186x.2016.1198354>
- Martins C, Araújo F, Gomes MJ, Fernandes C, Nunes R, Li W, Santos HA, Borges F, Sarmento B (2018) Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy. *Eur J Pharm Biopharm*. <https://doi.org/10.1016/j.ejpb.2018.01.014>
- Martins JP, Torrieri G, Santos HA (2018) The importance of microfluidics for the preparation of nanoparticles as advanced drug delivery systems. *Expert Opin Drug Deliv* 15(5):469–479. <https://doi.org/10.1080/17425247.2018.1446936>
- Gdowski A, Johnson K, Shah S, Gryczynski I, Vishwanatha J, Ranjan A (2018) Optimization and scale up of microfluidic nanolipomer production method for preclinical and potential clinical trials. *J Nanobiotechnol* 16(1):12–12. <https://doi.org/10.1186/s12951-018-0339-0>

19. Elsabahy M, Wooley KL (2012) Design of polymeric nanoparticles for biomedical delivery applications. *Chem Soc Rev* 41 (7):2545–2561. <https://doi.org/10.1039/c2cs15327k>
20. Martins C, Sousa F, Araújo F, Sarmento B (2017) Functionalizing PLGA and PLGA derivatives for drug delivery and tissue regeneration applications. *Adv Healthc Mater* 7:1701035. <https://doi.org/10.1002/adhm.201701035>
21. Mout R, Moyano DF, Rana S, Rotello VM (2012) Surface functionalization of nanoparticles for nanomedicine. *Chem Soc Rev* 41 (7):2539–2544. <https://doi.org/10.1039/c2cs15294k>
22. Feng L, Zhu C, Yuan H, Liu L, Lv F, Wang S (2013) Conjugated polymer nanoparticles: preparation, properties, functionalization and biological applications. *Chem Soc Rev* 42 (16):6620–6633. <https://doi.org/10.1039/C3CS60036J>
23. Bastakoti BP, Liu Z (2017) Multifunctional polymeric micelles as therapeutic nanostructures: targeting, imaging, and triggered release. In: Ficaí A, Grumezescu AM (eds) *Nanostructures for cancer therapy*. Elsevier, Amsterdam, pp 261–283. <https://doi.org/10.1016/B978-0-323-46144-3.00010-6>
24. Shi J, Xiao Z, Kamaly N, Farokhzad OC (2011) Self-assembled targeted nanoparticles: evolution of technologies and bench to bedside translation. *Acc Chem Res* 44 (10):1123–1134. <https://doi.org/10.1021/ar200054n>
25. Pfister D, Morbidelli M (2014) Process for protein PEGylation. *J Control Release* 180:134–149. <https://doi.org/10.1016/j.jconrel.2014.02.002>
26. Chen Y, Xianyu Y, Wu J, Yin B, Jiang X (2016) Click chemistry-mediated nanosensors for biochemical assays. *Theranostics* 6(7):969–985. <https://doi.org/10.7150/thno.14856>
27. Q-s X, H-m D, Ma Y-q (2017) Can dual-ligand targeting enhance cellular uptake of nanoparticles? *Nanoscale* 9(26):8982–8989. <https://doi.org/10.1039/C7NR01020F>
28. Zhu Y, Feijen J, Zhong Z (2018) Dual-targeted nanomedicines for enhanced tumor treatment. *Nano Today* 18:65–85. <https://doi.org/10.1016/j.nantod.2017.12.007>
29. Gao H (2017) Perspectives on dual targeting delivery systems for brain tumors. *J Neuroimmune Pharmacol* 12(1):6–16. <https://doi.org/10.1007/s11481-016-9687-4>
30. Xiong L, Du X, Kleitz F, Qiao SZ (2015) Cancer-cell-specific nuclear-targeted drug delivery by dual-ligand-modified mesoporous silica nanoparticles. *Small* 11(44):5919–5926. <https://doi.org/10.1002/smll.201501056>
31. Liu Y, Hui Y, Ran R, Yang G-Z, Wibowo D, Wang H-F, Middelberg APJ, Zhao C-X (2018) Synergetic combinations of dual-targeting ligands for enhanced in vitro and in vivo tumor targeting. *Adv Healthc Mater* 7 (15):1800106. <https://doi.org/10.1002/adhm.201800106>
32. Elias DR, Poloukhine A, Popik V, Tsourkas A (2013) Effect of ligand density, receptor density, and nanoparticle size on cell targeting. *Nanomedicine* 9(2):194–201. <https://doi.org/10.1016/j.nano.2012.05.015>
33. Nicolas J, Mura S, Brambilla D, Mackiewicz N, Couvreur P (2013) Design, functionalization strategies and biomedical applications of targeted biodegradable/biocompatible polymer-based nanocarriers for drug delivery. *Chem Soc Rev* 42(3):1147–1235. <https://doi.org/10.1039/c2cs35265f>
34. Khmelnitsky YL, Belova AB, Levashov AV, Mozhaev VV (1991) Relationship between surface hydrophilicity of a protein and its stability against denaturation by organic solvents. *FEBS Lett* 284(2):267–269
35. Sousa F, Fonte P, Cruz A, Kennedy PJ, Pinto IM, Sarmento B (2018) Polyester-based nanoparticles for the encapsulation of monoclonal antibodies. In: Picanço-Castro V, Swiech K (eds) *Recombinant glycoprotein production*, vol 1674. Springer, New York, NY, pp 239–253. https://doi.org/10.1007/978-1-4939-7312-5_20
36. Clark AJ, Davis ME (2015) Increased brain uptake of targeted nanoparticles by adding an acid-cleavable linkage between transferrin and the nanoparticle core. *Proc Natl Acad Sci U S A* 112(40):12486–12491. <https://doi.org/10.1073/pnas.1517048112>
37. Matsumoto A, Matsukawa Y, Suzuki T, Yoshino H (2005) Drug release characteristics of multi-reservoir type microspheres with poly (dl-lactide-co-glycolide) and poly(dl-lactide). *J Control Release* 106(1):172–180. <https://doi.org/10.1016/j.jconrel.2005.03.026>
38. Dragan U, Magdalená S (2009) Poly(lactide-co-glycolide)-based micro and nanoparticles for the controlled drug delivery of vitamins. *Curr Nanosci* 5(1):1–14. <https://doi.org/10.2174/157341309787314566>
39. Kennedy PJ, Ferreira I, Ferreira D, Nestor M, Oliveira C, Granja PL, Sarmento B (2018) Impact of surfactants on the target recognition of Fab-conjugated PLGA nanoparticles. *Eur J Pharm Biopharm* 127:366–370. <https://doi.org/10.1016/j.ejpb.2018.03.005>

40. Chiesa E, Dorati R, Pisani S, Conti B, Bergamini G, Modena T, Genta I (2018) The microfluidic technique and the manufacturing of polysaccharide nanoparticles. *Pharmaceutics* 10(4). <https://doi.org/10.3390/pharmaceutics10040267>
41. Lin J, Pan X, Yin Z, Ku X (2016) Solution of general dynamic equation for nanoparticles in turbulent flow considering fluctuating coagulation. *Appl Math Mech* 37(10):1275–1288. <https://doi.org/10.1007/s10483-016-2131-9>
42. Liu D, Zhang H, Fontana F, Hirvonen J, Santos HA (2017) Microfluidic-assisted fabrication of carriers for controlled drug delivery. *Lab Chip* 17(11):1856–1883. <https://doi.org/10.1039/c7lc00242d>
43. Stroock AD, Dertinger SK, Ajdari A, Mezic I, Stone HA, Whitesides GM (2002) Chaotic mixer for microchannels. *Science* 295(5555):647–651. <https://doi.org/10.1126/science.1066238>
44. Sharp KV, Adrian RJ (2004) Transition from laminar to turbulent flow in liquid filled microtubes. *Exp Fluids* 36(5):741–747. <https://doi.org/10.1007/s00348-003-0753-3>



Glioblastoma immuno-endothelial multicellular microtissue as a 3D in vitro evaluation tool of anti-cancer nano-therapeutics

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