

Faculdade de Engenharia da Universidade do Porto



**3D-printing of magnetic particles containing
hydrogels for smart drug release combined with
cancer phototherapy**

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Resumo

O cancro de pele não melanoma é o cancro mais comum no mundo, com previsões de um aumento contínuo na sua incidência até 2044. A descoberta de tratamentos alternativos é necessária, uma vez que as terapias atuais, incluindo cirurgia, quimioterapia e radioterapia, ainda mostram baixa eficácia e efeitos secundários significativos. A fototerapia é um tratamento baseado na aplicação de luz, sendo uma abordagem promissora para o tratamento de várias doenças, incluindo o cancro de pele. A terapia fototérmica (PTT) é uma técnica minimamente invasiva na qual são utilizados agentes fototérmicos que permitem a conversão da luz infravermelha próxima (NIR) em calor. Uma vez que os agentes fototérmicos se acumulam no tecido canceroso, estes permitem o aumento da temperatura apenas na área-alvo durante a irradiação, possibilitando a seletividade do tratamento. Os nanomateriais de carbono (CNMs) apresentam excelentes propriedades mecânicas e elétricas, alta condutividade térmica, área de superfície elevada e absorção de NIR, o que os torna excelentes potenciais agentes fototerapêuticos. Os materiais à base de grafeno (GBM) destacam-se dos restantes CNMs devido à grande área de superfície, versatilidade na funcionalização química, estabilidade em água e excelentes propriedades ópticas, bem como boa biocompatibilidade.

Neste trabalho, o óxido de nanografeno (GOn) foi produzido utilizando o método modificado de Hummers e foto-reduzido com luz ultravioleta (UV-C) para produzir o óxido de nanografeno parcialmente reduzido (p-rGOn), que foi então magnetizado através de um processo de magnetização hidrotérmica utilizando cloreto de ferro para obter óxido de nanografeno reduzido funcionalizado com partículas de óxido de ferro (@rGOn). A microscopia eletrónica de transmissão (TEM) mostra que o GOn é constituído por folhas individuais bem exfoliadas com um comprimento médio de 147.9 ± 82.2 nm, enquanto o @rGOn exibe um comprimento médio de 298.4 ± 67.7 nm. A bem-sucedida funcionalização com nanopartículas de óxido de ferro foi demonstrada, com essas partículas apresentando um tamanho médio de 12.9 ± 9.3 nm na superfície do material. A magnetização bem-sucedida foi confirmada, uma vez que todas as partículas se dirigiram para um íman colocado fora do frasco. A espectroscopia de infravermelho com transformada de Fourier (FTIR) revela que vários grupos funcionais contendo oxigénio estão presentes na superfície do GOn, tais como carbonilos, carboxilos, hidroxilos, éteres e epóxidos. No entanto, após o processo de redução e magnetização, essas funcionalidades quase desaparecem, confirmando o sucesso do procedimento. A análise termogravimétrica (TGA) mostra que o GOn perde 31.89% da sua massa até 300 °C, correspondente

aos grupos funcionais de oxigênio lábeis. Por outro lado, o @rGOn tem apenas uma pequena perda de peso de 4% entre 500-640 °C, revelando a sua grande estabilidade térmica e reduzido número de grupos de oxigênio. Outra evidência da eficácia do método de redução utilizado é o aumento em 7 vezes da absorção no infravermelho próximo (NIR, 750-850 nm) para o @rGOn, comparando com o GOn.

O GOn foi incorporado em hidrogéis de pectina em diferentes concentrações (0.625, 1.25, 2.5 wt%). O objetivo inicial era desenvolver hidrogéis para a impressão 3D de *scaffolds* a serem aplicados após cirurgia de tratamento de cancro de pele, para terapia fototérmica simultânea (destruindo quaisquer células cancerígenas remanescentes e prevenindo recorrências) e regeneração de tecidos (para melhorar os resultados estéticos). As propriedades magnéticas do @rGOn poderiam eventualmente ser usadas para mediar a libertação de substâncias/medicamentos ativos para prevenir recorrências ou infecções, mas também para acelerar a regeneração. A libertação poderia ser controlada externamente utilizando um campo/equipamento magnético adequado. Além disso, a hipertermia magnética também poderia ser uma possibilidade devido às propriedades do @rGO. No entanto, devido a limitações de tempo, o trabalho centrou-se na produção de um hidrogel a ser aplicado na falha pós-cirúrgica para realizar terapia fototérmica e promover a regeneração de tecidos. Portanto, 50 °C foi definida como a temperatura que deveria ser atingida para demonstrar que os hidrogéis teriam potencial para serem usados em terapia fototérmica. Além disso, estes deveriam manter a sua integridade estrutural após serem incubados durante 24 horas a 37 °C em meio de cultura celular (DMEM), e as suas propriedades magnéticas deveriam permanecer intactas.

A eficiência dos hidrogéis de pectina/GBM na conversão de luz NIR em calor foi avaliada colocando-os num forno pré-aquecido a 37 °C (temperatura corporal) e, posteriormente, irradiando-os com os nossos sistemas LED NIR personalizados (810 nm, 1000 mW/cm²). Os hidrogéis de pectina apenas aumentaram a sua temperatura até um máximo de 42 °C. Para os hidrogéis contendo grafeno, as curvas de aquecimento foram muito semelhantes, e todos os hidrogéis conseguiram aumentar a temperatura do DMEM no poço até 50 °C. Além disso, os hidrogéis de pectina/@rGOn (1.25 e 2.5 wt.%) mantiveram a sua eficiência fototérmica após 5 dias e 3 ciclos de irradiação NIR e aquecimento até 50 °C, bem como a sua integridade estrutural e propriedades magnéticas. Para avaliar o potencial dos hidrogéis para serem usados na impressão 3D de uma estrutura que poderia corresponder às falhas causadas pela cirurgia de cancro, a sua capacidade de impressão foi estudada. Os parâmetros de impressão ainda necessitam otimização adicional, mas foi claro que as tintas de hidrogel têm potencial para impressão 3D, uma vez que as estruturas obtidas apresentam uma aparência uniforme e propriedades mecânicas e estruturais suficientes para serem manipuladas após a impressão.

A citotoxicidade dos hidrogéis foi avaliada através de um ensaio de exposição a extratos, analisando se partículas de @rGOn seriam liberadas das amostras de hidrogel e causariam alterações na atividade metabólica das células. No primeiro dia, o valor mais baixo obtido foi igual ao controlo, indicando que o hidrogel não apresenta citotoxicidade para as células HFF-1. No sétimo dia, para concentrações de 0%, 1.25% e 2.5% de @rGOn, os valores de atividade metabólica obtidos foram de

98%, 89% e 84%, respetivamente. Apesar de uma diminuição significativa na atividade metabólica para todas as concentrações de @rGOn, os valores obtidos estão acima do limite estabelecido pela norma ISO 10993-5:2009(E).

Para simular a aplicação final dessas estruturas, ou seja, o contacto com células normais da pele (HFF-1) e células cancerígenas (A-431), e mediar a destruição das células cancerígenas, mantendo as células normais intactas e eventualmente promovendo a regeneração tecidual, foi desenvolvida uma nova estrutura para permitir a acomodação de uma matriz celular 3D durante os ensaios de contacto direto. Os ensaios de resazurina para células HFF-1 incorporadas num hidrogel 3D de GelMA revelaram que não houve diferenças significativas entre todas as condições testadas, com valores de atividade metabólica celular variando entre 94-100%. Para células A-431 incorporadas num hidrogel 3D de GelMA em contato apenas com os hidrogéis de pectina, após irradiação NIR, a atividade metabólica diminui para 81%. No entanto, esse valor ainda está acima do limite de toxicidade de 70% (ISO10993-5(E)), e não são observadas diferenças estatisticamente significativas. Curiosamente, amostras de A-431 em contato com hidrogéis de pectina/@rGOn a 1.25 wt% reduziram a sua atividade metabólica para 19%. Esse valor diminui para 10% quando irradiação NIR é realizada. No entanto, o teste precisa de ser repetido antes de tirar quaisquer conclusões, uma vez que estes são apenas resultados preliminares. Finalmente, um ensaio de células vivas/mortas foi realizado e permitiu concluir que as células HFF-1 e A-431 proliferam dentro da matriz de GelMA colocada em contato com hidrogéis de pectina e pectina/@rGOn a 1.25 wt%, apresentando morfologia celular normal. O efeito da irradiação NIR precisa ser explorado mais a fundo antes de tirar quaisquer conclusões.

Abstract

Non-melanoma skin cancer is the most common cancer in the world, with predictions for a continuous increase in incidence until 2044. The discovery of alternative treatments is necessary as current therapies, including surgery, chemotherapy, and radiation therapy, still show low effectiveness and significant side effects. Phototherapy is a treatment based on the application of light as a promising approach to the treatment of various diseases, including skin cancer. Photothermal therapy (PTT) is a minimally invasive technique in which photothermal agents are employed, allowing the conversion of near-infrared light (NIR) into heat. Since photothermal agents accumulate in cancer tissue, enabling the increase of temperature only in the target area during irradiation, allowing for treatment selectivity. Carbon nanomaterials (CNMs) present excellent mechanical and electrical properties, high thermal conductivity, surface area, and NIR absorption, placing them as excellent potential phototherapeutic agents. Graphene-based (GBM) materials stand out from the remaining CNMs due to large surface area, versatile chemical functionalization, water stability, and excellent optical properties, as well as good biocompatibility.

In this work, graphene oxide (GOn) was produced using the modified Hummers method, and photo-reduced using ultraviolet light (UV-C) to produce partially reduced graphene oxide (p-rGOn), which was then magnetized following a hydrothermal magnetization process using iron chloride to obtain reduced graphene oxide functionalized with iron oxide particles (@rGOn). TEM imaging shows that GOn is constituted by individual well exfoliated sheets with an average length of 147.9 ± 82.2 nm, while @rGOn displays an average length of 298.4 ± 67.7 nm. The successful functionalization with iron oxide nanoparticles has been demonstrated, with those particles presenting an average size of 12.9 ± 9.3 nm across the surface of the material. Successful magnetization was confirmed since all particles headed towards a magnet placed outside the vial. Fourier-transform infrared spectroscopy (FTIR) reveals that several oxygen-containing functional groups are present at GO's surface, such as carbonyls, carboxyls, hydroxyls, ethers, and epoxides. However, after the reduction and magnetization process, those functionalities almost disappear, confirming the success of the procedure. Thermogravimetric analysis (TGA) shows that GOn loses 31.89% of its mass until 300 °C, correspondent to labile oxygen functional groups. Opposingly, @rGOn has only a small step of 4% weight loss between 500-640 °C, revealing its great thermal stability and reduced number of oxygen-

groups. Another evidence of the efficiency of the reduction method used is the fact that near infrared (NIR, 750-850 nm) absorption increased 7-fold for @rGOn, comparing with GOn.

GOn was incorporated in pectin hydrogels at different loadings (0.625, 1.25, 2.5 wt%). The initial goal was to develop hydrogels for 3D-printing of scaffolds to be placed after skin cancer surgery, for simultaneous photothermal therapy (destroying any remaining cancer cells and preventing recurrence) and tissue regeneration (to improve aesthetic outcomes). The magnetic properties of @rGOn could eventually be used to mediate the release of active substances/drugs to prevent recurrence or infection, but also to speed up regeneration. The release could be controlled externally using a suitable magnetic field/equipment. Furthermore, magnetic hyperthermia could also be a possibility due to @rGO properties. However, due to time limitations, the work was focused on producing an hydrogel to be applied in the post-surgery defect to deliver photothermal therapy and promote tissue regeneration. Therefore, 50 °C was defined as the temperature that should be achieved to demonstrate that the hydrogels would have potential to be used for photothermal therapy. Furthermore, those should maintain their structural integrity after being incubated for 24h at 37 °C in cell culture media (DMEM), and their magnetic properties should be intact.

The efficiency of composite hydrogels to convert NIR light into heat was evaluated by placing them in an oven for pre-heating at 37 °C (body temperature), and subsequently irradiating them with our custom-built LED NIR systems (810 nm, 1000 mW/cm²). The pectin hydrogels only increased their temperature to a maximum of 42 °C. For the hydrogels containing graphene, the heating curves were very similar, and all hydrogels were able to increase the temperature of the DMEM in the well until 50 °C. Furthermore, pectin/@rGOn hydrogels (1.25 and 2.5 wt%) maintain their photothermal efficiency after 5 days and 3 cycles of NIR irradiation and heating until 50 °C, as well as their structural integrity, and magnetic properties. In order to evaluate the potential of the hydrogels to be used to 3D-print a structure that could match defects caused by cancer surgery, their printability was studied. The parameters still need to be optimized, but it is clear that the hydrogel inks have potential for 3D printing as the structures obtained exhibit a uniform appearance and sufficient mechanical and structural properties to be manipulated post-printing.

The hydrogel cytotoxicity was assessed through an extract exposure assay, analysing if any @rGOn particles would leach out of the hydrogel samples and cause alterations in cells' metabolic activity. For the day 1, the lowest value obtained was equal to the control, showing that the hydrogel presents no cytotoxicity to HFF-1 cells. At day 7 and for 0, 1.25, and 2.5 wt% @rGOn, the values of metabolic activity obtained were 98, 89 and 84%, respectively. Despite a significant decrease in metabolic activity for all @rGOn concentrations, the values obtained are above the limit set by the ISO 10993-5:2009(E).

In order to simulate the final application of these structures, namely to be in contact with normal (HFF-1) and cancer (A-431) skin cells, and mediate destruction of cancer cells, while keeping normal cells intact and eventually promote tissue regeneration, a new structure was developed to allow the accommodation of a 3D cell matrix during the direct contact assays. Resazurin assays for

HFF-1 cells embedded within a GelMA 3D hydrogel revealed that there was no significant difference between all conditions tested, with cell metabolic activity values being between 94-100 %. For A-431 cells embedded within a GelMA 3D hydrogel in contact with pectin hydrogels only, after NIR irradiation, metabolic activity decreases to 81%. However, this value is still above the toxicity limit of 70% (ISO10993-5(E)), and no statistically significant differences are observed. Interestingly, A-431 sample in contact with pectin/@rGOn 1.25 wt% hydrogels, reduce their metabolic activity to 19%. This value decreases to 10% when NIR irradiation is performed. However, the test needs to be repeated before any conclusions are withdrawn, since those are just preliminary results. Finally, a live/dead assay was performed that allowed to conclude that HFF-1 and A-431 cells proliferate inside the GelMA matrix placed in contact with pectin and pectin/@rGOn 1.25 wt% hydrogels, presenting normal cell morphology. The effect of NIR irradiation needs to be further explored before taking any conclusions.

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Abbreviations, Acronyms and Symbols

2D	Two-dimensional
3D	Three-dimensional
5-FU	5-fluorouracil
5-Ala	Aminolevulinic acid
ALP	Alkaline phosphatase
AM	Additive manufacturing
BCC	Basal cell carcinoma
BSA	Bovine serum albumin
CH	Chitosan
CM	Carbon materials
CNM	Carbon nanomaterials
CNF	Carbon nanofibers
CNT	Carbon nanotubes
COL	Collagen
CVD	Chemical vapor deposition
DAPI	4',6'-diamino-2-fenil-indol
DCM	Dichloromethane
DET	Direct-electron-transfer
DLP	Digital light processing
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ECM	Extracelular matrix
IDE	Interdigitated electrode
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GBM	Graphene-based materials
Gel	Gelatin

GelMA	Gelatin methacrylate
GO	Graphene oxide
GOn	Nanographene oxide
HA	Hydroxyapatite
HBSS	Hanks' balanced salt solution
KC	Keratinocyte carcinoma
LAA	L-Ascorbic acid
LAB	Laser-assisted bioprinting
MWCNT	Multi-walled carbon nanotubes
NIR	Near-infrared radiation
nHA	Nanohydroxyapatite
NMSC	Non-melanoma skin cancer
OCN	Osteocalcin
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PDI	Polydispersity index
PDT	Photodynamic therapy
PEGDA	Poly(ethylene glycol) diacrylate
PFA	Paraformaldehyde
PIC	Polyion complex
PLA	Poly(lactic acid)
PLG	Poly(lactide-co-glycolide)
p-rGOn	Partially reduced nanographene oxide
PpIX	protoporphyrin IX
PS	Photosensitizer
PTT	Photothermal therapy
ROS	Reactive oxygen species
RSF	Regenerated silk fibroin
RT	Room temperature
SCC	Squamous cell carcinoma
SLA	Stereolithography
SWCNT	Single-walled carbon nanotubes
TCP	Tricalcium phosphate
TE	Tissue engineering
TEM	Transmission electron spectroscopy
TGA	Thermogravimetric analysis
TPU	Thermoplastic polyurethane
UV	Ultraviolet

Chapter 1

Introduction

Skin cancer accounts for the uncontrolled growth of abnormal cells in the epidermis mainly provoked by chronic ultraviolet (UV) B exposure, achieved by sun contact and tanning beds. It's primarily divided into two categories: melanoma skin cancer (MSC) and nonmelanoma skin cancer (NMSC). The last one can be divided mainly into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), named after the types of skin cells where the cancer develops. Despite melanoma skin cancer being recognized as the most fatal due to its characteristic spreading, this form only accounts for 2% of malignant skin cancer [1]. For this work, the focus will be on SCC.

Squamous cells are flat cells located near the surface of the skin. Usually, this type of cancer is caused by UV exposure, inducing DNA damage in keratinocytes. Keratinocytes are the skin cells responsible for shielding the UV exposure. The condition affects predominantly Caucasians due to the lack of melanin. Melanin is able to absorb UV waves, preventing the DNA damage in the keratinocytes. Due to changes in lifestyle and clothing, skin UV exposure has increased. Factors such as ozone depletion enhance some populations UV exposure, including Australia and Northern Europe. Also, the growth of elderly population increases the incidence of SCC due to accumulation of damage in the DNA. This type of cancer develops mainly in areas with constant sun exposure such as face, scalp, ears, and chest [2].

A study published in 2023 based on the Netherlands, Scotland and two federal states of Germany (Saarland/Schleswig-Holstein), determined an annual percent increase of SCC incidence ranged between 2.4% and 5.7%, between 1990 and 2020. The highest increase occurred in the age groups above 60 years, especially in men aged ≥ 80 years, with a three to 5-fold increase. Extrapolations up to 2044 showed an unrestrained increase in incidence rates in all countries investigated [3].

The tendencies urge for new and more efficient forms of treatments, as current ones fail to fill all requirements. Traditional treatments for cancer include surgery to physically remove the

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cancer, chemotherapy to apply anti-cancer drugs systemically and radiotherapy to kill cancer cells with ionising radiation and charged particle beams. However, the presented therapies exhibit strong side effects caused by neoplastic factors failure to distinguish cancer cells from healthy cells. Some of the side effects with conventional techniques include nausea, kidney toxicity, fatigue, abdominal pain, dermatitis, hepatitis, and infection. The diverse side effects of traditional therapies encourage the search for novel therapies for cancer cells

Phototherapy has been studied for thousands of years, remoting to ancient Indian, Chinese and Egyptian civilizations, where sunlight was used to treat diseases including skin cancer. Phototherapy can be categorized in photothermal therapy (PTT) and photodynamic therapy (PDT) where thermal and chemical damage causes tumour ablation through the incision of light, respectively [4].

PDT relies on the use of photosensitizer (PS) agents, that can be applied topically and get absorbed by the skin, orally, and through intravenous route. The PS enable the production of reactive oxygen species (ROS) that allow elimination of cancer cells when a light source is applied. Some components of the tumour cells, like the membrane, proteins, and DNA are harmed by the ROS [5]. PDT allows selectivity during treatment as the PS and light source can be implemented only in the cancer area, restricting ROS to cancer cells only [6]. PDT also exhibits minimal side effects once the therapy is performed in multiple cycles of treatment [7].

Regarding PTT, photosensitive agents can absorb visible or near infrared radiation (NIR), converting the energy from the light into heat, which will cause local temperature increase. Just like PDT, this process is selective since the agents tend to accumulate in the tumour. NIR irradiation (700 - 1350 nm) is preferred for the cancer treatment as allows a more effective penetration of tissues than visible light (400 - 700 nm) [8-10], so, together with a PTT agent, it can be used in cancer phototherapy to provoke photothermal ablation.

The damage caused by the increase of temperature differs as this difference increases. The increase of temperature up to 42 - 52 °C constitutes mild hyperthermia, causing unrecoverable damages to the tumour cells taking place at 42 °C, like enhanced membrane permeability, cellular uptake, dysfunctional membrane transport and, metabolic suppression [8],[11]. At 46 - 52 °C, cell death occurs because of microvascular thrombosis and ischemia. Photothermal ablation, at temperatures higher than 60 °C, causes protein denaturation and plasma membrane destruction which will lead to instantaneous cell death [8]. The employment of combined phototherapy is also possible, using both PDT and PTT. Whilst PDT has been approved by the Food and Drug Administration (FDA) for about 40 years [10], for treatment of other skin diseases, only more recently it has been applied to NMSC treatment. PTT requires more investigation as it shows great potential given that it is a much more recent cancer treatment than PDT [8], and it is the therapy around which this work will be centred.

Ideal agents for phototherapy should present high radiation absorbance and need to be able to convert light into ROS and/or heat. The main advantage of phototherapy is that the lesions are

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selectively destroyed while there is minimal to no damage to the surrounding healthy tissue. The localized approach plays an important role in skin cancer treatment and hydrogels unique characteristics present them as the perfect platform to carry and nest the PS. Hydrogels fill crucial requirements including desirable biocompatibility and tuneable physicochemical properties. At the moment hydrogels have been extensively studied for cancer therapy, drug delivery, and tissue engineering due to their unique properties and their potential to address various challenges conventional techniques can't overcome.

Pectin is a natural, biocompatible polymer, which possesses tuneable mechanical and rheological properties [12, 13]. To modulate the rheological behaviour of pectin, the addition of controlled concentrations of common divalent or trivalent ions, like Ca^{2+} , Sr^{2+} , or Al^{3+} , can be explored, taking advantage of the inherent ionic gelation ability of pectin [12]. In order to increase pectin's mechanical properties, the incorporation of GBM has been studied and a significant improve of the mechanical behaviour has been documented [14]. Pectin has been implemented in different areas due to the unique characteristics that displays.

Natural polymers, including pectin, are suitable for the design and production of hydrogels for wound healing purposes regarding their biodegradability, biocompatibility, durability, low cost, and low skin toxicity and sensitivity. Hydrogels based on the combination of both pectin and sodium alginate (SA) are widely used due to their good biocompatibility, biodegradability, and strong film-forming properties [15, 16].

Regarding drug delivery, one of the important precursors for the synthesis of drug delivery carriers is polysaccharide-based hydrogels such as pectin and their derivatives due to their non-poisonous quality, biocompatibility, and biodegradability. Pectin based hydrogels are widely used in targeted therapies such as colon-targeted antitumor drug delivery [17, 18].

Finally, tissue engineering is heavily influenced by hydrogel scaffolds due to properties including compatibility, microporous structure with adjustable porosity, and suitable pore size. Natural polymers that have attractive properties such as biodegradability, biocompatibility, and the ability to imitate the native extracellular matrix (ECM) are, by themselves or in combination with other macromolecules, widely used in the formation of tissue engineering hydrogels. The presence of numerous functional groups on the pectin surface has made it very capable of chemical modification and gelation. Pectin-based scaffolds are commonly used as a cell carrier to promote cell proliferation [19, 20].

Incorporating nanoparticles into hydrogel-based systems provides a versatile platform for tailoring cancer therapy to specific patient needs and tumour characteristics.

Carbon nanomaterials (CNM) gained interest due to high absorption capability in the visible light and NIR spectra [7]. Graphene has an array of functional characteristics that make this material widely investigated and used in biomedical applications, including its chemical and physical properties, remarkable thermal and electrical conductivity, high surface area and small particle size. Graphene

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oxide (GO) possesses oxygen-containing functional groups, which improve polarity and water solubility. Plus, the presence of functional groups facilitates a wide range of modifications and surface functionalization, and has shown reduced cytotoxicity [21]. However, the polarity and reactivity of these oxygen groups lessens the optical absorption properties and the thermal stability of GO [22]. The reduction of GO enables the combination of some of the original properties of graphene, like its thermal and electrical conductivity and its optical absorbance, whilst maintaining graphene oxide's functionalization [21]. Reduced graphene oxide (rGO) can be obtained by methods such as thermal, chemical or photo-reduction of GO. By having higher absorption in the NIR region, rGO can be used for PTT, but its hydrophobicity presents a challenge for its use in biomedical applications [9]. In this work a pectin hydrogel is combined with a novel GBM, characterized by its nanometric size, exhibiting enhanced photothermal properties compared with rGO, with additional magnetic behaviour.

Additive manufacturing (AM), commonly referred to as 3D-printing, consists of a wide range of technologies where 3D objects are fabricated via the deposition of materials in a layer-by-layer fashion. AM technologies have been employed in the biomedical field, having the possibility of creating biomimetic constructs partially mimicking the architecture and features of certain tissues and organs [23]. Bioprinting is a specific branch of AM, where biomaterials, cells and/or bioactive molecules are bioprinted towards the fabrication of tissue and organ analogues with biological function. For bioprinting, the deposited formulation can be a hydrogel, without the use of cells, or a bioink, a cell-containing formulation usually made of hydrogel precursors. Several biomaterials have been applied in bioprinting, such as hydrogels, resins, thermoplastic polymers, or a combination of the previously presented materials. However, at times, these biomaterials cannot sustain the assigned biological function and the printability without the addition of another material. CNM have been used to complement the materials used for bioprinting, since they have the capability of tuning multiple key properties that can make an ink more suitable for a wide range of applications [24].

1.2 Motivation

Non-melanoma skin cancer has a high recurrence rate, and the most common treatments are invasive, and at times result in poor aesthetic outcomes. The precarious quality of life during treatment mainly caused by the variety of side effects characteristic to conventional cancer treatments, requires the research of alternative treatment approaches, like PTT. Graphene-based materials possess unique characteristics, like nanometric sizes, allowing for good biocompatibility, high surface area, making GBM usable for drug-delivery, and high NIR absorption, improving the phototherapeutic potential [7]. The objective of this dissertation was to develop a biomaterial-based approach based on an improved GBM and pectin and evaluate its properties in PTT for skin cancer treatment. The improved GBM has higher NIR absorption properties compared to graphene oxide, whilst exhibiting magnetic properties. The incorporation of this GBM in pectin hydrogels with LED based systems for PTT constitutes a novel approach. Most research done in PTT for cancer therapy

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implements laser irradiation. However, in this the LED based system was used to increase the safety of the process, as the heating occurs in a more slow and controlled matter. As the heating is slower using the LED system, the risk for burns is significantly lower, reducing the probability of an operator's error. Multiple works have used GBM for the treatment of cancer with phototherapy [9]. However, the use of hydrogels with GBM for PTT is lacking in the treatment of skin cancer. Also, selectivity of the treatment regarding effectively killing only cancer cells, without affecting healthy ones is yet to be shown on the same study. Moreover, even the possibility of printing GBM-hydrogels to be administered for localized PTT, allowing for selective cancer eradication, while lessening the eventual damages to surrounding healthy tissues is lacking from literature.

1.3 Goal

The main goal of this dissertation work was to develop innovative GBM-containing pectin hydrogels that can be 3D-printed to generate personalized constructs for localized photothermal therapy of skin cancer. The specific goals of this work were:

- Collection of information and organization of a literature review regarding state-of-the-art studies on 3D printing of nanoparticle-containing hydrogels and bioinks for cancer phototherapy, magnetic hyperthermia, and tissue engineering;
- Development and characterization of GBM with suitable physicochemical properties and photothermal effect;
- Optimization and production of pectin hydrogels loaded with GBM;
- Optimization of the pectin/GBM hydrogel inks and evaluation of printability;
- Characterization of the physical and photothermal properties of the produced hydrogels;
- Understanding the effect and phototherapeutic potential of pectin/GBM hydrogels on healthy human skin fibroblasts and on skin cancer cells, in the presence or absence of NIR irradiation.

This dissertation is divided in six chapters besides this introduction. Chapter 2 reviews the state-of-the-art on carbon nanomaterials in 3D-printed biomedical devices, presenting the most used polymers and nanoparticles in hydrogels and bioinks for biomedical applications, bioprinting techniques, and different applications of such biomedical devices. Chapter 3 presents a description of the materials and methods used in the experimental work, whilst Chapter 4 describes the obtained results. Chapter 5 consists of a discussion of the most relevant results, in the light of current literature. Chapter 6 presents the main conclusions withdrawn out from the work performed.

Chapter 2

3D printing of nanoparticle-containing hydrogels and bioinks for cancer phototherapy, magnetic hyperthermia, and tissue engineering

1. Introduction

Approximately 19-20 million people are diagnosed with cancer annually worldwide and this disease still causes approximately 10 million deaths annually [25]. Thus, there is a need for consistent efforts to develop novel therapeutics. Cancer treatment has now a variety of therapies with the purpose of controlling and/or eliminating the abnormality. Conventional methods including surgery, chemotherapy, and radiation therapy have proven successful especially combined, however these cause a variety of side-effects being one of the major ones the lack of specificity. Because of it, methods like chemotherapy and radiation therapy cause harm to the tumour cells, but also to healthy tissues and organs [26, 27]. The rising of nanotechnology in areas of diagnosis and treatment enable the creation of novel minimal invasive nanomedicines (MINs), minimizing or irradiating side effects such as pain, using small or no incision, and effectively target abnormal cells, tissues and/or organs, improving bioavailability and biosafety of drugs. Nanoparticles exhibit large surface-to-volume ratio and flexible surface modification, enabling the surpassing of barriers in aspects including bioavailability, biosafety, and therapeutic efficacy. Some MINs use including photothermal therapy (PTT) and magnetic hyperthermia (MHT) use outside stimuli including light to maximize therapeutic efficacies through targeted therapy enable the decrease in dosages and side effects. Nanomedicines can specifically accumulate in the targeted tissue, for example the tumour mass, and when exposed to the stimuli activate to exert therapeutic effects. Magnetic hyperthermia uses as exogenous stimuli an alternating magnetic field (AMF) to produce local increase of temperature by magnetically

mediated heating, through power absorption by magnetic nanoparticles used as nano heaters. This therapy increases the tumour's mass temperature above 40°C, for which the nanoparticle exhibit preferentiality, whilst preserving the environment tissue's temperature. The heating causes disruption in cancer cell's essential activities, leading to irreversible damage and, eventually, cell death. The heating capacity depends on the magnetic and physicochemical properties of the magnetic nanoparticles. Alternatively, photothermal therapy requires light to activate its specific photothermal agents, capable of absorbing near-infrared radiation (NIR) light to increase the tumour's temperature. However, the surrounding tissue will remain with minimal to no-damage. The ideal PTT nanoparticle exhibits high photothermal conversion efficiency, and strong absorbance and good photostability in the NIR region, without compromising its biosafety and biocompatibility. Magnetic hyperthermia therapy and photothermal therapy have been widely used in the treatment of different tumours and achieved good therapeutic efficacies using nanoparticles with magnetic and photothermal properties, respectively. Advances in nanomedicines presenting low toxicity, contrasting agent properties, tailorable characteristics, target/stimuli-response delivery potential, and precise control over behaviour have revolutionised tissue engineering field. It is estimated that annually around 310 million major surgical procedures are performed annually [28], the majority involving either repair or replacement of damaged tissues/organs. Conventionally, these surgeries require autografts and/or allografts, causing additional trauma to the patient or the possibility of rejection by the immune system, respectively [29, 30]. Additionally, current techniques face drawbacks including lack of cell growth, difficulty to mimic physiological architecture, and inefficiency in stimulating cell communication and proliferation. To address these challenges, the field of TE has emerged and benefited from important advances in multidisciplinary fields, including nanotechnology. The use of nanoparticles for enhancement of mechanical properties and osteogenic stimulation creates crucial improvements in this field and the acceptance of tissue engineered constructs, such as three-dimensional (3D) scaffolds, has changed over the last few years.

The preferential technique for the manufacturing of the 3D scaffolds and other nanoparticle containing systems is the extrusion-based 3D printing (3DP). Extrusion-based 3D printing is one of the most used methods for the printing of structures for biological and non-biological applications owing to its affordable price and tailoring construction. Multi-material extrusion-based printing has recently been developed, which is a requirement for biomedical applications to mimic natural tissues and structures. This method benefits from wide adoption, ease of use, precision printing of complex geometries via computer-aided design (CAD), and multiple solidification methods, despite requiring materials with specific printability characteristics [31-33]. It includes a temperature-controlled cartridge extruding the desirable material from a fine nozzle with various forms of extrusion forces. Usually, the driving force can be a pressure-driven, pneumatic-driven, force and displacement-driven, or mechanical-driven force [34], being the pneumatic the most common. These common extrusion methods enable compatibility with many materials, but all feature a curing step that can be for

example photoactivated. Because hydrogels are one of the most practicable classes of ink materials for 3D printing, its use has increased with the advances of this field. The use of hydrogels and/or hydrogels-based bioinks, being the last of greatest importance as contains living cells and biomaterials that mimic the extracellular matrix environment, allow the supporting of crucial events including cell adhesion, proliferation, and differentiation after printing.

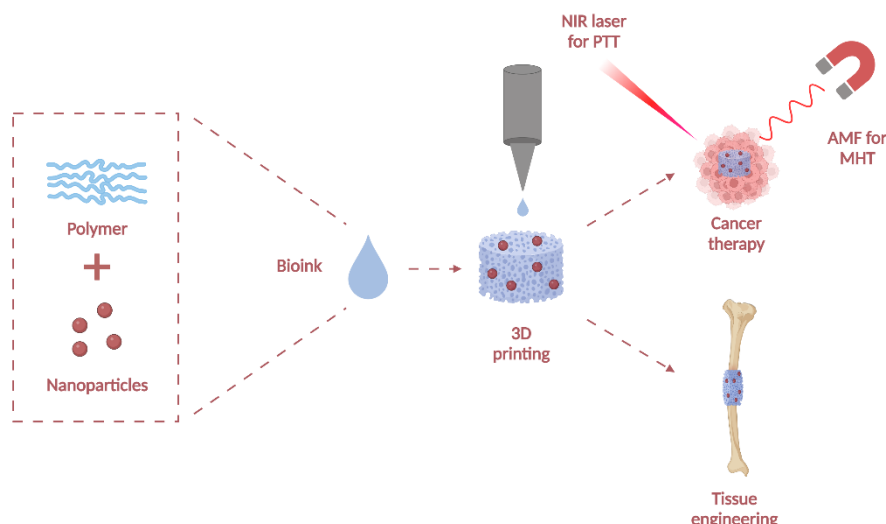


Figure 1. Production of 3D-printed hydrogels and their application in cancer photothermal therapy and magnetic hyperthermia therapy. Hydrogels have been studied for wide range of biomedical applications where polymers conjugated with nanomaterials absorb light and convert it into heat, in the case of PTT, or an alternative magnetic field, in the case of MHT, leading to cancer ablation or cell death. The incorporation of cells in hydrogels forming bioinks creates a structure for new tissue proliferation. After degradation the result will be only healthy tissue. Abbreviations: PTT, photothermal therapy; MHT, magnetic hyperthermia therapy. (A colour version of this figure can be viewed online.)

In this review, we aim to comprehensively provide an overview of the current state-of-the-art in 3D printed nanoparticles-containing hydrogels and bioinks for PTT, MHT, and TE, while presenting the most used fabrication techniques. First, an overview on polymers, nanoparticles and their properties, as well as a summary of methodologies and fabrication techniques is provided, to appropriately focus the reader on the landscape of the field. Then, the fabrication of 3D nanoparticle-hydrogel system is reviewed. The most recently available literature reports are included to highlight main applications in the biomedical field. Finally, the main conclusions together with the future perspectives, are presented.

2. Nanoparticles properties and applications

Nanoparticles, defined as ‘solid colloidal particles ranging in size from 10 to 1000 nm (1 μm) [35], are used in biomedical applications as they offer many advantages to larger particles such as increased surface to volume ratio and increased magnetic properties [36, 37]. Nanoparticles may be classified into different nanosystems.

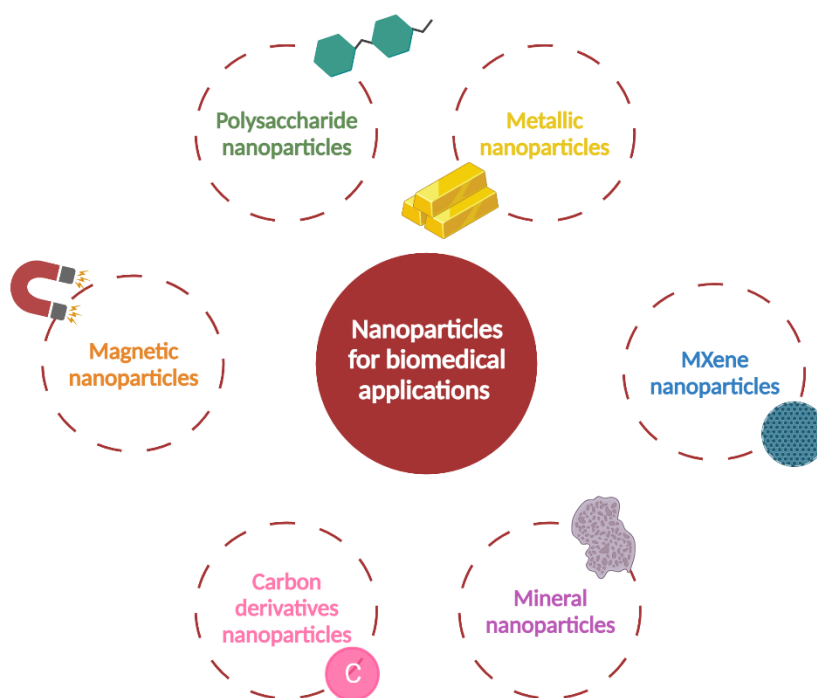


Figure 2. Representative nanoparticles for biomedical applications for enhancing their biological, drug delivery, and phototherapeutic properties. Polysaccharide nanoparticles (NPs) have played a vital role in the field of biomedical research in last few decades. In addition to high loading efficiency, good target ability, biodegradability, and biocompatibility, the NPs have gained significant attention due to their abundance, easy processing and sustainable feedstocks. Metallic NPs attract increasing attention in biomedical field due to their significant inert nature, and nanoscale structures, with size similar to many biological molecules. These are mainly applied in targeted drug delivery, sensing, photothermal and photodynamic therapy, and imaging. MXenes fascinating physicochemical properties have shown their potential for various biomedical applications, including biosensing, bioimaging, therapeutic diagnostics, implants and antibacterial agents. Hydroxyapatite is the most used mineral nanoparticle for biomedical applications in areas including sustained drug and gene delivery, bio-imaging, magnetic resonance, cell separation, and hyperthermia treatment due to their promising biocompatibility. The carbon nanoparticle family has unique physicochemical properties. The most researched carbon nanoparticles include graphene and graphene oxide. The main direction of use involves medical diagnostics, which includes bioimaging and the detection of chemicals or metabolites present in the body. Since the question of nanoparticle toxicity has not been fully answered, the use of nanoparticles in the fields of therapeutics (drug delivery), regenerative medicine (cell scaffolding, tissue engineering), and vaccine production is still under research and many in vivo studies are ongoing. Magnetic NPs have been widely used in hyperthermia, target drug delivery system, imaging, and extraction of biomolecules due to their unique surface chemistry, nontoxicity, biocompatibility, and particularly their inducible magnetic moment. Created with Biorender.com. (A colour version of this figure can be viewed online.)

The most commonly used nanoparticles are metal and metal-oxide nanoparticles [38]. Due to their biocompatibility and unique magnetic properties, ferrites are the the most widely studied, especially superparamagnetic iron-oxide nanoparticles (SPIONs). These exhibit unique properties such as superparamagnetism, surface-to-volume ratio, and high surface area. Superparamagnetic

nanoparticles become magnetized when exposed to a magnetic field, however this process is reversible in the absence of an external magnetic field. The use of nanoparticles, especially metals and derivatives, in a biological context requires good colloidal stability in order to minimize the risk of nanoparticles' aggregation in the blood [39]. Additionally, the biodegradability of SPIONs appears as one of the most important features for clinical use[40-42]. Iron oxides can be present in the form of maghemite ($\gamma\text{-Fe}_2\text{O}_3$), hematite ($\alpha\text{-Fe}_2\text{O}_3$), and magnetite (Fe_3O_4)[43]. Magnetite's unique magnetic, catalytic, and biochemical properties make it the most promising SPION, from a medical point of view [44]. Fe_3O_4 nanoparticles have a large surface area needed for adsorption and immobilization of molecules or drugs. They can be easily modified to improve absorption [45] and their size and shape can be easily controlled by using different synthesis methods [46]. It is possible to synthesize multifunctional platforms using Fe_3O_4 nanoparticles, in which other compounds cover magnetite nanoparticles, such as biodegradable polymers[47-49].

Calcium phosphate is a family of minerals containing calcium cations and phosphate anions. Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) is a naturally occurring form of calcium phosphate, being the main mineral component of bones and teeth. HA possesses exceptional biocompatibility and bioactivity properties, with respect to bone cells and tissues, probably due to its similarity with the hard tissues of the body. Calcium phosphate biomaterials have been widely applied in interdisciplinary field of sciences including chemistry, biology, and medicine in the form of powders, granules, dense and porous blocks, and various composites [50]. Nanotechnology rising in the medical area has led to the development of a wide range of synthesis techniques in producing nano-sized materials, including HA nanoparticles (nHA) with reduced sizes, increased surface areas and controlled morphology[51, 52], regulating the physical, chemical and surface properties of synthetic nHA. Synthetic nHAs has shown to have tremendous potential as a carrier for delivery of drugs and other therapeutic agents, as they overcome many of the inherent problems associated with systemic administration of drugs or other therapeutic agents [53-57] including proteins anticancer drugs [58].

Two dimensional (2D) nanomaterials have gained interest, owing to their ultrathin structure and remarkable properties. Various 2D materials like graphene and its derivatives [59-61] are nanoparticles that have been extensively explored for its biomedical applications. Among the 2D nanomaterials, ceramic based MXenes have become the material of the current focus for a variety of applications. These materials exhibit a general formula of Mn+1AX_n , where 'M' denotes an early transition-metal, 'A' group 13 or 14 element, 'X' carbon or nitrogen, and 'ene' the presence of graphene [62]. After synthesis, several surface terminations like hydroxyl (-OH), oxygen (-O), and/or fluorine (-F) were observed and were represented by 'T' in the modified formula, $\text{Mn+1X}_n\text{T}_x$. These surface terminations provide room for further surface modifications [62]. 2D titanium carbide (Ti_3C_2) derived from Ti_3AlC_2 is the pioneer of MXenes, and over seventy different MAX phases and twenty MXenes have been effectually discovered since then. Compared to other 2D materials, MXenes possess abundant functional groups on their surface making modifications more flexible. MXenes contain

complete metal atomic layers and tunable composition as well as hydrophilicity, great for biomedical applications. Also, the cost-effective scale-up production of MXenes may be a superiority. So far, the main applications of MXenes in biomedical field include bioimaging, antibacterial, biosensing, drug delivery, tissue engineering, and various therapeutic methods [63].

In the last decade, the properties of graphene materials have been intensively studied in order to develop their potential in various biomedical applications. Graphene is a single layer (in thickness) of carbon atoms arranged in hexagonal rings of an aromatic electron structure [64]. Graphene oxide and reduced graphene oxide are the graphene derivatives characterized by unique chemical structures composed of small sp² carbon domains surrounded by sp³ carbon domains and the presence of oxygen functional groups on the graphene surface and, therefore, by the presence of defects in the graphene hexagonal rings as well [65]. Such disorder in the graphene structure leads to many unique properties of GO and RGO. The surface of both GO and RGO is easily modified by different molecules, including biological ones, enable the synthesis of composites with strictly controlled and desired properties. GO and rGO, due to their large active surface areas, can serve as a platform for biological molecules (both organic and inorganic) to be safely introduced into the organism without the risk of uncontrolled and undesirable spreading into the surrounding tissues, a great advantage in the case of anticancer therapy, where cytotoxic compounds are introduced in the tumour's area [66-70]. In many biomedical applications, materials are required to be nontoxic to human cells. In terms of this requirement, GO and slightly reduced GO seem to be suitable materials. The biocompatibility of these two graphene derivatives has been confirmed by many publications in the literature [22].

Metallic nanoparticles (MNPs) have shown tailorable shape, size, charge, and surface in a controlled manner. Metallic nanoparticles such as gold (Au) possess excellent electronic and optical properties as well as chemical inertness and its ability for surface functionalisation [71-73]. Gold's unique electronic and optical properties have been applied in the areas of biosensors [74-76] and bioimaging [77-81] as well as photothermal therapy [79-83]. Gold and its compounds have been used for medicinal purposes since its discovery over 5000 years ago, having massive research made regarding its biomedical applications. Gold's ease at functionalisation with organic molecules allows for conjugation with ligands, antibodies, or drug molecules for active or passive drug delivery [84-88] and its chemical inertness allows for good biocompatibility in vitro and in vivo. Silver (Ag) and its compounds like gold have been used for medicinal purposes since discovery. Silver nanoparticles present unique physicochemical properties including high electrical conductivity, thermal conductivity, chemical stability, catalytic activity, antibacterial and enhanced optical properties [89, 90]. Because of silver nanoparticles extraordinary antimicrobial activity, it is mainly used in the medical industry for wound dressings and device coatings. However, the use of silver nanoparticles in biomedical applications such as biosensors [91-93] photothermal therapy and drug delivery [94-104] is increasing.

Melanins are well-known biopolymers that are abundant in nature, distributed widely in microorganisms, plants, and animals, and play significant physiological roles [105][83]. Poly(dopamine) (PDA), synthesized using L-DOPA or DHI as precursors, gives place to a synthetic melanin similar to natural eumelanin. It can be applied in many fields, such as energy, environmental, and biomedical, and it has potentially broad application prospects.[106, 107]. Melanin-like materials (including PDA) have been widely applied in many fields because of their excellent biocompatibility and biodegradability.

Cellulose is formed from the repeating units of D-glucose, linked through glycosidic linkages $\beta(1\rightarrow4)$ [108]. Nanocellulose comprises properties of cellulose and nanomaterials combined with high aspect ratio, improved mechanical properties, renewability, and biocompatibility enabling its use for advanced and novel applications in tissue engineering, drug delivery, and wound healing [109]. Nanocellulose is generally considered as a potential reinforcing filler for manufacturing composites as they are renewable, possess lightweight and is cost effective. These nanoparticles have found immense applications in drug delivery systems and as scaffolds in tissue engineering. There are three main classes of nanocellulose (a) cellulose nanocrystals (CNCs) or cellulose nanowhiskers (CNWs) also known as nanocrystalline cellulose (NCC), (b) cellulose nanofibrils (CNFs) or nanofibrillated cellulose (NFC) or microfibrillated cellulose, and (c) bacterial cellulose (BC or microbial cellulose) [110].

Nanoparticles possess attractive and tuneable properties, and they have developed rapidly in the biomedical field. To meet the increasing demand for biomedicine, these can be modified physically or chemically to improve their dispersity and biocompatibility. Surface modification can also increase the chemical stability and targeting ability and broaden the applications of 2D nanomaterials. Physical or chemical modification with PEG has been widely used to improve the dispersity and biocompatibility of 2D nanomaterials [111, 112] as other biocompatible polymers such as polyvinylpyrrolidone (PVP), polydopamine (PDA), and biological molecules such as bovine serum albumin (BSA) are also applied in the modification of 2D nanomaterials [113]. Reasonable modification can also improve the targeting ability of 2D nanomaterials in cancer therapy. For example, the implementation of molecules able to target overexpressed makers in many malignant tumours, which has been used for the modification of 2D nanomaterials [114]. 2D nanomaterials modified by some functional materials have broader applications in the medical field.

The application of 2D nanomaterials for PTT has been explored by many groups over the past decade. To further improve PTT efficiency, 2D nanomaterials have been tailored for stronger light absorption. For example, Robinson *et al.* reported that reduced graphene oxide (rGO) shows a significant increase on NIR laser absorption when compared with GO [115]. The integration of plasmonic metal nanomaterials with 2D nanomaterials is another approach for enhanced light absorption via the plasmonic near-field effect, compared with its individual component. Besides strong absorption in the NIR region, another crucial property for nanoparticles implemented in the biomedical field is good biocompatibility. For rapid excretion after systemic administration, the size of nanoparticles must be tailored to ensure that the glomerular filtration apparatus allows the passage of nanoparticles [116].

In this regard, Sun Z. *et al.* confirmed that the preparation of ultrasmall nanoparticles exhibited efficient renal filtration[117]. However, they suffered from attenuated enhanced permeability and retention (EPR) effects, which led to the reduced accumulation in tumours. An alternative is the design of biodegradable large-sized nanoparticles to balance tumour targeting and efficient clearance. For this ultrasmall nanoparticles are encapsulated in biocompatible polymers allowing a prolonged blood circulation and enhanced tumour accumulation, with nontoxic biodegradation products. Chemotherapy is a major cancer treatment method. However, low cellular uptake in tumours, severe side effects and multidrug resistance pose great challenges for chemotherapy. As a result, multifunctional nanocomposites for PTT-synergized chemotherapy have received great attention because of their potential synergistic effects. NIR light can realize drug release triggered at the desired location, and improve cellular uptake of the drug, leading to the lower side effects and higher anti-tumour efficiency. To improve the efficiency of PTT synergized chemotherapy, ligands including NH₂-PEG-Apt where the amine group is able to bioconjugate targeting molecules including antibody, aptamer, protein, and peptide, for active targeting [118].

3. Polymers properties and applications

Hydrogels produced from a single polymer using natural and synthetic polymers have been widely investigated. Natural polymers such as polysaccharides (e.g., chitosan, alginate and chondroitin sulfate) and proteins (e.g., collagen and gelatin) are obtained from natural resources and exhibit desirable intrinsic properties such as biocompatibility, low toxicity and biodegradability. Compared with natural polymers, synthetic polymers (e.g., polycaprolactone, polylactic-co-glycolic acid and polyvinyl alcohol) possess better reproducible physicochemical properties and tunable degradation profiles, which are critical for hydrogel production. Synthetic polymers are often cheaper than natural polymers and can be produced uniformly in large quantities with a longer shelf life.

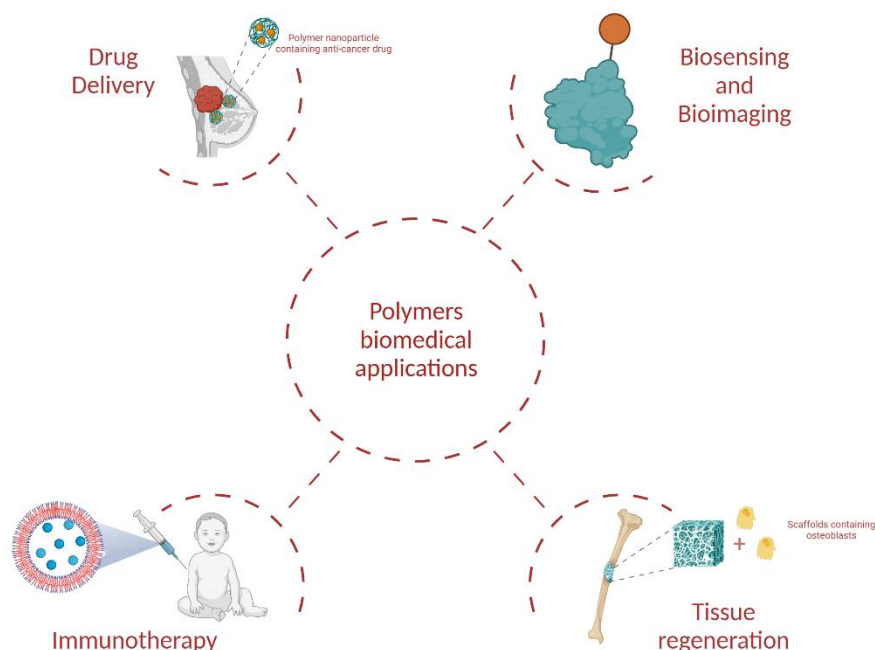


Figure 3. Areas of polymers application in the area of biomedics. Polymers possess great processing flexibility, biocompatibility, and biodegradability and are one of the most widely used scaffolding biomaterials. Scaffolds combine several functions including biocompatibility with host tissues, tunable biodegradation rate, nontoxic degradation products, and suitable porosity for the transportation of nutrients and wastes, mechanical strength, and sterilization. Polymers have become an integral part of drug delivery systems due to their improved pharmacokinetic properties. They have better circulation time than conventional small drug molecules thus target tissue more specifically. Polymers used in drug carrier systems show great advantage in drug delivery systems because of optimized drug loading and releasing property. These offer protection against chemical degradation and the development of responsive polymer structures that react to atmospheric stimulus like change in temperature, pressure, and pH, are extremely beneficial for targeted drug delivery. Polymers became the material of choice for the development of biosensors since properties and chemistry can easily be tuned and customized for each receptor type. Particular interesting are conductive polymers which can facilitate electron transfers with enzymes, change the electronic signature and thus serve as transducer element. Polymeric carriers are beneficial for enhancing immunotherapeutic approaches because of their ability to be modified with various physical properties, encapsulants, and surface ligands. Meanwhile, polymeric nanoparticles can be designed to co-deliver multiple therapeutic agents to cancer or immune cells. Polymeric nanoparticles used in the clinic are biocompatible and have excellent stability. They have the potential to improve therapeutic effects while significantly reducing off-target toxicity. Created with Biorender.com. (A colour version of this figure can be viewed online.)

3.1. Natural polymers

Alginate is a family of linear copolymers containing blocks of (1,4)-linked β -D-mannuronate (M) and α -L-guluronate (G) residues. Only the G-blocks of alginate are believed to participate in

intermolecular cross-linking with divalent cations to form hydrogels. The composition (i.e., M/G ratio), sequence, G-block length, and molecular weight are thus critical factors affecting the physical properties of alginate and its resultant hydrogels. Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed and has been extensively investigated and used for many biomedical applications, due to its biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+} [119]. Alginate hydrogels can be prepared by various cross-linking methods, and their structural similarity to extracellular matrices of living tissues allows wide applications in wound healing, delivery of bioactive agents such as small chemical drugs and proteins, and cell transplantation. Drug molecules, from small chemical drugs to macromolecular proteins, can be released from alginate gels in a controlled manner, depending on the cross-linker types and cross-linking methods. In addition, alginate gels can be orally administrated or injected into the body in a minimally invasive manner, which allows extensive applications in the pharmaceutical arena. Alginate gels are also promising for cell transplantation in tissue engineering.

Chitosan is a linear copolymer of β -(1-4) linked 2-acetamido-2-deoxy- β -D-glucopyranose and 2-amino-2-deoxy- β -D-glycopyranose. It is obtained by deacetylation of its parent polymer chitin, a polysaccharide widely distributed in nature (e.g. crustaceans, insects and certain fungi). Due to chitin's poor solubility in aqueous solution and organic solvents, it does not find practical applications whereas chitosan as an artificial variant of chitin is more suitable for useful bioapplications [120]. Chitosan polymers are semi-synthetically derived aminopolysaccharides that have unique structures, multidimensional properties, highly sophisticated functionality and a wide range of applications in biomedical and other industrial areas [121-123]. They have become interesting not only because they are made from an abundant renewable resource but because they are very compatible and effective biomaterials that are used in many applications [124, 125]. The positive facets of excellent biocompatibility and admirable biodegradability with ecological safety and low toxicity with versatile biological activities such as antimicrobial activity and low immunogenicity have provided ample opportunities for further development [126-130].

Gelatin (Gel) is a combination of denatured proteins obtained by acid or alkaline partial hydrolysis of collagen (type A and type B, respectively) [131, 132], which is a fibrous protein found mainly in the connective tissues within the body [133]. The most abundant sources of gelatin are pig skin (46%), bovine hides (29.4%), pig and cattle bones (23.1%), and fish (1.5%) [134]. The polymer chains of gelatin are composed of repetitive Gly-Xaa-Yaa tripeptide units [133, 134]. Gelatin is widely used in biomedical applications due to its excellent biocompatibility, biodegradability, non-immunogenicity, cell-interactivity and commercial availability [132, 135, 136]. Gelatin can absorb 5-10 times their mass in water and is the main ingredient in hard and soft capsules in the pharmaceutical industry because gelatin melts above 30 °C and easily releases bioactive compounds, nutrients and drugs in the human digestive tract. In addition, gelatin

contains arginine-glycine-asparagine (RGD)-like sequence in its polymer structure, which encourages cell adhesion and migration[137].

Silk is one of the most abundant naturally derived polymers. There are more than 23 independently evolved lineages of insect silks, but silks produced by silkworms and spiders are most widely used and researched. Silk satisfies every criterion that a biomedical polymer exhibits, that is, biological inertness, biocompatibility, biodegradability, bioresorbability, and sufficient mechanical strength to bear day-to-day operations[138]. Due to these inherent advantages, silk fibroin (SF) has emerged as a suitable biomaterial for drug delivery, tissue engineering, regenerative medicine, wound dressing, matrices for enzyme immobilization, and biosensor applications. SF has higher tensile strength, stiffness, and compressive modulus, which makes silk rigid and hard. The rigid and hard materials are most suited for tissue engineering of bones, cartilage, and chondrocyte tissues and other similar applications which require hard and rigid support for cells and tissue growth but may not be suitable for growing soft tissues such as the brain, heart, skin, etc., and thereby limiting applicability of SF. The functionality of SF or any other material could be altered by preparing a composite of SF with other material to generate properties unique to both polymers.

3.2. Synthetic polymers

Biodegradable synthetic polymers have a very wide spectrum of biomedical applications in tissue engineering and regenerative medicine. Polyesters like poly(lactic acid) (PLA) and poly(ϵ -caprolactone) (PCL), constitute an important group of biodegradable polymers. Their rate of degradation depends on various properties such as chain length, crystallinity, extent of branching as well as the form and the medium in which the polymer is introduced. All these can be achieved by using different polymerization techniques, polymerization conditions, reactants and monomers during the production of the final polymer or by modifications of the obtained polymer. This gives us polymers with a wide range of properties such as stiffness, extensibility, solubility, and T_g and T_m values (glass transition and melting temperatures, respectively).

PCL is a polyester obtained by ring-opening polymerization of ϵ -caprolactone monomers, which can proceed via anionic, cationic, coordination or radical polymerization mechanisms [139]. These reactions were performed under different conditions such as polymerization temperature and duration, types and concentrations of catalysts, monomer to solvent ratio, etc. By changing one or more of these variables, it was possible to produce PCLs having different molecular weights (M_n) and polydispersity indices (PDI) [140], which in turn led to differences in the degradation behaviour and mechanical strength. PCL has many good qualities for being considered as a biomaterial. Although its biocompatibility is very low, its rubbery property, adjustable biodegradability, ease of forming of blends, composites and copolymers make it a desirable material for use as supporting device especially for hard tissue, a good scaffold material to be

used in tissue engineering, and a desirable material for surgical sutures and micro and nano drug delivery vesicles[141]. Also its tailor ability of surface roughness and hydrophilicity provides preferable surfaces and interfacial characteristics for the tissue[142]. Some of the devices composed of PCL have already been approved by the Food and Drug Administration (FDA) to be used in human.

PLA is thermoplastic polyester. PLA is obtained from LA and converted back into its monomer (LA) when hydrolytically degraded. LA is a naturally occurring organic acid traditionally produced by fermentation of sugars obtained from renewable resources. Therefore, PLA can be produced and used as an environment-friendly material. Although there are multiple ways to synthesize PLA, none of them are simple or easy to execute. PLA synthesis requires the use of catalysts under rigorously controlled conditions (temperature, pressure and pH) and long polymerization times, which leads to high energy consumption[143]. In 1970, PLA products have been approved by the US Food and Drug Administration (FDA) for direct contact with biological fluids. The development of biomaterials with the required characteristics to help in the recovery of damaged tissues is one of the greatest research challenges in the fields of medicine and engineering. PLA has an extensive mechanical property profile and is thermoplastic with high biocompatibility and biodegradability[144].

PVA is the oldest synthetic polymer and one of the most common used to fabricate hydrogels [145] mainly due to its high biocompatibility and its ability to form crosslinked structures without the incorporation of cross-linkers agent toxic, which can be easily tailored depending on the application purpose. Polyvinyl alcohol (PVA) is a water-soluble linear synthetic polymer produced by polymerization of vinyl acetate to polyvinyl acetate, and a successive partial or full hydrolysis of acetate groups [146-148]. The hydroxyl groups of PVA could be crosslinked by physical (freeze-thawing process), chemical methods with crosslinking agents (e.g., glutaraldehyde) or high energy irradiation[149]. Thus, physicochemical properties of PVA hydrogels will depend on the percentage of hydrolysis, molecular weight and type of crosslinking process[145]. In freeze-thawing process, mechanical properties of the hydrogel increase with increasing temperature, number of freezing/thawing cycles, polymer solution concentration and PVA molecular weight. On the other hand, PVA hydrogels lack cell-signaling and cell-interactive motifs to biomedical applications, being intrinsically bio-inert like many synthetic hydrogels.

PNIPAAm-based system due to its phase transition between ambient and body temperature and copolymerization of PNIPAAm with different types of monomers, remains to be one of the most commonly used thermosensitive materials to formulate hydrogels. PNIPAAm exhibits an LCST around 32 °C, making it most suitable polymer for in situ hydrogel [150]. At room temperature it is a free-flowing solution, once the temperature is raised (body temperature) it solidifies into an elastomeric hydrogel. Moreover, crosslinked PNIPAAm, owing to its highly swollen nature allows injectability even through small gauge needles. Poly(NIPAAm) undergoes gelation by physical cross-linking. At temperatures below its LCST, the polymer chains are hydrophilic and thus soluble

in the aqueous environment. Gradual increase in hydrophobicity is/was observed as the temperature of the polymer chain is increased above its LCST. Shrinkage of the chains is due to the dispersion of the water present between chains to form a gel [151]. Here, the sol-gel transition state is rapid and reversible too. With such fast transition to temperature stimuli, drugs can be quickly released from the hydrogel, exhibiting on-off switching release system [152]. PNIPAAm along with its copolymers is extensively used for biomedical applications, including as embolic agents, for drug delivery, as a nucleus pulposus replacement [150], as an injectable multifunctional scaffold for tissue engineering applications and for the treatment of ocular diseases [153]. PNIPAAm, due to its structural function, preferably is not in its pure form and due to its poor mechanical behaviour, and PNIPAAm based hydrogels exhibit low compressive modulus with poor elastic recovery after drug loading [154-156]. In addition, PNIPAAm based hydrogel suffers from limited amount of drug released with respect to change in temperature [157]. The crosslinked bond in PNIPAAm hydrogel is non-biodegradable, resulting in the formation of a non-biodegradable hydrogel. A way of solving this problem is free radical copolymerization of NIPAM with hydrolyzable monomers. Hydrolysis of these groups increases the overall hydrophilicity of the copolymer, which in turn results in an increase of the LCST. When the LCST passes body temperature, the polymer becomes soluble in body fluids and can potentially be secreted by the kidneys when the molecular weight is below a threshold of 45 kDa [155].

Poly(ethylene glycol) (PEG), otherwise known as poly(oxyethylene) or poly(ethylene oxide) (PEO), is one of the most widely used hydrogels in medicine and biomedicine. EG-based hydrogels are characterized by their high biocompatibility, lack of toxic influence on surrounding tissue and solubility in water, which makes them good candidates for drug delivery system applications [158, 159]. Poly(ethylene glycol) (PEG) based copolymers play a crucial role as a biomedical material for biomedical applications, because of its biocompatibility, biodegradability, thermosensitivity and easy controlled characters. PEG hydrogel drug release systems are stimuli sensitive and react in the presence of a physical or chemical (biological) agent. Because of their unique properties, hydrogels used for these kinds of drug release systems are called “smart” or “intelligent gels”. Physical stimuli include temperature, solvent, light, radiation, pressure, a magnetic, acoustic or electrical field, while the chemical and biological stimuli include pH, specific ions, and molecular recognition events [160]. Chemically cross-linked PEG hydrogels are used in the manufacture of synthetic, biologically active hydrogel scaffolds for protein recombination and functional tissue production. But the main field of PEG hydrogel applications is in DDSs, as drug carriers in the efficient and controlled release systems of drugs, proteins and biomolecules, and growth factor distribution [161, 162].

Pluronic are made up of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) in which PPO is the hydrophobic segment and contributes for 30% of the block copolymer whereas, PEO is a hydrophilic segment contributing 70% of the block copolymer. Among various types of pluronics, pluronic F127 (PF127) has gained considerable attention due to its wide

range of biomedical applications. The PPO segment of PF127 comprises of a hydrophobic core as a microenvironment for incorporation of lipophilic drugs whereas, PEO segment of PF127 prevents the adsorption and aggregation of incorporated protein [163]. PF127 in situ gel system has attained significant consideration due to ease of preparation, having no use of organic solvents, site-specific delivery and improved patient compliance [164, 165]. The pluronic system is thermosensitive in nature which exists as liquid at low temperature and becomes semi-solid gel at higher temperatures. This reversible transition behavior of PF127 in situ gel system has been applied in different fields of drug delivery of various bioactive agents [166-170]. PF127 alone can partly solubilize the hydrophobic drugs due to which the in vivo release of incorporated drugs can only last for several hours to few days [168]. Another shortcoming of PF127 gel system is the inconstant drug release rate as the drug loaded in hydrophilic phase (PEO) part releases at the initial phase before the dissolution of the gel system while the release of drug loaded in the PPO part of PF127 requires the dissolution of the gel system. Besides acting as a carrier for drug delivery, PF127 can directly translocate into cells and by interacting with various cellular functions it may alter processes like mitochondrial respiration, apoptotic signal transduction, activity of drug efflux transporters and gene expression [163]. However, few weak mechanical properties of PF127, its rapid dissolution in contact with aqueous media or biological fluid and short residence time were found to be some critical challenges for its applications [171, 172][22]. Owing to the presence of terminal hydroxyl group in PF127 that may be easily chemically and/or covalently conjugated to achieve desired therapeutic outcomes.

4. 3D printing of nanoparticle-containing hydrogels and bioinks

Traditional methods in the areas of cancer therapy and tissue engineering have major clinical challenges. Conventional cancer treatments are related with overdosage and lack of specificity, while tissue engineering appears connected with problems such as donor shortage for transplantation, secondary injury, and transplant rejection. To surpass these, hydrogels have been widely applied, as they can function as mechanical support for tissue engineering and emerging drug carriers for cancer treatment (e.g. scaffolds). The necessity to create individualization of treatments can be met by the use of three-dimensional printing (3DP) or additive manufacturing (AM), allowing personalization of the patient care. It is possible to create products with a variety of purposes including drug delivery systems where a precisely control amount of dosed drug is released, based on patient characteristics like age, lifestyle, disease stage, and so on. Regarding tissue engineering, it also enables the production of complex geometries and structures including prostheses and porous scaffolds, with a considering enhancement in terms of precision when compared with traditional techniques. The use of a computer aided (CAD) software combines an increase in accuracy, when designing shape and geometry, with enabling reproducibility [173, 174]. In the biomedical field, many AM techniques are applied including material extrusion (e.g., fused deposition modelling (FDM), Gel or paste extrusion),

vat polymerization (e.g., stereolithography (SLA), digital light projection (DLP)), powder bed fusion (e.g., selective laser sintering (SLS)), binder jetting (e.g., powder bed inkjet printing), material jetting (e.g., inkjet printing, polyjet), and sheet lamination (e.g., laminated object manufacturing). Usually, mentioned methods differ from each other by device construction and selection of suitable material for each method, layer bonding methods, efficiency of production, but also characteristics of the obtained object (e.g., geometric accuracy, surface finish, structure, mechanical properties). It is possible to implement these elements in various sectors of industry depending on the materials used (i.e., polymers (natural and synthetic), metal, ceramic, resins, or even living cells, but also merger of basic materials with additions like nanomaterials (e.g., carbon nanofibers, carbon nanotubes, graphene)). The ability to produce a complex structure without compromising the precision of manufacturing as enable the recent expansion of 3DP to the biomedical field.

Material extrusion designates the selective extrusion of a wire shape thermopolymer or a gel/paste, in case of fused deposition modelling or gel and paste extrusion, respectively. Material extrusion techniques have been applied in a wide range of biomedical applications including customized medicine for drug delivery, customized patient specific implants and prostheses, and scaffolds for tissue engineering and regenerative medicine. All resin-based 3D printing, in which a liquid photopolymer resin is stored in a vat (as opposed to being ink-jetted through a nozzle), is collectively known as vat polymerization. SLA and DLP differ from each other by the UV light source used to cure the resin. In case of SLA a laser, precisely controlled by rotating mirrors to “draw” out each layer being printed, is implemented while DLP uses UV projectors, which work by using micromirrors to control the projected light. The cross-sections of each layer are projected in a single go, curing everything in the layer at once. These have been applied in the fabrication of drug-loaded scaffolds, cell containing hydrogels, and spatially patterned tissue engineering scaffolds. SLS consists of the sintering or melting of a powder polymer with a laser, being mostly used in the dental field. Powder Bed Inkjet printing bonds a powder material with selectively jetted liquid binder and has been used to manufacture surgical templates, drug delivery devices, and resorbable devices. Polyjet and inkjet printing work in a similar way, but while the last consists of injecting droplets, the first injects layers of liquid photopolymer into a tray that harden when exposed to ultraviolet rays. Both appear mostly applied in the dental field. Finally, laminated object manufacturing, where occurs lamination of sheet photopolymer material in a layer-by-layer by laser, is mostly used for anatomical models [175].

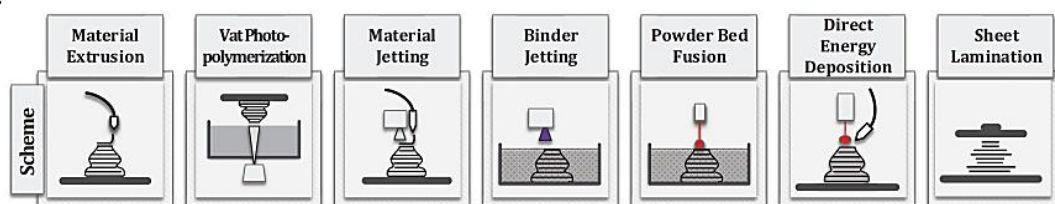


Figure 4. Visualization of different 3D printing methods [149].

The possibility of using several biomaterials simultaneously decreases the production time and enables customization of drug dosage in a single medication. AM technology also enables the precise manufacturing of the most important scaffold factor – open porosity, which allows the flow of nutrients and metabolites, as well as enables tissue growth and provides appropriate mechanical properties [176]. Biomaterials in AM technology, especially used in tissue engineering, ought to be biocompatible, easy biodegradable and sterilizable. For biomedical applications, the most commonly used materials are natural and synthetic polymers. Not all materials have suitable properties for human body applications. Hydrogels usually exhibit excellent swelling properties, but poor mechanical properties. For cancer treatment, hydrogels' swelling ability makes them ideal as a drug carrier for localized cancer therapy. Regarding tissue engineering, hydrogels can be modified by crosslinking and provide a good environment for the cell growth and tissue regeneration. By controlling process parameters in AM technology and proper selection of the material, it is possible to achieve desired mechanical properties, where the properties of the implanted scaffold should be similar to this of the host tissue and should maintain its mechanical properties until it is replaced by new growing tissue. Research concerning the development of new materials exhibiting relevant characteristics and their compatible chemical and mechanical properties are the main aims of the revolution in the biomedical field. For the purpose of this review, a variety of materials printed through material extrusion for cancer photothermal therapy, magnetic hyperthermia, and tissue engineering, will be presented.

Zhang, J., et al. manufactured mesoporous bioactive glass (MBG) scaffolds to be implemented in magnetic hyperthermia. For this, a mixture of MBG powders and magnetic Fe_3O_4 nanoparticles were ground and passed through 400 mesh sieves, resulting in a particle size of less than $37\text{ }\mu\text{m}$. To improve the mechanical properties of the Fe_3O_4 /MBG/PCL scaffolds, PCL was added as a binder, with the purpose of secure the MBG powders and Fe_3O_4 nanoparticles together. The mixture was quickly stirred at room temperature until it had formed a paste for injection. Finally, the prepared paste was introduced into a polyethylene injection cartridge that was fixed onto the 3D Bioplotter™ printing device. Square block models ($6\times6\times6\text{mm}$) were loaded on the CAD software and the scaffold was plotted by layer-by-layer, through the extrusion of the paste as a fibre, up to 29 layers. The architecture was changed by plotting fibres with 0 and 90 angle steps between two successive layers, the dosing pressure to the syringe pump was 1.5-3.0 bar and the speed of the dispensing unit was 1.5-5 mm/s, with a nozzle size of 0.25 mm. Lastly, the obtained Fe_3O_4 /MBG/PCL scaffolds were dried in an oven at 37°C for 2 days to evaporate the chloroform. Figure 5. confirms the role of the PCL binder as the surfaces of each type of scaffolds are presented very smooth, and very small amount of powders can be observed on the surfaces, embedded in PCL matrix.

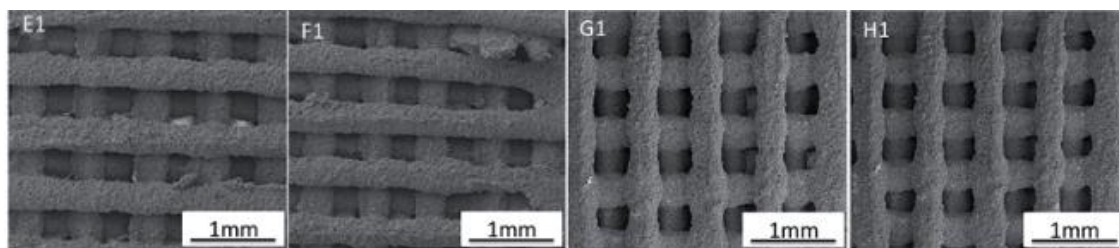


Figure 5. Photograph of the (E1) MBG/PCL, (F1) 5Fe₃O₄/MBG/PCL, (G1) 10Fe₃O₄/MBG/PCL and (H1) 15Fe₃O₄/MBG/PCL scaffolds printed by a 3D Bioplotter™; Magnification: ×100 [177].

The nanoparticles were characterized by TEM, ranging in size between 15 to 20 nm, indicating superparamagnetic behaviour, as sizes are below a critical value, generally around 20 nm. Photograph and SEM observation showed that scaffolds with 0 and 90 angle steps between two successive layers were successfully manufactured by the 3D Bioplotter™, exhibiting microporous structure (~400 μm). The Fe₃O₄ increased the structure resistance to applied compressive strength as the compressive strength of the MBG/PCL, 5Fe₃O₄/MBG/PCL, 10Fe₃O₄/MBG/PCL and 15Fe₃O₄/MBG/PCL scaffolds were estimated at 13.1 ± 1.6 MPa, 13.9 ± 1.4 MPa, 15.7 ± 4.0 MPa, and 16.6 ± 1.9 MPa, respectively. The porosities of the scaffolds did not present significant differences as the percentages were estimated at 59.6 ± 5.6 %, 62.9 ± 6.7 %, 61.7 ± 5.4 % and 61.7 ± 5.4 %, respectively. After soaking in SBF for 7 days, the MBG/PCL, 5Fe₃O₄/MBG/PCL, 10Fe₃O₄/MBG/PCL and 15Fe₃O₄/MBG/PCL scaffolds maintained high compressive strength, which were 10.9 ± 0.6 MPa, 11.8 ± 0.3 MPa, 13.2 ± 0.8 MPa and 14.0 ± 1.0 MPa, respectively [178].

Through gel extrusion for the same application, Aluri et al. fabricated a nanocomposite hydrogel based of Pluronic F127 and PEGylated magnetic iron nanoflowers (NFs) with integrated GO flakes. Pluronic provide a tuneable ‘soft’ shear-thinning matrix with is usually injectable or printable at room temperature, with Pluronic F127 (or F127, of average formula PEO101-PPO56-PEO101, with the sol-gel transition at 4 °C and excellent room-temperature shear-thinning) a particularly common choice. Suspensions of unmodified as-produced, or bare, NFs (here named BNFs) are not stable at physiological pH of 7 due to the absence of net surface charge, as is most often required for biomedical applications. Polyethylene glycol (PEG) is a linear polymer with chemically active hydroxyl groups at both ends, making it easy to conjugate with functional groups. Biomolecules or nanoparticles can be conjugated with linear PEG via functional groups, called the PEGylation process [179]. The GO flakes were implemented to mediate F127-induced NF aggregation, maintaining NF separation, and recovering the high hyperthermic potential of NFs. After stabilizing at pH 7, GO and PEGylated nanoflowers (PNFs) were mixed using a vortex and made into nanocomposite suspension (sPNF_GO) using HPLC grade H₂O. Pluronic P127 (40 w/v%) was added to the suspension and homogenized by cycling from room temperature to 4 °C (2 min) to room temperature till it dissolved. The hydrogel printing was performed by a dualhead Allevi-2 bioprinter. In order to load inks into printing syringe, samples were kept in the fridge until loading, removed from refrigerator and

immediately transferred as liquids (4 °C) in the syringes. Then, prior to printing, syringes were finally rested at 19 °C for 30 mins. Each gel was printed into a 15x15mm grid, using a straight stainless-steel needle with 152mm inner diameter. Different head velocities 200-300 mm.min⁻¹ and pressures 5.24-5.65 bar was used for different formulations. It was observed that samples with and without GO provided smooth prints using a standard extrusion format without significant optimization, and the deposited material showed good structural integrity. In this way, as it will be discussed further later in this review, PEGylation and nanocompositing with GO contribute to this improvement in the applications relevant format, without compromising printability for pluronics-based hydrogels. [180].

Qian, K.-Y., et al. produced an injectable hydrogel to implement in magnetic hyperthermia ablation of deep tumour. For this, iron oxide nanoparticles were manufactured and once again PEG stabilized in the form of iron nanocubes (iONS) to integrate silk fibroin hydrogel to confer deeper reach of the treatment. Silk fibroin hydrogels have been successfully designed as drug carriers and bioinks for 3D printing due to the injectability[181]. For this, iron(III) acetylacetonate was added to a mixture of oleic acid and benzyl ether. The mixture solution was degassed at room temperature for 1 h and then heated to 290 °C at the rate 20 °C/min under vigorous magnetic stirring. The reaction mixture was maintained at this temperature for 30 min. After cooling to room temperature, a mixture of toluene and hexane was added to the solution. The solution was then centrifuged to precipitate the Fe₃O₄ nanocubes. The separated precipitate was washed using chloroform, and coated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000](DSPE-PEG2000) in order to obtain a well-dispersed water suspension. To fabricate injectable magnetic hydrogel, IONCs suspension was mixed with silk fibroin (SF) solution at varying ratios, and then subjected to sonication for 3 min. Finally, the mixture was filled into the syringe and cultivated at 37°C until gelation was completed. As shown in Figure 6. the FSH could be injected through both large and small needles (18G and 22G) of syringes and its linear shape could be maintained in normal saline and fetal bovine serum (FBS).



Figure 6. Photographs of injection process of FSH into the solution of normal saline (room temperature) and fetal bovine serum (37 °C), respectively [182].

Through SEM imaging the microstructure of the magnetic composite hydrogel was accessed and it was shown a porous interconnected network structure. Although the iron oxide nanocubes could not be clearly observed in SEM image due to the extremely small size and well-dispersity in silk fibroin

network, the uniform distribution of iron in the composite hydrogel was confirmed in the EDS mapping image. Notably, lots of nanocubes could be clearly identified in the TEM image of the lyophilized FSH sample. The obtained injectable FSH showed quick response to applied magnet, and magnetic nanoparticles had been totally confined in the polymeric matrix in the magnetic manipulation process. According to the rheological results, the addition of the iron oxide nanocubes at the low concentration in this composite hydrogel rarely affected the injectable ability of silk fibroin hydrogel [182].

A novel fabricated structure was presented by Tang, J., et al., where a programmable shape transformation hydrogel of poly(N-isopropylacrylamide) (PNIPAm) embedded in Fe_3O_4 nanoparticles, able to undergo abrupt volume collapse due to magnetothermal effect, was fabricated through direct ink writing (DIW). DIW consists of the deposition of liquid-phase “ink” out of small nozzles under controlled flow rates on digitally defined paths to fabricate 3D structures layer-by-layer, a method heavily applied in micro-scales. It was required two types of inks, the magnetic hydrogel one and an elastomer one. Among the smart polymers, thermoresponsive polymer poly(N-isopropylacrylamide)(PNIPAM) is shows great potential as its temperature response is close to the temperature of the human body and can be finetuned as well. Mechanical properties are critical for the performance of stimuli responsive hydrogels in diverse applications. However, native PNIPAM hydrogels are very fragile and hardly useful for any practical purpose. Intense researches have been done in recent decade to enhance the mechanical features of PNIPAM hydrogel. With the purpose of bettering the polymer’s mechanical properties, nanoclay (physical crosslinker and rheology modifier) was added to the hydrogel ink. Finally the the mixture was stirred for 4 hours to form an uniform hydrogel precursor. Then, magnetic particles (3 wt%, 5 wt%, 7 wt%, 10 wt%, 15 wt%, 20 wt%) were added into the precursor, forming a transitional ink. Before printing, TEMED and APS solution were injected into the transitional ink as catalysts for the polymerization process. Finally, the ink was mixed and degassed by a planetary mixer (Thinky ARE-300) at 2000 rpm for 3 min to form a homogeneous printing ink. The ink of elastomer has a base of Ecoflex, a material with very low viscosity which can be stretched many times its original size without tearing and will rebound to its original form without distortion [183]. To increase viscosity the ink was prepared using nanosilica and a photoinitiator (benzophenone) was added to induce strong interface adhesion.

The shape transformation structure was manufactured using direct ink writing (DIW) of the magnetic hydrogel ink (15 wt% Fe_3O_4) and the Ecoflex elastomer ink (4 wt% of nanosilica). The printing system consists of two machines: a printer modified from a MakerBot ReplicatorTM 2X and an air pump system. The composite was manufactured by alternative printing, using one syringe loaded with the ink of magnetic hydrogel and another syringe loaded with the ink of elastomer with a printing speed of 30 mm/s and a printing pressure of 10~20 kPa for magnetic hydrogels, and 15~25 kPa for elastomers. The nozzle diameter is 0.51 mm for magnetic hydrogels and 1.2 mm for elastomers. During printing, the ink was loaded into a syringe connected with the two machines and squeezed out using an appropriate pressure onto a piece of glass placed on the printing platform. The elastomer ink is first extruded onto the heated printing platform (~110 °C) for in situ polymerization. The ink of

magnetic hydrogel is then printed onto the cured elastomer. The integrated structure is irradiated by a UV lamp for 4 h in a glove box of nitrogen atmosphere. During cure, the initiator can induce the formation of covalent interlinks between the polymer networks of magnetic hydrogels and elastomers. To demonstrate the strong interface adhesion between magnetic hydrogel and elastomer, a panda was printed and stretched vigorously in multiple directions. The panda was able to recover to its initial shape without any interface failure. The tensile properties of magnetic hydrogels were measured by varying the content of magnetic nanoparticle and nanoclay. The tensile strength increases from 208 ± 60 kPa to 379 ± 102 kPa, when the content of magnetic nanoparticle increases from 0 to 10 wt%. Nanoclays also show a strengthening effect on both the modulus and the tensile strength of magnetic hydrogels. Using the pure shear test it was determined that the highest toughness is ~ 9000 J m⁻² when the clay content is 10.6 wt%, among the highest of reported magnetic hydrogels. Interface adhesion is vital for the deformation of the composite structure. A 90-degree peel test was carried to measure the adhesion energy between magnetic hydrogels and elastomers. The adhesion energy increases from 0 to ~ 900 J m⁻², when the concentration of the initiator increases from 0 to 0.1 wt%. For the case of 0% benzophenone, the measured force is below the resolution of the peel test machine (± 0.01 N). It is noted that the intrinsic adhesion between hydrogels and elastomers is usually very weak (on the order of 1 J m⁻²), because of lacking covalent bonds. Here, adding the initiator into the volume of the elastomer ink induced covalent interlinks on the interface. In this way, the adhesion energy is markedly enhanced [184].

Still in the cancer treatment field, material extrusion has experienced modifications and has been applied in other sources of treatments, including photothermal therapy. In this area, Wei, X., et al. produced core-shell hydrogel fiber scaffolds including polydopamine (PDA)/alginate (Alg) as the shell part and gelatin hydrogels as the core part. Polydopamine (PDA) can greatly affect polymer's properties, due to the chemical and physical interactions between the polymers and PDA. PDA has demonstrated the ability to adjust the pore structures and increase the mechanical properties of alginate hydrogel-based scaffolds [185]. To prepare the stimuli-responsive material DA was dissolved in Tris-HCl solution and alginate was added stirring until it created a homogenous paste. Then, the prepared Alg-PDA inks and gelatin inks were loaded into two printing tubes. The core/shell fibers and scaffolds were fabricated by injection and extrusion-based 3D printing with GUI-based control software using co-axis core/shell nozzles with different sizes (15/20, 16/22 and 18/25 G). The scaffolds with designed shape (lattice) and size ($12 \times 12 \times 6$ mm in 8 layers and $6 \times 8 \times 3$ mm in 4 layers) were fabricated. The printing temperature was set at 25 °C and 60 °C for PDA/Alg and gelatin inks, respectively, with a speed of 5 mm/s, and pressure around 300-400 kPa. Afterwards, the prepared core/shell fibers and scaffolds were crosslinked by soaking them in 1M CaCl₂ solution for 5 min, followed by washing with deionized water three times. The size of the core-shell fibers could be easily controlled by adjusting the diameter of the used core-shell nozzles. Meanwhile, the structures of the printed core-shell fiber scaffolds could be tailored by computer-assisted design (CAD) to perfectly match the tissue defect caused by surgery. It should be noted that the concentration of

alginate inks used in this system was 15.3wt% because the highly concentrated alginate inks contributed to much dense wall of the shell fiber for preventing drug leakage from the fiber wall [186]. Lou et al. had demonstrated in previous studies that the highly concentrated alginate inks were excellent for 3D printing of scaffolds without deformation and collapse, especially for 3D printing of hollow fiber and core-shell fiber scaffolds [187-189].

With the same material base, Luo, Y., et al. executed a different approach by incorporating PDA in a dopamine-modified alginate and polydopamine scaffold through 3D Printing of inks based on the mixtures of Alg-DA and DA. First, Alg-DA was synthesized by standard carbodiimide coupling chemistry, followed by the addition of synthesized Alg-DA in 2mL of alkaline Tris-HCl solution (10 mM, pH 8.5) containing 0, 1, 5, and 10 mg/mL DA, respectively. The DA was oxidized and subsequently underwent self-polymerization into PDA nanoparticles. During this process, PDA nanoparticles were partly knotted with Alg-DA by chemical and physical interactions. Then, the mixture was stirred vigorously to form homogeneous paste and the prepared inks were transferred to a printing tube. The scaffolds were generated through conical plastic nozzles (22 G) using a 3D printer in layer-by-layer fashion, and the structures and macropores were designed and controlled by CAD and computer-assisted manufacturing (CAM). The printing speed was set at 4 mm/s with the dosing pressures ranging from 270 to 380 KPa (according to the content of DA in the inks). After the scaffolds were fabricated, they were transferred to 0.5 M manganese chloride tetrahydrate solution at room temperature and incubated for 10 min, followed by washing with deionized water three times. Mn^{2+} crosslinking not only acted in process of the gelation of alginate, but also coordinated with the PDA moieties including o-quinone, amino, and phenolic groups to form Mn cation complexation, thereby increasing the structural stability of the Alg-PDA scaffold. In addition, the interaction among the catechol groups in Alg-DA and PDA nanoparticles also contributed to the desirable flexibility and structural integrity of the printed Alg-PDA scaffold. SEM images showed the submicropores in pure alginate and Alg-PDA scaffolds with pore size smaller than 1 mm) and macropores with designed pore size of approximately 1 mm. The modulus of the fabricated scaffolds (2.08 ± 0.24 kPa) was of the same order of magnitude as that of human breast tissues (3.25 ± 0.91 kPa). Furthermore, the compressive strength and modulus of scaffolds were able to be tailored by the porosity, pore size, and the concentration of the hydrogels. In addition, cyclic compression tests were also performed on the fabricated scaffolds, and the results showed that the scaffolds could maintain the mechanical strength even after 20 loading cycles (40% compression). These results indicated that the fabricated scaffolds with adequate flexibility and mechanical stability could be applied as a breast filler from the view of mechanical properties, granting a second function besides the photothermal properties explored later [190].

Combining photothermal therapy with smart drug delivery Liu, C., et al. developed a DOX/PCL core/shell scaffold coated with polydopamine (PDA) in an attempt of enhancing its photothermal effect. The anthracycline doxorubicin (DOX) is widely used in chemotherapy due to its efficacy in fighting a wide range of cancers such as carcinomas, sarcomas and hematological cancers [191]. The core gel scaffolds were firstly fabricated through 3D printing by paste extrusion. A gelatin solution

(30 wt%) was prepared by dissolving gelatin powers in 80 °C hot water under magnetic stirring (500 rpm). Then, doxorubicin hydrochloride (DOX) (2 mg/mL) was added into the solution under magnetic stirring. Afterwards, alginate powders were added, and the mixture was thoroughly stirred until homogeneous printing pastes obtained. The printing pastes were loaded into printing tube equipped with 18 G dosing needle, and the alginate/gelatin scaffolds were fabricated by 3D printing system according to computer assisted design (CAD) with a printing speed of 8 mm/s and dosing pressure of 300-500 KPa. After the scaffolds were fabricated, the scaffolds were crosslinked by incubating in 0.1M CaCl_2 solutions for 10 seconds. Then, these were rinsed by deionized water for three times and partly dried at room temperature for 1 h. PCL coating alginate/gelatin scaffolds (Gel/PCL core shell scaffolds) were fabricated by soaking the printed hydrogel scaffolds in a viscous PCL/chloroform solution (10%, 20% and 30 wt%, respectively). Afterwards, the scaffolds were removed from the chloroform solution and placed in a ventilated place for the evaporation of chloroform. The hydrogel scaffolds were soaked in 30 wt% PCL solution for 5 min with PCL coating thickness of ~220 μm . Then, the scaffolds were immersed in ethanol/water (75%) mixture for 1 h to remove any residual chloroform. Finally, PDA coated Gel/PCL core/shell scaffolds were fabricated by soaking the Gel/PCL core/shell scaffolds in dopamine solution (5 mg/mL in Tris buffer, pH = 8.5) for 24 h. Afterwards, the scaffolds were rinsed by deionized water for three times and kept in 4 °C for further use.

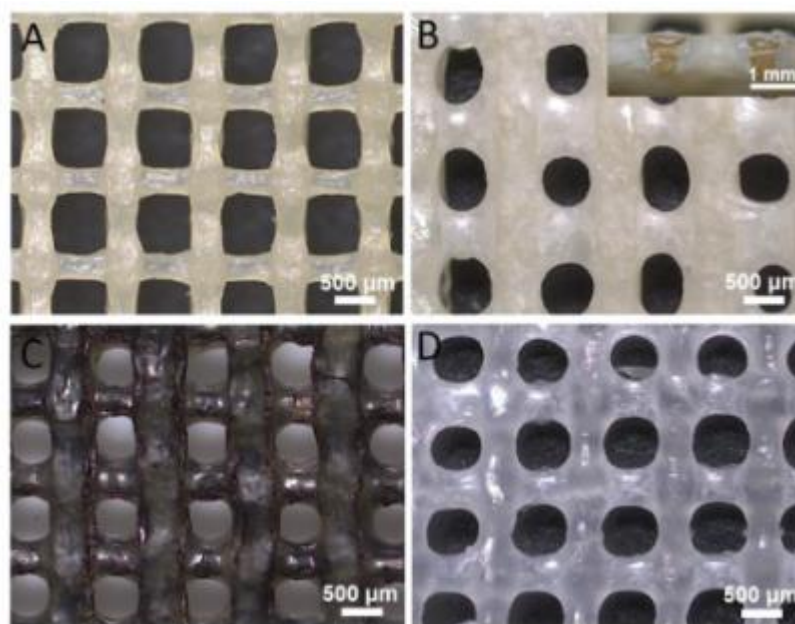


Figure 7. Photographs of 3D printed alginate/gelatin scaffold with regular morphology (A); Gel/PCL core/shell scaffold with a complete PCL coatings on the surface (B), and the insert image showing that PCL could cover the entire surface of the printed Gel filaments; Gel/PCL/PDA core/shell scaffold with a deep brown surface in color, indicating the homogeneous PDA coating on the surface (C); and the remained pure PCL scaffold after removal of the core hydrogels (D) [186].

SEM analysis showed that the the surface of the Gel/PCL core/shell scaffold contained a large number of micropores (pore size around 1-5 μm), which were generated by phase separation during

solvent evaporation. The generated micropores could greatly increase the specific surface area of the scaffolds and promoting cells adhesion and accelerate the biodegradation of scaffolds. The mechanical properties of the scaffolds were measured, showing a tensile strength and Young's modulus of 59 ± 11.3 KPa and 0.53 ± 0.24 MPa, respectively. While, after soaking the gel scaffolds in 30% PCL solution for 30 min, the tensile strength and modulus of the Gel/PCL scaffolds were 253 ± 51.1 KPa and 2.98 ± 0.2 MPa, respectively. The PDA coatings showed no significant impact on the mechanical properties of the scaffolds since the PDA coating thickness on the surface of scaffold was no more than 50nm. The printed alginate/gelatin scaffolds, as well as the homogeneous PCL coatings both maintained good structural integrity, which could contribute to the enhanced mechanical properties. The scaffolds' mechanical properties could be tailored by the thickness of the PCL coatings and the pore size. The degradation of the scaffolds was studied by incubating the scaffolds in PBS (pH = 7.4) at 37 °C. These showed a fast degradation rate, with only ~15% weight remaining after 7 days of incubation, possibly because only the alginate was partly crosslinked by CaCl_2 , while the gelatin was not crosslinked. The degradation of PCL scaffolds also could be tailored by the molecular weight of PCL and the thickness of the PCL coatings [192].

In tissue engineering, material extrusion remains an important tool to fabricate complex 3D constructs with favourable cell adhesion and proliferation allied with desirable mechanical properties. In this area, Luo, Y., et al. fabricated highly concentrated alginate/GelMA bioinks via co-axial 3D printing (coaxial nozzle composed of an outer and an inner needle) and in situ mineralization, under mild conditions. Alginate and GelMA are widely used hydrogel materials for tissue engineering and 3D bioprinting because of their excellent biocompatibility, great printability and easy gelation in mild conditions [187]. Alginate and GelMA based hydrogels have also been applied for bone tissue engineering, while hydrogels were generally mechanical weak and lack of osteogenesis for bone repair. Therefore, concentrated alginate/GelMA were prepared as the bioinks for 3D printing scaffolds, as well the biomimetic nano apatite was introduced on the surface of the hydrogel scaffold for bone tissue engineering.

The printing inks were prepared using GelMA and Na_2HPO_4 solution (0.5 M) mixed under stirring, followed by adding alginate in the solution, and the mixture was stirred vigorously until homogeneous pastes were obtained. The hollow fiber scaffolds with mineral coatings (HFM) were fabricated by extruding the prepared bioinks through core/shell (co-axial) printing nozzles via 3D printer. The printing speed was 5 mm/s and dosing pressure was 520-550 kPa. Afterwards, the printed scaffolds were firstly crosslinked by UV light (365 nm, 150 mW, 10 min), and then they were transferred to 1 M CaCl_2 solution (pH = 9) and kept for 30 min. Finally, the scaffolds were washed with deionized water and dried at room temperature. As control, non-hollow scaffolds with mineral coatings (NHM) were fabricated by extruding the prepared inks (with 0.5 M Na_2HPO_4) through normal printing nozzles. Hollow fiber scaffolds (HFS) and non-hollow fiber scaffolds (NHS) without mineral coatings were fabricated by extruding inks (GelMA/alginate mixing with 0.5 M NaCl instead of Na_2HPO_4 solution) through core/shell printing nozzles and normal nozzles, respectively. After irradiating by UV

light, the control scaffolds were also incubated in 1M CaCl_2 solution (pH = 9) for 30 min, followed by washing and drying for further use. Nano apatite coatings with thickness of 10-80 μm were coated on both the inner and outer surfaces of HFM scaffolds, homogeneously.

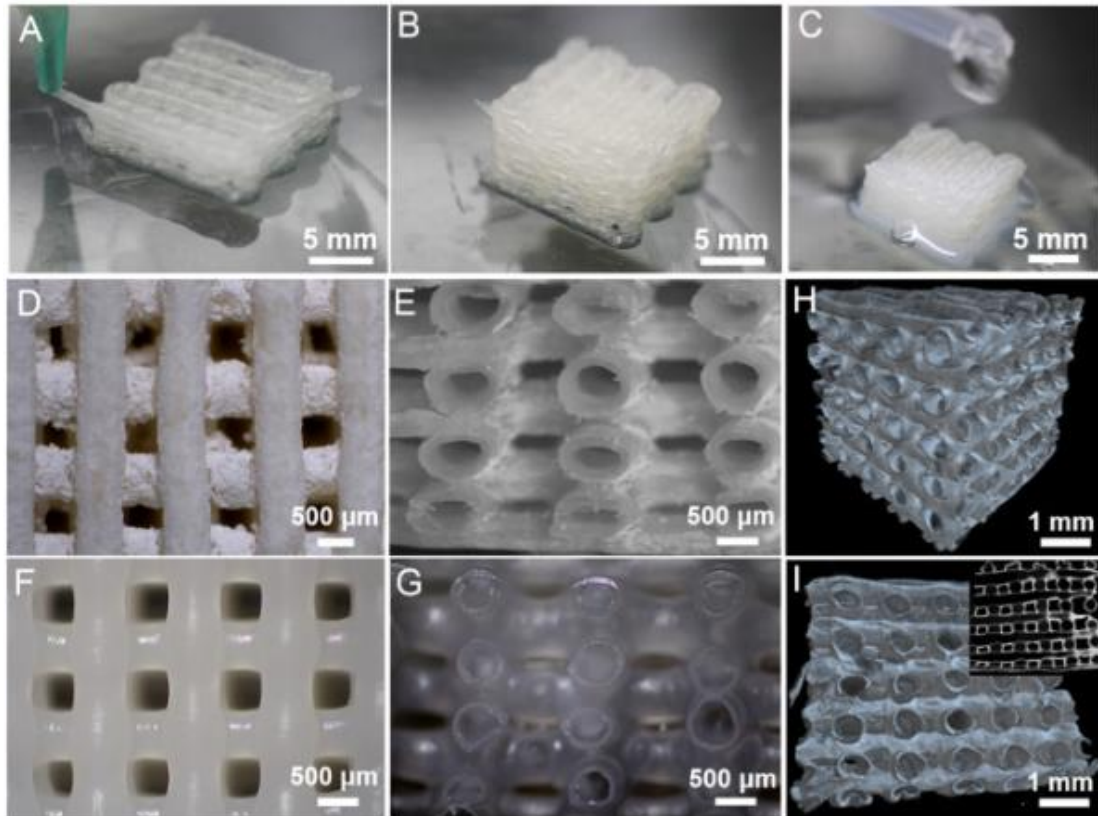


Figure 8. The photographs of 3D printing hollow scaffold using co-axial nozzles (A) and the printed scaffold with 20 layers could maintain the structural integrity (B), and white minerals produced on the surface of the scaffold after dripping CaCl_2 solution to the scaffold (C). The microscopic images of 3D printed HFM (D, E) and HFS (F, G) scaffolds in top (D, F) and vertical sections (E, G). The micro-CT images of the 3D printed HFM scaffolds, and the insert image is a two-dimensional slice of HFM scaffold (H, I) [193].

Microscopic images showed a homogeneous layer of white minerals covering on the surface of HFM scaffolds, while no apparent minerals were observed on the surface of HFS scaffolds. It was also observed that HFM and HFS scaffolds not only had macro pores, but also possessed hollow channel structures. Additionally, micro-CT analysis further confirmed the interconnected macro pores, opened hollow channels and the mineral coatings on the entire surface of the HFM scaffold. SEM images showed a layer of nano minerals coating on both the outer and inner surfaces of the hollow fibers, homogeneously, with layer thickness of $\sim 10\mu\text{m}$ in inner surface and $\sim 80\mu\text{m}$ in outer surface tightly. The porosity of HFS and HFM scaffolds were $85.1 \pm 3.8\%$ and $78.7 \pm 3.2\%$, respectively. However, the porosity of NHM scaffold was only $37.2 \pm 3.5\%$, meaning the porosity was doubled when the hollow channel structures were produced in scaffolds. Therefore, the porosity of scaffolds for tissue engineering could be significantly increased by introducing hollow channels in 3D printed struts instead of enlarging the pore size, enabling it to match the optimal range for new tissue formation.

The compressive strength and modulus of 3D printed scaffolds in wet states were tested and showed that the compressive strength and modulus of HFM scaffolds prepared by 15/20 G nozzles were 0.136 ± 0.017 MPa and 0.42 ± 0.08 MPa, respectively. As expected, the strength and modulus of HFM scaffolds were significantly lower than those of NHM scaffolds (0.324 ± 0.04 MPa, 1.24 ± 0.18 MPa, respectively), as the hollow channels increased the total porosity of scaffold and decreased the density of the struts. In addition, the compressive strength of HFS scaffolds prepared by 15/20 G nozzles were 0.104 ± 0.003 MPa, which is lower than that of HFM scaffolds prepared with same size of nozzles. The nano apatite coatings with thickness of 10-80 μ m demonstrated potential for enhancing mechanical properties. However, nano mineral coatings did not greatly increase the compressive strength and modulus of the HFM scaffolds probably due to the porous structures of the nano apatite layer. On the other hand, the compressive strength and modulus of printed HMA scaffolds were higher than those of hydrogel scaffolds prepared with low concentration of alginate, probably because the HMA scaffolds prepared by concentrated hydrogel inks had dense structures [193].

Another application of the GelMA combined with alginate was the production of a 3DP composite hydrogel scaffold GelMA/B-TCP/sodium alginate (Sr²⁺)/MXene (Ti₃C₂) (GTAM) by Nie, R., et al. Gelatin methacrylate (GelMA) is gelatin modified with photopolymerizable methacrylic anhydride (MA) groups, which not only possesses temperature-induced physical crosslinking through gelatin but can also be photo-crosslinked permanently under mild conditions in the bioprinting process [194]. Compared to gelatin, photo-crosslinked GelMA is more stable and controllable at body temperature, and at the same time, it has similar biocompatibility [195]. B-TCP is a common bioceramic material for bone tissue engineering. It has been used as a synthetic bone substitution in orthopedics and dental maxillofacial application [196]. Furthermore, the interconnected microporosity of B-TCP is beneficial to osteoconduction capacity. Sodium alginate (Alg) can be crosslinked with divalent cations such as calcium (Ca²⁺) or strontium (Sr²⁺) to form hydrogels with stronger mechanical and biological performances. Interestingly, Ti₃C₂ has also been proved to accelerate bone tissue regeneration after osteosarcoma therapy [197].

The bioinks were prepared using GelMA (5% w/v) dissolved in photoinitiator LAP (0.25% w/v) PBS solution and immersed in deionized water at 50 °C for 30 min without light. After that, B-TCP (1% w/v) was added to the GelMA solution and stirred for 2h. Subsequently, Alg (2% w/v) and MXene (Ti₃C₂) dispersions (0.1 mg.ml⁻¹, 0.3 mg.ml⁻¹ or 0.5 mg.ml⁻¹) were mixed into the GelMA-TCP precursor solution respectively at 50 °C for 2h in the dark. Finally, 5 kinds of bioinks were prepared according to the above-mentioned methods: GelMA-TCP (GT), GelMA-TCP-Alg (GTA), GelMA-TCP-Alg-0.1mg.ml⁻¹ MXene (GTAM0.1), GelMA-TCP-Alg-0.3mg.ml⁻¹ MXene (GTAM0.3), GelMA-TCP-Alg0.5mg.ml⁻¹ MXene (GTAM0.5). The bioinks were kept at 4 °C prior to 3D bioprinting. The overall dimensions were 10×10×1.5 mm (in vitro) or 5×5×1.5mm (in vivo) through a 200 μ m diameter nozzle. The printing temperature was set at 20 °C to dispense the bioinks smoothly. The cooling plate was precooled to 4 °C for the solidification and stabilization of the printed hydrogel. In the course of 3D bioprinting, the printing parameters were set as 0.15mm layer thickness, 10 layers, 1mm line space, 10mm.s⁻¹

printing speed. Scaffolds were crosslinked by 405nm blue light for 15s on a cooling plate for the preliminary photo-crosslinking solidification of the hydrogel. Eventually, the samples were permanently crosslinked with SrCl_2 (2% w/v) solution. The Ti_3C_2 was purchased and prepared by etching Al from MAX-phase Ti_3AlC_2 ceramics with LiF and HCl. Subsequently, the multilayered Ti_3C_2 was subjected to sonication. The printed structures are present in Figure 9.

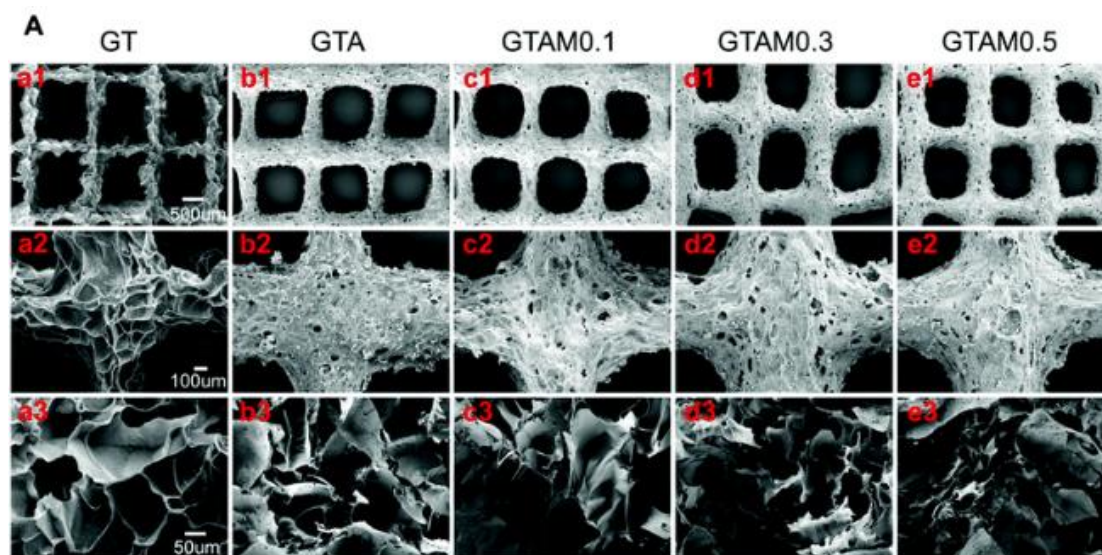


Figure 22. Characterization properties of 3D printed scaffolds. (A) SEM images of GT (a1-a3), GTA (b1-b3), GTAM0.1 (c1-c3), GTAM0.3 (d1-d3) and GTAM0.5 (e1-e3) scaffolds.

TEM and AFM images both revealed the 2D ultrathin structure of Ti_3C_2 . The thickness of the layered Ti_3C_2 nanoflake was about 4nm. MXene dispersions in different concentrations were mixed into the precursor hydrogel bioinks to form GTAM0.1, GTAM0.3 and GTAM0.5. Scaffolds were photo-crosslinked and Sr^{2+} ionic-crosslinked after printing. The structure was more stable because of more entanglement between polymer chains. Mechanical tests showed that compressive strength and compressive Young's modulus were significantly higher in GTA and GTAMs as compared to GT scaffolds, and GTAM0.3 and GTAM0.5 were the highest meaning that hydrogels with added Alg (Sr^{2+}) and Ti_3C_2 notably increased the mechanical strength by increasing the interactions between the polymeric network in both physical and chemical crosslinking. In addition, the concentration of Ti_3C_2 was proportional to the mechanical strength. In the UV-vis absorption spectrum the MXene dispersion liquid exhibited a distinct absorption peak at in the range of biological window NIR-I (750 nm- 1000 nm) around 800 nm, which made MXene a desirable candidate for photothermal agents for PTT. The zeta potential decreased as the concentration of MXene increased meaning the MXene conferred stability to the system. The degradation rate of bone tissue engineering scaffolds should match the speed of new bone formation. GT scaffolds were completely degraded at 17 days in PBS. However, the remaining rate ratio of GTA and GTAMs were about 40% at 8 weeks. GTAM0.3 and GTAM0.5 had the highest retention rates. The degradation of hydrogels was due to the limited long-term stability of photocrosslinked GelMA and ionic-crosslinked Alg in physiological conditions. The results also

demonstrated that the reinforcement of Alg (Sr^{2+}) and Ti_3C_2 nanosheets in scaffolds reduced the degradation rates due to the increased crosslinking density, which were matched to the rate of osteogenesis[198].

In another study, Dutta, S.D., et al. fabricated a 3D-printed hybrid biodegradable hydrogels composed of alginate, gelatin, and cellulose nanocrystals (CNCs) by DIW for bone tissue engineering. CNCs are well-known for their ultra-high mechanical strength and low thermal expansion coefficient [199]. The 3D-printed hybrid biodegradable hydrogels composed of alginate, gelatin, and cellulose nanocrystals (CNCs) were prepared to provide a favorable environment for cell proliferation, adhesion, nutrients exchange, and matrix mineralization for bone tissue engineering (BTE) applications. For this, the CNCs gel was diluted in $1\times$ phosphatebuffered saline (PBS) solution to obtain a final concentration of 0.5, 1, 2, and 4% (w/v). The required amounts of Na-alginate powder were added into the homogenous solution of CNCs with continuous mechanical stirring at 70°C for 20min, followed by incorporating the calculated amounts of gelatin in the solution at 45°C . The solution was kept in a refrigerator at 4°C overnight to initiate gelation. The hydrogels were stored at 4°C and incubated at 37°C before use. All the five hydrogels containing different concentrations of CNCs (0, 0.5, 1, 2, and 4%) were designated as Alg/Gel, 0.5% CNC/Alg/Gel, 1% CNC/Alg/Gel, 2% CNC/Alg/Gel, and 4% CNC/Alg/Gel, respectively. The hydrogels were incubated at 37°C before the printing and carefully taken into plastic printing cartridges. The printing was conducted using CELLINK BIO-X, and the structures were printed directly into sterile petri-plates on a precooled print bed ($4\text{--}6^\circ\text{C}$). The structures were designed by the Solidworks software. The square ($5\times 5\times 0.8\text{mm}$), rectangular ($50\times 15\times 0.8\text{mm}$), and round ($5\times 5\times 0.8\text{mm}$) structures of five perpendicular layers were printed carefully using a 27G (0.2 mm) metalhead needle, with needle size of 27G, pressure ranging from 100kPa to 500kPa, for different cellulose concentrations, and a speed of 5mm/s. The scaffolds were treated with or without CaCl_2 (100mM). After 1h of crosslinking, the printed scaffolds were washed with $1\times$ PBS (3 times) to remove the excess calcium from the scaffolds. The morphologies of the crosslinked scaffolds were evaluated by FE-SEM, and the images showed that the crosslinked scaffolds exhibited a smooth morphology, exhibiting a good dispersion of the CNCs. This is attributed to the presence of Ca^{2+} , which facilitates the strong interaction between the hydrogel components. The enhanced crosslinking was observed in the composite hydrogel compared to the pure polymer hydrogel, and this tendency was further increased by adding the crosslinking agents in the printed scaffolds. The Alg/Gel scaffold exhibited higher swelling potential compared to CNCs incorporated Alg/Gel scaffolds. It is well-known that the swelling potential of the scaffolds is profoundly affected by the availability of hydroxyl groups, amorphous regions, the degree of crosslinking, and crystallinity. The lower swelling potential of the composite scaffolds compared to the Alg/Gel scaffold is associated with their improved crosslinking ability in the presence of CNCs, which hindered their water uptake potential. A decrease in the expansion ratio was observed in composite scaffolds with increasing CNCs concentrations, showing their semi-solid behaviour causing the reduction in the swelling potential.

The presence of CNC also induced improvements in the mechanical strength of the printed hydrogels, including the increase of the G' value (storage modulus). This alteration points to a better interaction between the polymer matrix and incorporated CNC, which restricts the motion of the polymer chains. The restriction in motion of the polymer chains in the presence of CNC facilitates the crystalline structure in the scaffolds and subsequently increased the G' values compared to the pure polymer hydrogels. The integrity factor of both un-crosslinked and crosslinked samples was evaluated after 5 layers of printing and compared with control samples as described in the previous literature. Unlike previous parameters, CaCl_2 crosslinking significantly improved the layer integrity of the printed scaffolds. The structure of the printed scaffolds was maintained after the freeze-drying, indicating their stability. [200].

Heo, D.N., et al. developed a novel functionalized hydrogel in order to satisfy the high biomimetic requirement for success in bone tissue regeneration. Using the biodegradable thermoplastic polylactic acid (PLA) as the 3D printed microstructure in combination with photo-curable gelatin hydrogels as the encapsulation matrix for the incorporation of cyclicRGD conjugated GNPs (RGNP) the 3D printed microstructure was manufactured via fused deposition modeling (FDM). Combining PLA, which has emerged as an important polymeric material for biomedical applications on account of its properties such as biocompatibility, biodegradability, and mechanical strength, with GelMA applied to induce excellent cell attachment and cell proliferation of various cell types, these requires might be met [201]. Synthesized GelMA was dissolved in dulbecco's phosphate-buffered saline (DPBS) containing 0.1 wt% photo-initiator at 8 wt%. Subsequently, 1 mL of these mixed solutions were injected in the 3D PLA microstructure and exposed to UV light for 60 sec. To compare the functionality of the GNPs and RGNPs, 1 mL of aqueous GNPs and RGNPs solution were centrifuged, and the pellets were then added into 4 mL of the above mixture and labelled as 'Gel-GNP' and 'Gel-RGNP'. All samples were incubated in DPBS at 37 °C for 24h before further experiments. For embedding the hydrogel inside the 3D PLA microstructure, the CAD based architectural models with the square open pore were designed with various fiber spacing from 0.6 mm to 1.4 mm and printed using a FDM 3D printer. The 3D PLA microstructures were composed of parallel aligned fibers measuring 200 μm thickness at a 250 μm layer height. The resultant scaffolds had porosities varying from 74.2 % to 86.8 %. All 3D PLA scaffolds had multi layers of parallel aligned fibers with orthogonal orientation. AFM images showed that the synthesized RGNPs have a near-spherical shape with well-dispersion in the range of 20-40 nm. All PLA scaffolds with 0.6 mm fiber spacing demonstrated compressive modulus similar to human mandibular bone (431.0 ± 217.0) being collected 401.0 ± 29.2 MPa for PLA scaffold, 378.9 ± 27.7 MPa for PLA infilled with Gel, 402.0 ± 26.9 MPa for PLA infilled with Gel/GNP. These results corroborate the combination of GelMA with PLA in order to acquire the needed mechanical properties [202].

In another approach of simulating the bone tissue mechanical properties, Radhakrishnan, S., et al. synthesised silver nanoparticles (AgNPs) in polycaprolactone (PCL) solution by in-situ reduction and further extruded into PCL/AgNps filaments. PCL solution (10% w/v) was prepared by dissolution

in solvent mixture (7:3 volume ratio of THF and DMF respectively) at 90°C. Silver nitrate with 2 different percentages (1% and 3% w/v) was added into the PCL solution and the temperature of the solution was maintained at 90°C. Resultant solution was refluxed for 3h to facilitate the reduction of silver nitrate into silver nanoparticles. Brown polymer suspension was obtained which was cooled and precipitated using 300 mL of ethanol and dried at room temperature. PCL/AgNps composites prepared using 1% and 3% silver nitrate are denoted as C1Ag2 and C3Ag2. PCL, C1Ag2 and C3Ag2 were extruded into filament by Noztek pro extruder at 120°C. 2.3. PCL/AgNps scaffolds were fabricated using FDM technique, using DesignSpark Mechanical® 2.0 software. The extruded filament was cast into 3D printed matrix by using FDM based 3D printer (PRUSA i3) and with a nozzle diameter of 0.4mm, extrusion width of 0.45mm, layer height of 0.05mm at a speed of 20-30mm/s. Furthermore, the scaffold made from the filaments of PCL, C1Ag2 and C3Ag2 are denoted as PCL, 1Ag2 and 3Ag2.

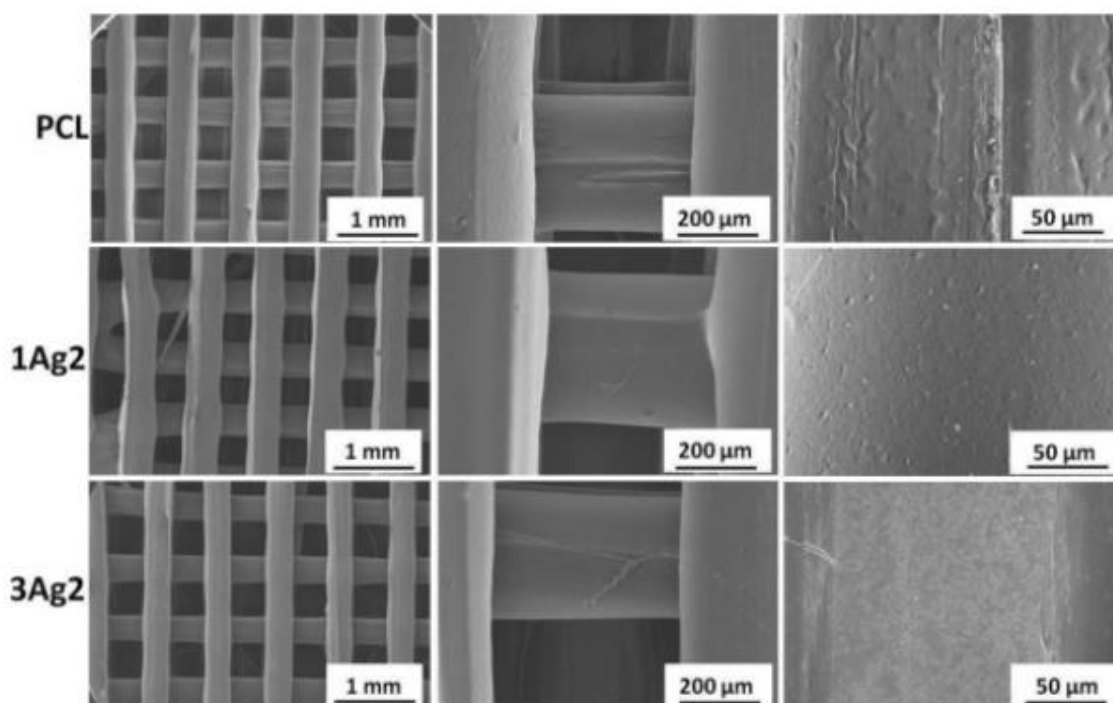


Figure 23. SEM analysis of 3D printed scaffolds.

SEM micrographs revealed that the PCL and PCL/AgNps 3D scaffolds matrix displayed uniform porous structures with open and interconnected pores. The scaffolds obtained displayed pore size larger than 300μm, suitable for osteoblast cell infiltration. SEM micrographs of 1Ag2 and 3Ag2 revealed homogeneous filament diameters which is crucial in order to obtain scaffolds with uniform architectures. High magnification SEM micrograph reveals wrinkly surface morphology for PCL and almost smooth and uniform surfaces for 1Ag2 and 3Ag2. Here, AgNps act as the inorganic filler leading to the increased stiffness, which is evident from the increased Young's modulus. In situ synthesis and distribution of AgNps in the 3D printed PCL matrix was analyzed using transmission electron microscopy (TEM) along with EDX analysis. TEM bright field micrographs of 3Ag2 clearly reveal the

distribution of AgNps as spots throughout the PCL matrix with sizes distributed in the range of 20 to 150 nm exemplifying wide dispersity of the synthesized AgNps. Furthermore, EDX analysis confirms the distribution of AgNps in the PCL polymer matrix. The mechanical properties of 3D printed pristine PCL and PCL/AgNps were characterized using tensile test analysis. Firstly, the Poisson's ratio for PCL was 0.38 and remains constant with the addition of AgNps. The Young's modulus of PCL was found to be 0.35GPa increasing to from 0.35 (PCL) to 1 GPa (3Ag2). In this study, AgNps are also acting as fillers which decrease the elastic behavior of PCL and improve its rigidity. Furthermore, increasing the silver concentration enhanced the Young's modulus of the PCL/AgNps (1Ag1 and 3Ag2) composite scaffolds. Here, the values of Young's modulus of the composites are between that of the cancellous bone (0.05-0.1 GPa) and cortical bone (3-30 GPa) [203].

In the work performed by Li, Y., et al., hydrogel composite scaffolds of polyvinyl alcohol/sodium alginate/hydroxyapatite (PVA/SA/HA) are manufactured with the purpose of combining bone regeneration with tumour treatment. PVA hydrogels present bio-adhesive, non-carcinogenic and non-toxic characteristics combined with very high swelling on contact with water and a rubbery texture, hence, they closely simulate natural body tissue. Nano-hydroxyapatite (HA), one of the major components of human bone, makes implants more osteoconductive, thus improving tissue compatibility, while improving the mechanical strength properties of hydrogel composites [204]. These were prepared at low temperature via AM. To prepare the GO/Fe₃O₄ nanoframework, graphene oxide powder was dispersed ultrasonically in deionised water. FeCl₃·6H₂O and FeCl₂·4H₂O were mixed at a molar ratio of nFeCl₃: nFeCl₂ = 2:1 in deionised water under a stream of N₂. This reaction continued at 80 °C with magnetic stirring until the solids were completely dissolved. Then, the GO suspension was mixed into the solution and reacted for 30 min at 80 °C until a dark-brown precipitate was obtained. To ensure MGO's purity, centrifugation was carried out multiple times with anhydrous ethanol and distilled water respectively, then dried in a vacuum for 12h. Then the MGO particles were separated by the magnetic field. To prepare hydrogel scaffolds, different contents of MGO nanocomposites were suspended in 10.0mL of deionised water by ultrasonic vibration for 5 min to form a uniform MGO/water suspension. To prepare the alginate-PVA composites, 1.5wt.% Na₂HPO₄ sodium alginate was added to 5wt.% PVA solution until there were no alginate clumps left. The solution was further mixed with 5wt.% HA and different contents of MGO/water suspension, then kept in a water bath at 80 °C for 30 min. After that, the liquid was transferred to the extrusion nozzle of the 3D-printing machine (3D Discovery, regenHU, Switzerland). This 3D printer used "a 3D honeycomb", a porous prototype with 3D interconnectivity. A computer aided design (CAD) design was used with printing parameters: road width of 0.51mm, road gap of 1.50mm, slice thickness of 0.40mm, lay-down pattern 0/90°, nozzle tip 0.51mm, mould size 16.0mm×16.0mm×4.0mm. The printed MGO/PVA/SA/HA hydrogel scaffold was then immersed in CaCl₂ solution for cross-linking. After that, the cross-linked samples were washed with distilled water and then dried. The scaffolds were loaded with various concentrations of magnetic graphene oxide (MGO), i.e. graphene oxide coated with Fe₃O₄ nanoparticles, with a smaller size than 50nm. According to the detailed observation

of the SEM images and elemental mapping, the MGO scaffold had good porosity with a pore size of about 300 μ m, which is conducive to the growth of cells and nutrient transportation. The surface of the scaffold with MGO enhanced the cell adhesion, probably due to the anionic functional groups from the MGO, such as single-bond COOH or single-bond OH. In addition, the elemental mapping shows that MGO diffused into the PVA/SA/HA hydrogel composite scaffolds [205].

Lastly, Olate-Moya, F., et al. developed bioconjugated hydrogel nanocomposite inks for 3D printed scaffold fabrication through a micro-extrusion process. The hydrogel is based on a photocrosslinkable alginate bioconjugated with both gelatin and chondroitin sulfate (ACG) in order to mimic the cartilage extracellular matrix, while the nanofiller is based on graphene oxide (GO) to enhance the printability and cell proliferation. To fabricate the scaffolds by 3D printing, three inks were formulated: ACG composed by ALG 10mg.mL⁻¹ + ALG-MA 6mg.mL⁻¹ + CS-MA 6mg.mL⁻¹ + GEL-MA 6mg.mL⁻¹ + PI 0.5mg.mL⁻¹, ACG/GO0.1 with the ACG composition + GO 0.1mg.mL⁻¹ and ACG/GO1 with the ACG composition + GO 1mg.mL⁻¹. The 3D printing of scaffolds was carried out in a fourth generation 3D Bioplotter (EnvisionTEC, Germany). By employing Materialise Magics software, a scaffold of 30×30×1 mm³ (length × width × height) was drawn, and the obtained STL file was processed with Perfactory Software Suite in order to divide the designed structure into four layers. Finally, a mesh-like inner pattern with 1.5mm of thread spacing was set with Visual Machine software from the 3D Bioplotter. To print the scaffolds, polyethylene cartridges of 30cm³ were filled with the inks. The cartridge having the ink was placed in a low-temperature dispensing head at 5°C, and the ink was extruded through a 25G needle into a Petri dish at 2°C. The ink was physically crosslinked by an ionotropic process with a CaCl₂ 100mM solution (ALG chains) and by a thermotropic process (GEL chains). The 3D printed scaffolds were irradiated with a Blak-Ray UV lamp with a wavelength of 365nm and intensity of 9mW.cm⁻² for 5 min (at 20cm of distance) to photocrosslink the methacrylated biopolymers. Finally, the scaffolds were freeze-dried and stored at -20°C before further experiments. The incorporation of GO into the ink formulation enhanced its printability, obtaining scaffolds with higher shape fidelity and resolution than ink without GO, due to an enhanced thixotropic behaviour. Threads printed with ACG/GO1 result in anisotropic fibers, probably induced by liquid crystalline properties of GO, with interesting projections for tissue engineering where cellular alignment is required.

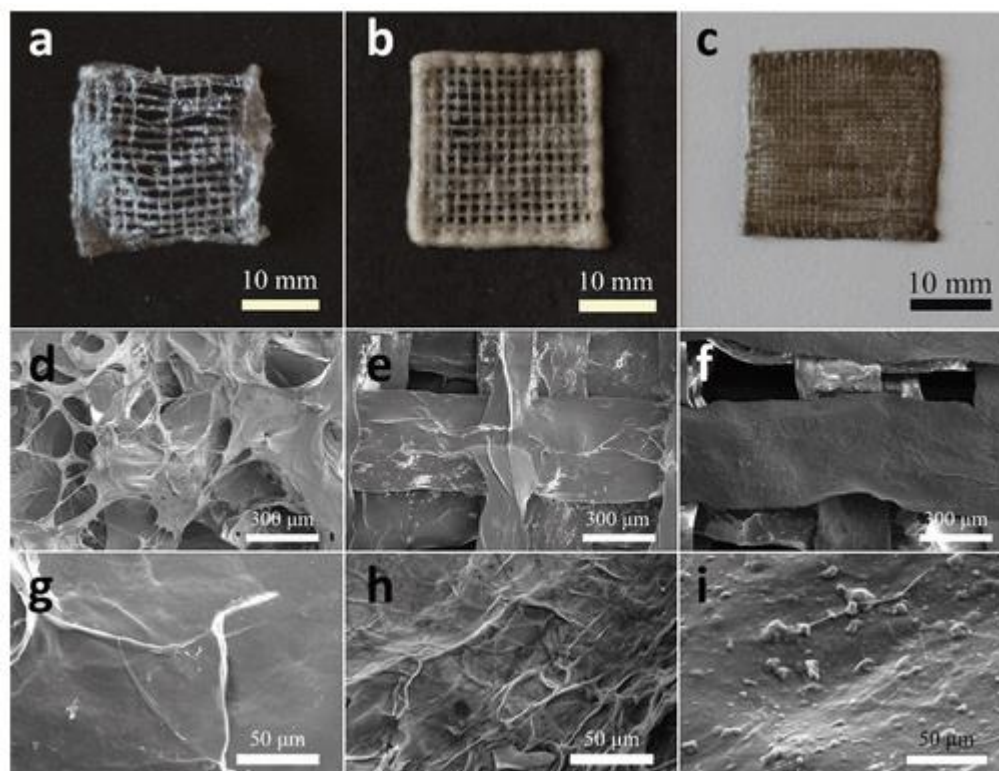


Figure 24. Full-size appearance of lyophilized scaffolds 3D printed with (a) ACG, (b) ACG/GO0.1, and (c) ACG/GO1 inks. FE-SEM images of thread surfaces: (d,g) ACG, (e,h) ACG/GO0.1, and (f,i) ACG/GO1 3D printed scaffolds.

FE-SEM image analysis shows a flattening of the thread in the scaffolds having GO after the freeze-drying process. The morphologic analysis of the surface on strands shows an increment in the roughness when GO is added. While a smooth surface is observed in the scaffold with ACG composition, fibrillar and bumped micro-topography are observed on the surface of 3D printed scaffolds using ACG/GO01 and ACG/GO1 inks, respectively. The compressive modulus in ACG/GO1 is ~60% higher than hydrogels without and with a low concentration of GO. This increase can be attributed to the improved mechanical behaviour of the GO nanofiller further forming bridges that reinforces the three-dimensional structure of the hydrogel scaffolds [206].

5. Nanoparticles-containing hydrogels and bioinks for biomedical applications

Cancer treatment has evolved due to vast research on the area, creating a wide range of techniques to diagnose, control and cure the condition. Conventional methods including surgery, chemotherapy, and radiation are still highly implement, however the combination and/or substitution of these with minimal invasive therapy (MIT) has gained researcher's interest. Surgical intervention has side effects such as severe pain, deformations, and possible disease recurrence. Chemotherapy and radiation therapy have characteristically low specificity, causing harm to healthy cells/tissues. Nevertheless, these therapies can be applied producing quality results, especially when combined [26, 27].

However, nanotechnology is an ascendent area in the field of diagnosis and treatment of various diseases, one being cancer. Nanoparticles large surface-to-volume ratio and flexible surface modification, enables the surpassing of barriers in aspects including bioavailability, biosafety, and therapeutic efficacy. Combining the advantages of nanomedicines and MITs, minimal invasive nanomedicines (MINs) can minimize or eliminate side effects such as pain, using small or no incision, and effectively target abnormal cells, tissues and/or organs, improving bioavailability and biosafety of drugs. Implementing outside stimuli such as light and magnetic field, stimuli-responsive MINs enable real-time control, minimizing dosages and side effects, whilst maximizing therapeutic efficacies. Once administrated, nanomedicines can specifically accumulate at the site of the lesion through passive or active targeting effect, and then be activated to exert the therapeutic effects under the action of a minimally invasive exogenous stimulus, which is different from the traditional therapy. For example, magnetic hyperthermia therapy (MHT) and photothermal therapy (PTT) have been widely used in the treatment of different tumours and achieved good therapeutic efficacies using nanoparticles with magnetic and photothermal properties, respectively.

Advances in nanomedicines with low toxicity, contrasting agent properties, tailorable characteristics, target/stimuli-response delivery potential, and precise control over behaviour have made it possible to overcome obstacles in tissue engineering. Current TE techniques face drawbacks including lack of cell growth, difficulty to mimic physiological architecture, and inefficiency in stimulating cell communication and proliferation. The use of nanoparticles for enhancement of mechanical properties and osteogenic stimulation creates crucial improvements in this field.

5.1. Nanoparticles-containing hydrogels and bioinks for magnetic hyperthermia therapy

Magnetic hyperthermia uses an alternating magnetic field (AMF) to produce local increase of temperature by magnetically mediated heating, through power absorption by magnetic nanoparticles used as nano heaters. MHT enables hyperthermia of the tumour's mass, meaning increasing its temperature above 40 degrees, while maintaining the environment temperature through preferentiality of the nanoparticles for the targeted tissue. The heating causes disruption in cancer cell activities, culminating in irreversible damage. MHT increases safety and effectiveness through selective cell killing. The heating capacity depends on the magnetic and physicochemical properties of the magnetic nanoparticles.

For MHT, multiple studies have applied iron oxide (Fe_3O_4) nanoparticles with different processing. For example, Zhang, J., et al. manufactured Fe_3O_4 /MBG/PCL scaffolds and reported enhanced magnetic properties. The magnetic properties of the NPs were tested exposing the material to an AMF of 180G in strength at 409 kHz frequency for 30 minutes. Magnetic heating property of Fe_3O_4 /MBG/PCL scaffolds was evaluated using an optical fibre temperature sensor to

measure the temperature. The ground magnetic scaffolds powder was dispersed in water to a concentration of 10 mg/mL. The solution was heated in an alternating magnetic field of 180 G in strength at 409 kHz frequency for 30 min. The upper limited temperature was set up 80°C. The capacity of a magnetic material to absorb energy from an alternating magnetic field is quantified through the SAR index. The magnetization saturation of the Fe₃O₄/MBG/PCL scaffolds increased with increasing Fe₃O₄ content, and the magnetization of MBG/PCL, 5Fe₃O₄/MBG/PCL, 10Fe₃O₄/MBG/PCL and 15Fe₃O₄/MBG/PCL scaffolds were 0, 1.01, 2.02 and 2.90 emu/g, respectively. In the alternating magnetic field, the solution temperature of MBG/PCL scaffolds did not increase, whereas the solution temperatures of 5Fe₃O₄/MBG/PCL, 10Fe₃O₄/MBG/PCL and 15Fe₃O₄/MBG/PCL scaffolds increased rapidly. The 15Fe₃O₄/MBG/PCL scaffolds induced the temperature increase from 20°C to 43°C at the AC frequency of 409 kHz and magnetic strength of 180 G within 2 min. Furthermore, the scaffolds showed a positive correlation between heating rate and Fe₃O₄ content in the scaffolds. The SAR index of the MBG/PCL, 5Fe₃O₄/MBG/PCL, 10Fe₃O₄/MBG/PCL and 15Fe₃O₄/MBG/PCL scaffolds were 0, 1.4, 2.9 and 4.7 W/g, respectively. Further studies to investigate the effect of the magnetic heating on the behaviour of cancer cells were not conducted. However, the research demonstrated potential for the appliance of Fe₃O₄/MBG/PCL scaffolds in MHT [178].

To enhance iron oxide NPs magnetic properties, Rani Aluri, E., et al. conducted the addition of GO flakes to flowerlike structure iron oxide nanoparticles, iron nanoflowers (iNFs), aiming to better its heating properties. To evaluate the hyperthermic efficiency specific absorption rate (SAR) was determined at ν_{AC} 535 kHz, I_{AC} 24 mT. The GO flakes had no effect on the SAR, indicating a lack of disruptive mechanisms and associated inter-PNF interactions.

To evaluate gPNF (without GO) hyperthermic response, measurements were carried out on a Live Cell-Alternating Magnetic Field (LC-AMFTM) module connected to a NanoTherics NAN201003 MagneTherm™ system, which enables RF irradiation of a sample placed in a standard glass Petri dish. The AMF was applied at a frequency of 663 kHz and magnetic field strength of 16 mT. In the case of gPNF (without GO) the hyperthermic response was weak, with an increase in temperature after 600s, of 0.4°C. Upon addition of GO a significant increase in response was observed, with an increase of temperature after 600s of 2.5°C at the plateau for gPNF_0.6GO and gPNF_1.08GO. A weaker response was observed for gPNF_0.8GO, even though its native SAR was slightly higher, due to greater dissipation associated with higher surface area. Interestingly, for the three GO-containing prints the extracted initial slope (the average for the whole grid area, blue curves) is independent of GO content, with an average value of $0.012 \pm 0.001^\circ\text{C s}^{-1}$ obtained. On the other hand, increasing GO content in the nanocomposite hydrogels progressively increased cohesive hydrophilic interactions, leading to recovery of SAR, as compared to the equivalent gels without GO, to values close to the native suspensions. Hence PEGylating and incorporation of GO provided nanocomposite hydrogels that retain significant greater hyperthermic efficiency than was

previously possible, enabling use of much lower (approximately 23- fold) NF concentration to achieve any target hyperthermic response (temperature jump) [180].

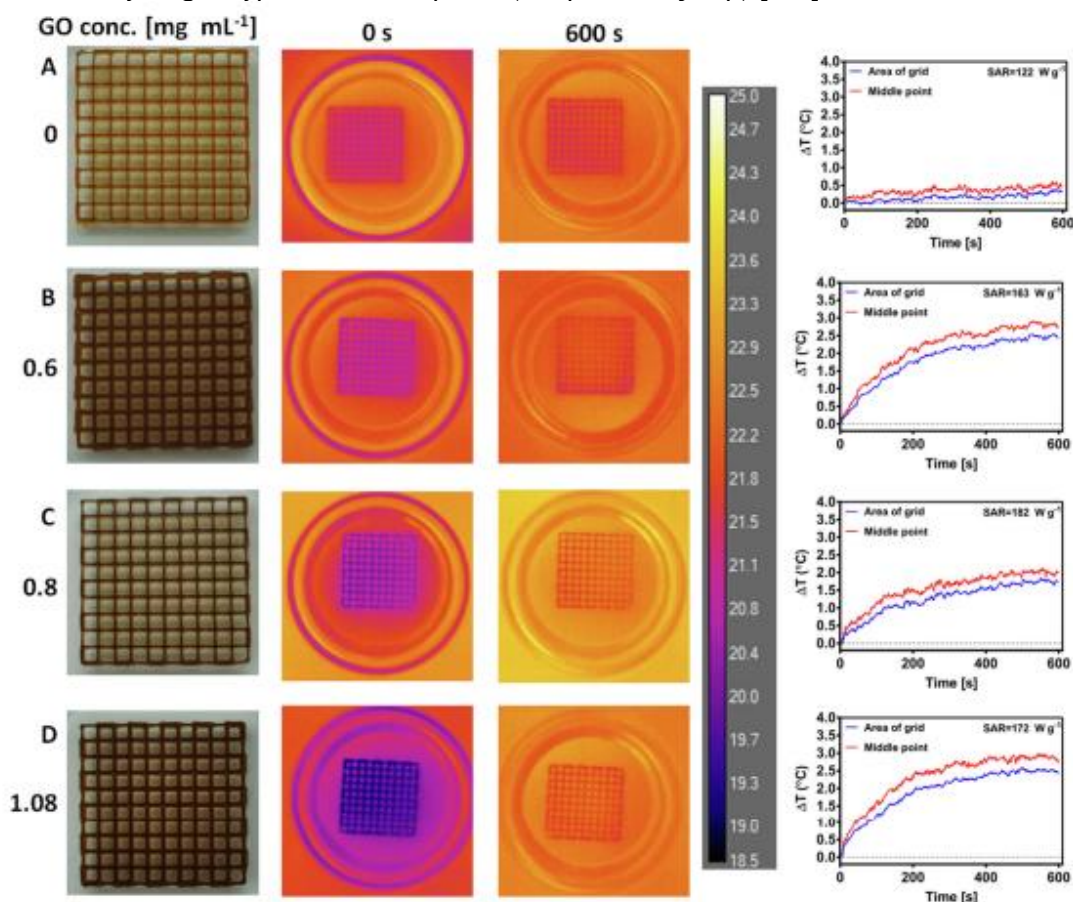


Figure 12. Thermographic evaluation of spatially patterned/3D printed gPNF_GO grids at $\text{Fe } 2.5 \pm 0.2 \text{ mg mL}^{-1}$; (A) gPNF; (B) gPNF_0.6GO; (C) gPNF_0.8GO, and; (D) gPNF_1.08GO. From the left; optical photographs of each $1.5 \text{ cm} \times 1.5 \text{ cm} \times 1 \text{ mm}$ (high) print; time resolved in-situ thermography of ‘open system’ LC-AMF stimulation (vAC 663 kHz, IAC 16 mT) of printed grids at the start (0 s, and 19°C in all cases) and at the end (600 s) of the irradiation, a single colour-coded thermal scale is used for all thermographs; the change in temperature (DT) as a function of irradiation time (as a whole grid average and at the centre point), the closed system SAR values of the gels are indicated. The error in temperature is $< 0.1^\circ\text{C}$ in all cases.

Qian, K.-Y., et al. implemented iron oxide nanoparticles in the form of iron nanocubes (iONS) to integrate silk fibroin hydrogel to confer deeper reach of the treatment. In vitro magnetic hyperthermia therapy effect tests were conducted using 4T1 cells after exposure to the FSHs with varying IONCs concentrations (50, 100 and $200 \mu\text{g Fe/mL}$) in the absence and under an AMF of 312 kHz, 30 kA/m, 15 min. The FSH at $50 \mu\text{g Fe/mL}$ showed a moderate hyperthermia effect on cancer cells. The injectable hydrogels at 100 and $200 \mu\text{g Fe/mL}$ IONCs induced 45% and 59% 4T1 cell death respectively, so the increase in IONCs concentration elevated the promoted therapy efficiency.

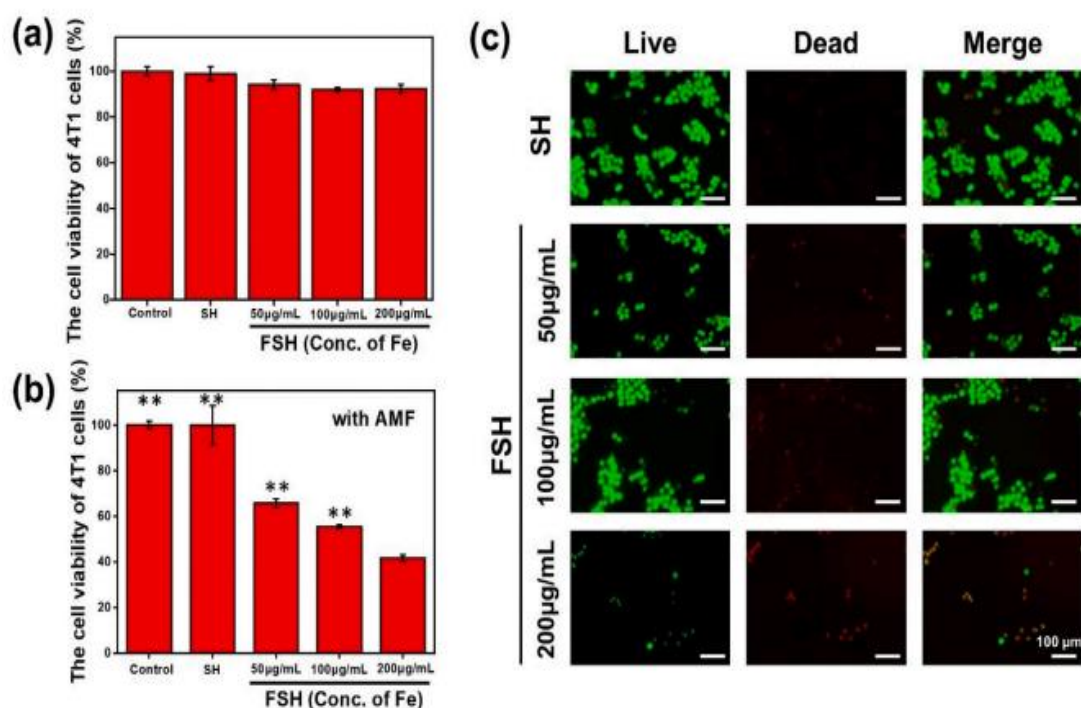


Figure 13. Cellular viability of 4T1 cells after exposing to the SH and FSHs in the (a) absence and (b) presence of an AMF (compared with the last group, * $p < 0.05$ and ** $p < 0.01$; Student's t-test). (c) The live-dead co-staining images of 4T1 cells after exposure to the SH and FSHs under an AMF. The concentration of Fe_3O_4 were defined as the content of iron oxide (μg) per volume of cell culture (mL).

For the in vivo test of induced therapeutic efficacy of the hydrogels, 4T1 tumour-bearing mice were divided into four groups, subjected to intratumorally injected with PBS, FSH, PBS (with AMF exposure) or FSH (with AMF exposure) respectively. The temperature changes of the tumour site were recorded by infrared thermal camera.

The in vivo therapy was initiated when the tumour volume reached approximately 100 mm^3 . Compared with the almost constant temperature of the PBS + AMF group, the temperature of FSH + AMF group raised quickly to 46.1°C within 3 min, and reached 49.6°C at 6 min. Such hyperthermia effect was reasonable to cause the tumour ablation. The tumour weight and images, mice photographs and H&E staining images of the tumour tissues demonstrated significant tumour ablation effect from FSH triggered magnetic hyperthermia effect under an AMF [182].

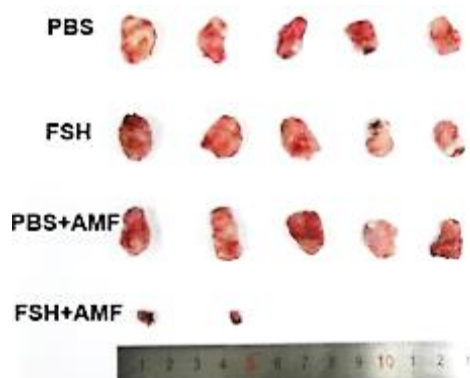


Figure 324. The excised tumour weight and its digital images of four groups after treatment.

Since magnetic hyperthermia depends on the capacity of the magnetic material to implement an increase of temperature through the whole tumour's mass, Tang, J., et al. created an innovative programmable shape transformation hydrogel of poly(Nisopropylacrylamide) (PNIPAm) embedded in Fe_3O_4 nanoparticles, able to undergo abrupt volume collapse due to magnetothermal effect. For the characterization of the hydrogel's magnetic properties, the content of magnetic nanoparticles was varied, and the hydrogels were exposed to an AMF. The rising temperature increases from 11.4°C to 86.5°C at 100 s, when the content increases from 3 wt% to 20 wt%. After confirming the magnetic properties of the hydrogel, the problem was exemplified by placing a small squared magnetic hydrogel under a large Petri dish containing cancer cells on top of gel, exposing only the center area of cells to heat. The heated magnetic hydrogel caused acute cell death at the center of the dish, with the outer cells unaffected. To create a solution for the fact that only the middle cells suffer the damage from the magnetic field, a multiple-arm structure was designed to encase and kill cancer cells. Under the actuation of the alternating magnetic field, the arms of the structure are heated to bend up to hold the hydrogel block. Due to the temperature increase of magnetic hydrogel in the presence of AMF, the cells embedded in GelMA will be killed simultaneously. The temperature of the magnetic hydrogel can be controlled by changing the AMF strength. Temperatures above 50°C can cause damage to cancer cells via thermoablation and it is seen that the AMF with an alternating current of 230A can induce a steady temperature above 50°C for 20 min. Both the number and fluorescence intensity of live cells are decreased after heated by the structure. Quantitative analysis shows that $\sim 50\%$ of cancer cells are killed by the heated structure. The results can be improved by regulating the actuation speed through the content of magnetic nanoparticles and/or using a different 3D printing technique to fabricate the structure. Fine structure with smaller feature size than that of extrusion printing, can be fabricated by other types of 3D printing strategy, such as digital light processing stereolithography. It is hoped that work will follow to apply the shape changing structure of magnetic hydrogels to in vivo situations in medicine and bioengineering [184].

Table 1. Selected recent studies on nanoparticle-hydrogel systems fabricated by 3DP for magnetic hyperthermia, along with the nanoparticles properties, magnetic field conditions, and biological effect in vitro and in vivo.

Materials	Nanoparticles modification	Nanoparticles properties	Alternative Magnetic Field Conditions	Biological studies In vitro	In vivo	Ref.
Polymer: MBG/PCL	—	15-20 nm, Superparamagnetic behaviour	180 G in strength at 409 kHz frequency	—	—	[178]
Nanoparticle: Fe ₃ O ₄						
Polymer: F127	PEGylation of NF suspensions	41 ± 4 nm	16mT in strength at 663 kHz frequency 10 mins	—	—	[180]
Nanoparticle: Fe ₃ O ₄ nanoflowers /GO						
Polymer: Silk fibroin	Coated DSPE-PEG2000.	<50 nm	30 kA/m in strength at 312 kHz frequency 15 mins	100 and 200µg Fe/mL IONCs induced 45% and 59% 4T1 (hepatocellular carcinoma) cell death	H&E staining images demonstrated significant tumour ablation	[182]
Nanoparticle: IONCs						
Polymer: PNIPAm	—	Fe ₃ O ₄ : - Nanoclay: 15 nm	230 A	~50% of A375 (human malignant melanoma cells) cell death	—	[184]
Nanoparticle: Fe ₃ O ₄ Nanoclay						

MBG, Mesoporous bioactive glass; PCL, Polycaprolactone; F127, Pluronic F127; Fe₃O₄, iron oxide; GO, graphite oxide; PEGylation, covalent conjugation with polyethylene glycol; NF, nanoflowers; IONCs, iron oxide nanocubes; DSPE-PEG2000, 1,2- distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy (polyethylene glycol)-2000]; PNIPAm, Poly(N-isopropylacrylamide); A435, Human skin melanoma (A375) cell line; 4T1, 4T1 murine breast cancer cell line .

5.2. Nanoparticles-containing hydrogels and bioinks for photothermal therapy (PTT) and chemo-phototherapy

Photothermal therapy (PTT) employs light-activated heating with a similar goal as MHT. The presence of natural light absorbers including water, hemoglobin, and melanin require an increase in specificity to reduce or avoid normal tissue damage. Therefore, the use of specific photothermal nanoagents, that can absorb NIR light to destruct the targeted tumour with minimal damage to the surrounding healthy tissues by converting NIR light to heat, have been widely developed and systematically investigated. The ideal PTT nanoparticle possesses high photothermal conversion efficiency, and strong absorbance and good photostability in the NIR region, while exhibiting biosafety and biocompatibility. PDA as a biocompatible material with many striking properties, has been widely used in the field of biomedical engineering and its excellent photothermal conversion effect make it highly applied in the PTT field.

PTT is often used in combination with one of the conventional cancer treatments such as surgery, chemotherapy, and radiation. For example, Wei, X., et al. produced core-shell hydrogel fiber scaffolds including PDA/alginate (Alg) as the shell part and gelatin hydrogels as the core part. This system allows the combination of photothermal therapy with chemotherapy, using light as a stimulus inducing the targeted drug release. In vitro tests were conducted to evaluate the photothermal properties of the core-shell fibers and its ability for drug release in a controlled manner. Under near infrared (NIR) laser irradiation (808 nm) with different power density (0.5, 0.6 and 0.8 W cm⁻²), the temperature of the core-shell fibers rapidly raised to 53°C and 60°C in 1 minute. This behaviour remained unchanged after five cycles of irradiation, indicating excellent photothermal stability, a requirement for repeatedly controlled drug release from the core-shell fibers. The core of the hydrogels was loaded with doxorubicin (DOX), an FDA-approved and common applied drug in cancer treatment, and its release was studied in the presence and absence of irradiation. DOX was dramatically released under laser irradiation, while nearly no DOX was released in the condition of no laser irradiation (no more than 6%). Regarding the therapeutic effect of the hydrogels, 4T1 cells were seeded in 24-well plate covered by drug-loaded core-shell fibers. The viability of cells in the groups of PDA/Alg + DOX fibers plus laser and PDA/Alg fibers plus laser was decreased by about 80% and 70%, while the viability of cells in groups of DOX loaded Alg fiber and DOX loaded PDA/Alg fibers without laser showed limited decrease. In addition, the drug loaded group plus laser showed higher cell-killing ability than the group of non-drug loaded fiber plus laser.

To evaluate the photothermal induced drug release of the drug-loaded core-shell fibers when implanted, these were placed into subcutaneous area of mice and subjected to the presence and absence of irradiation. Fluorescence images were used to review the before and after. The control sample (without laser irradiation) showed nearly no fluorescence signals in the implanted area,

indicating few DOX was released from the core-shell fibers in the situation of no laser irradiation. However, after each time of laser irradiation, the fluorescence signals from DOX were significantly increased in the open ends of the fibers, indicating a triggered release of DOX from the open ends of the core-shell fibers. The possibility of the fabricated core-shell fiber system for *in vivo* cancer treatment was evaluated after the fibers were implanted in tumor areas of mice. The photothermal effect of the coreshell fibers *in vivo* was tested under 808 nm laser irradiation in 5 min (0.6 W cm^{-2}). The temperature of PDA/Alg coreshell fibers rapidly raised to 51.3°C after 5 min irradiation, while the temperature of control group (laser only) was only increased by 5°C . The results confirmed that the PDA/Alg core-shell fibers exhibited good photothermal effect *in vivo*. Meanwhile, the lower density of laser used in our study could minimize the damage of laser to the skin tissues of mice. The *in vivo* tumour therapy study was carried out by treating the tumour-bearing mice as following groups: (1) without any treatment (control); (2) PDA/Alg with laser irradiation; (3) DOX loaded PDA/Alg without laser; (4) DOX loaded Alg/Gel fibers without laser; and (5) DOX loaded PDA/Alg plus laser. The volumes of the tumour in the group of DOX loaded PDA/Alg and PDA/Alg (no DOX) with laser irradiation were significantly reduced. As control, tumour in mice without any treatment grew fast in the following days, and the groups with DOX loaded core-shell fiber but no laser irradiation showed limited tumour inhibition effect. Hematoxylin and eosin (H&E) staining images from the major organs of mice after treatment showed no significant damage, which further confirming the negligible side effect of our core-shell fibers [186].

Another application of PDA was as a coating for the same hydrogel structure by Liu, C., et al.. Polydopamine (PDA) was coated on the Gel loaded with DOX/PCL core/shell scaffold in an attempt of enhancing its photothermal effect. The photothermal conversion capability of the scaffold was tested by irradiating the scaffold while in PBS with NIR laser (808 nm) in different power density (0.1, 0.2, 0.3 and 0.4 W/cm^2). The results showed that the PDA coated scaffold had a rapid temperature rise from room temperature (22°C) to 44°C under 0.2 W/cm^2 laser irradiation for 60s. However, the pure gel scaffolds and Gel/PCL scaffolds without PDA coating nearly had no temperature rise in the same conditions. Furthermore, the PDA coated scaffolds demonstrated good photothermal stability after suffering six cycles of NIR laser irradiation, crucial for controlled drug release. The *in vivo* photothermal effects of scaffolds were also evaluated by implanting the scaffolds with and without PDA coatings in the subcutaneous area of mice. After irradiating by NIR laser (0.5 W/cm^2) for 3 min, the temperature of the PDA coated scaffold was raised rapidly to 55°C , while the temperature of control sample (PCL scaffold) was only increased to 42°C . The lower density of laser could minimize the potential damage of laser to normal tissues. The ability to induce controlled drug release was evaluated both *in vitro* and *in vivo*. Drugs achieved a burst release in the situation of NIR laser irradiation. As control, less

drugs were released from the sample without laser irradiation. In vivo drugs release was studied by implanting the core/shell filaments in the subcutaneous area of mice, subjecting it to NIR irradiation for 5min, and followed by keeping the mice in the conditions of no irradiation for another 24h. The whole process was repeated for three times. The data showed that the fluorescence signals of DOX were observed in the end of the filament after laser irradiation, indicating the burst release of DOX from the open end of the filament. As control, the sample without laser irradiation showed nearly no fluorescence signals during the whole time. In vitro therapeutic effects of scaffolds were evaluated by determining the viability of 4T1 cells after different treatments. The data showed that more than 94% cells were effectively killed by DOX loaded scaffold plus laser irradiation and about 80% and 40% cells were killed in the treatment groups of DOX free scaffold plus laser and DOX loaded scaffold without laser irradiation, respectively. In vivo cancer therapy of the scaffolds was studied by implanting the scaffolds in the tumour region of mice. Then, the tumour therapy study was conducted by treating the tumour-bearing mice as four different groups: 1) DOX loaded scaffolds plus laser irradiation; 2) DOX loaded scaffolds without laser treatment; 3) scaffolds (without drugs) plus laser irradiation; and 4) control group (without any treatment). The tumour volume of the group treated by DOX loaded scaffold with laser irradiation was significantly reduced. The tumour-inhibition ability of the DOX loaded scaffold with laser was much better than the other groups. All mice treated by DOX loaded scaffold with laser showed complete tumour inhibition or even tumour ablation without recurrence. The scaffold in combination with chemo- photothermal therapy showed the most effective tumour inhibition [192].

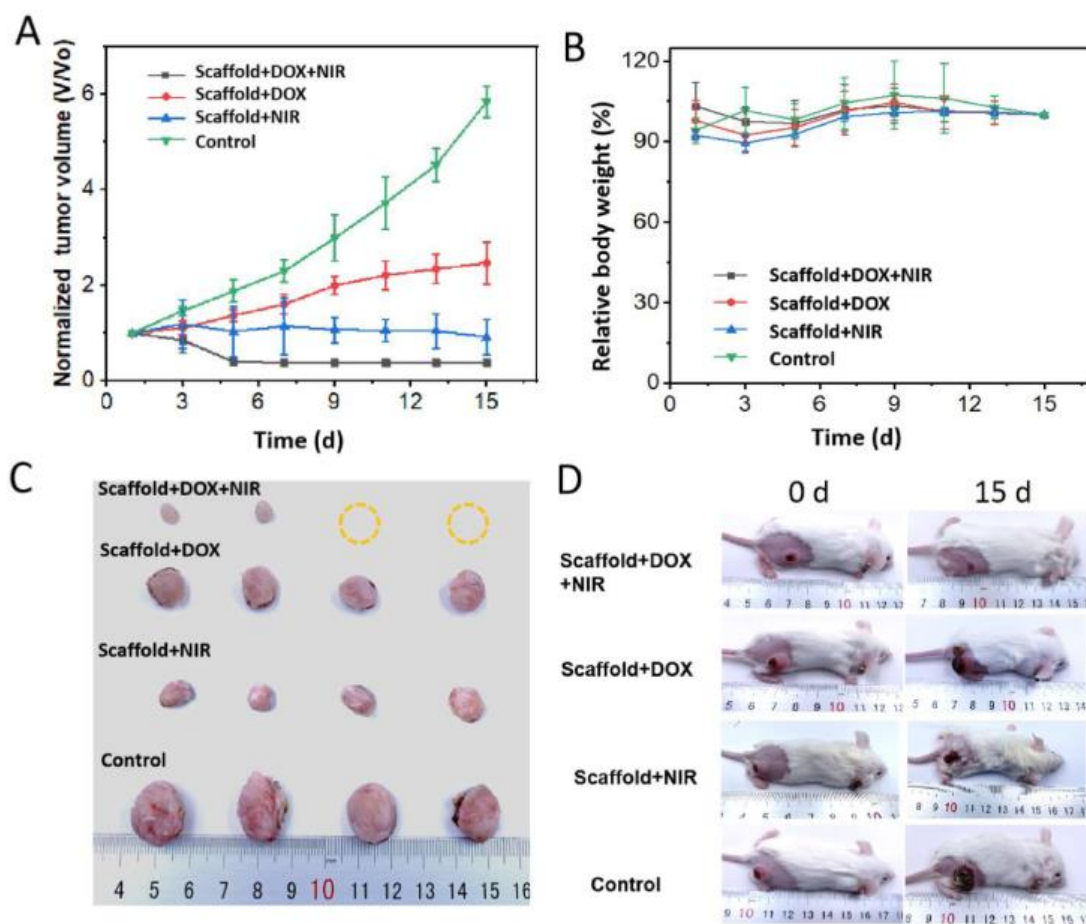


Figure 15. The tumour growth curves (A) and body weight (B) of tumour bearing mice with different treatments. Photographs of tumours collected from the mice with different treatments (C) and tumour bearing mice at day 0 (before treatment) and day 15 (after treatment) (D).

Finally, Luo, Y., et al. incorporated PDA in a dopamine-modified alginate and polydopamine (PDA) scaffold through 3DP. The photothermal effects of the scaffolds prepared with different original concentrations of DA were investigated by incubating the scaffolds in PBS under the irradiation of an NIR laser (808 nm) 0.5 W cm² laser. The data indicated that the printed scaffolds containing PDA exhibited excellent photothermal effect. When the concentration of DA in the printing ink was 10 mg/mL, the temperature of the scaffold rapidly increased from 25 to 53.6 °C under the irradiation for only 1 min. However, the temperature of pure alginate and Alg-DA scaffolds was increased only from 25 to 26.1 and 37.5 °C, respectively. The scaffolds maintained constant photothermal effect after five cycles of laser irradiation, demonstrating excellent photostability. To evaluate the effect of the PTT of the Alg-PDA scaffold on the viability of breast tumour cells, 4T1 cells were seeded in a 96-well plate covered by a scaffold. Cells showed adequate viability in the control (without any treatment), laser irradiation only, alginate scaffold plus laser irradiation (5 min), and Alg-PDA scaffold without laser irradiation groups. However, the cell viability was reduced by 65% in the Alg-PDA scaffold plus laser irradiation group (5 min). The photothermal effects of the fabricated scaffolds in vivo were evaluated by implanting them in tumour tissues of mice, followed by irradiation. The temperature of the Alg-PDA scaffold was

increased to 58.3 and 67.7 °C after NIR laser irradiation (808 nm) 0.5 W cm² for 1 and 5 min, respectively. However, the temperature increased only to 42.2 and 43.5 °C after irradiation for 5 min in laser only and alginate scaffold groups, respectively. Upon NIR laser irradiation, the volume of tumours in mice implanted with the Alg-PDA scaffold was significantly reduced, while tumour volume in laser only, alginate with NIR, and Alg-PDA without NIR treatment groups increased rapidly [190].

Table 2. Selected recent studies on carbon nanomaterials colloids for cancer photodynamic therapy nanoparticle-hydrogel systems fabricated by 3DP for photothermal therapy, along with the nanoparticles properties, irradiation conditions, and biological effect in vitro and in vivo.

Material	Nanoparticles production	Nanoparticles properties	NIR laser Irradiation Conditions	Biological studies In vitro	In vivo	Ref
Polymer: Alg/Gel Nanoparticles: PDA	Produced in the form of Alg/PDA ink	—	808 nm with different power density 0.5, 0.6 and 0.8 Wcm ⁻²	4T1 cells death: ~80% and 70%	tumour volumes were significantly reduced	[186]
Polymer: Gel/PCL Nanoparticles: PDA	Produced in the form of Alg/PDA ink	—	808 nm in different power density 0.1, 0.2, 0.3, 0.4 and 0.5 Wcm ⁻²	4T1 cells death: > 94% cells	tumour volumes were significantly reduced	[192]
Polymer: Alg Nanoparticle: PDA	Produced in the form of Alg/PDA ink	—	808 nm with power density 0.5 Wcm ⁻²	4T1 cells death: ~35%	tumour volumes were significantly reduced	[190]

Alg, alginate; Gel, gelatin; PDA, polydopamine; 4T1, 4T1 murine breast cancer cell line ; PCL, Polycaprolactone.

5.3. Nanoparticles-containing hydrogels and bioinks for TE

The growth of new tissues and/or organs from a base of cells and scaffolds has benefited from the implementation of nanotechnology in the field. The use of nanoparticles able to approximate the scaffold properties to the biological tissues it aims to replace or increasing cells affinity, viability, and proliferation, overcomes predominant obstacles in tissue regeneration. Three-dimensional grafts/scaffolds with hierarchically biomimetic features from nano to macro scale and high porosity are required for bone tissue engineering.

For example, Luo, Y., et al. fabricated highly concentrated alginate/GelMA bioinks through 3D printing and processing under mild conditions. Due to the mild processing conditions, proteins could be directly loaded in the materials pre-printing into scaffolds, and the proteins presented sustained release over 28 days. The impact of porosity and mineral coatings was studied using four conditions: 1) hollow fiber scaffolds with mineral coatings (HFM), 2) non-hollow scaffolds with mineral coatings (NHM), 3) hollow fiber scaffolds without mineral coatings (HFS), and 4) non-hollow fiber scaffolds without mineral coatings (NHS). The proliferation of osteoblasts on those four types of scaffolds showed no significant difference in the CCK-8 assay measurements. The results showed that cells on HFM and NHM scaffolds presented apparent higher ALP activity compared to that of cells on HFS scaffolds on both day 7 and day 14. The hollow channel structures had positive effects on cells adhesion and proliferation due to the increased porosity and surface area for scaffolds, while showed no significant effects on cells differentiation. Additionally, nano apatite coatings were able to enhance osteogenic differentiation of cells, probably because the nano apatite structures not only increased the roughness of the surface, but also provided bone-like microenvironments for cells. The in vivo bone repair functionality of the prepared scaffolds was further investigated by implanting the scaffolds in a larger femur defect model of rats for 4 and 12 weeks. After the scaffolds implanted in vivo for 4 and 12 weeks, microCT analysis was performed to evaluate to the bone repair ability. 2D and 3D images indicated that the HFM scaffolds showed better bone formation ability than the other groups. After implantation of HFM scaffolds over 12 weeks, the bone defects were almost filled with new bone. However, there were still defects existence in the other groups, especially for the control group. Bone mineral density (BMD) and bone volume/total tissue volume (BV/TV) data also showed that the BMD and BV/TV of HFM group were significantly higher than the control group [193].

Another example was the production of a scaffold using of MXene as a nanoparticle for a 3DP composite hydrogel scaffold GelMA/B-TCP/sodium alginate (Sr^{2+})/MXene (Ti_3C_2) (GTAM) by Nie, R., et al. One of the main concerns while implanting a biomaterial is decreasing or eliminating the risk of developing a bacterial infection. In this way, this work was produced with the purpose of exhibiting bifunctional properties: photothermal antibacterial and osteogenic abilities. To explore the photothermal effect of MXene composite scaffolds, the samples were immersed in PBS with NIR irradiation (808 nm, 1.5 W cm^{-2}) for 5 min. The temperatures of all GTAM scaffolds increased significantly with positive correlation with the MXene concentration. The temperature of GTAM0.3 could up to 56°C , while the temperature of GTA did not increase significantly. Studies have shown that *S. aureus* and *E. coli* could be killed at around 58°C , generally. As the temperature of GTAM0.3 could go up to around 56°C in wet conditions with NIR (808 nm, 1.5 W cm^{-2}), these were selected to complete the further studying. Bacterial growth kinetics curves of both *S. aureus* and *E. coli* showed that, with the extension of time, the concentration of bacteria co-cultured with the GTA with or without NIR irradiation both steadily increased, while the

bacteria growth was slightly slower in the GTAM group as compared with GTA and GTA + NIR groups. It is worth noting that the GTAM + NIR group showed an obvious and continuous inhibition of bacteria growth for 24h. In order to further investigate if GTAM scaffolds with NIR could kill proliferated bacteria to better mimic the in vivo experiment, bacterial colonies were counted after scaffolds were co-cultured with bacteria for 24h and then irradiated with NIR for 5 min. There was limited inhibition effect on the bacterial viabilities of *S. aureus* and *E. coli* in the GTAM group, while the *S. aureus* and *E. coli* viabilities significantly decreased to 1.4% and 1.1% in the GTAM + NIR group, meaning that the resistance to bacteria of the GTAM scaffold was the strongest under NIR irradiation. This result is consistent with the bacterial growth kinetics.

The in vitro osteogenic ability of rat bone marrow mesenchymal stem cells (rBMSCs) in scaffolds was explored by the relative mRNA expression levels of some osteogenic markers. RUNX2 (early specific transcription factor for osteogenic differentiation), ALP (early marker of osteogenic differentiation) and OCN (later marker of osteogenic differentiation) were tested through real-time. After osteogenic induction for 7 days and 14 days, both RUNX2 and ALP expression levels were upregulated in all the GTAM groups when compared to GTA. For OCN, at the 7-day mark there was an increase in GTAM0.5 when compared with GTA. After 14 days of osteogenic induction, the expression levels of OCN using GTAM0.3 and GTAM0.5 were significantly enhanced compared to the GTA group. The expression levels of the osteogenic proteins of ALP and OPN (later marker of osteogenic differentiation) were obtained via immunofluorescence staining at 14 d. The results revealed that compared to the GTA group, GTAM0.3 and GTAM0.5 had significantly higher ALP and OPN expression levels.

In the purpose of evaluating the bifunctional scaffolds in vivo, scaffolds of all groups were implanted into mandibular infected defect areas of rats. Scaffolds in GTA + NIR and GTAM + NIR groups were exposed to NIR laser 808 nm, 1.5 W cm^{-2} for 5 min. The temperatures of the GTA + NIR and GTAM + NIR groups were up to 34.2°C and 58.3°C , respectively at 5 min, indicating that GTAM scaffolds had an effective photothermal conversion ability in vivo. At 4 weeks post-operation, gross observation showed that almost all rats looked normal. After the rats were sacrificed, micro-CT 3D reconstruction images of the mandibles exhibited osseointegration in each group at 4 weeks and 8 weeks. Bone volume/tissue volume (BV/TV) represented the percentage of new-born bone tissue volume accounting for the entire defect area and was significantly higher in GTAM and GTAM + NIR as compared to other groups, while the GTAM + NIR group was the highest both at 4 weeks and 8 weeks. The GTAM + NIR group had an excellent bone regeneration effect among the 5 groups, indicating that NIR-induced hyperthermia did not exert a long-term negative influence on the osteogenesis process. H&E staining of the main organs (heart, liver, spleen, lung, and kidney) after 4 weeks post-operation showed that no histological change could be found in all groups [198].

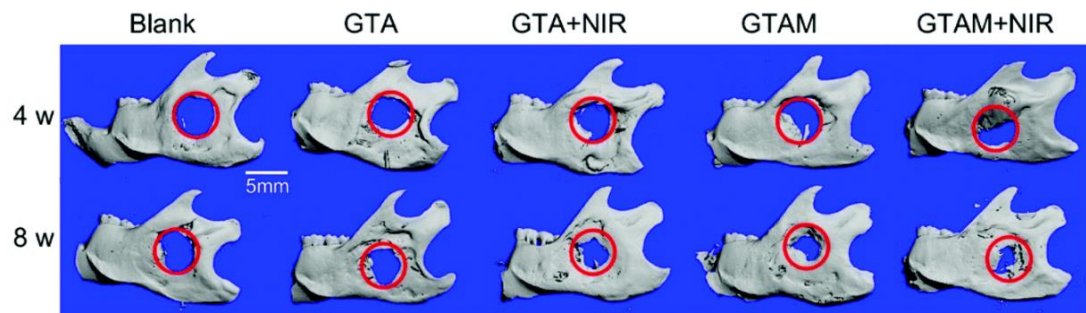


Figure 16. Micro-CT 3D reconstruction images of rats with infected mandibles after 4 weeks and 8 weeks of scaffold implantation.

3D-printed hybrid biodegradable hydrogels composed of alginate, gelatin, and cellulose nanocrystals (CNCs) were prepared to provide a favourable environment for cell proliferation, adhesion, nutrients exchange, and matrix mineralization for bone tissue engineering (BTE) applications by Dutta, S.D., et al.. Adhesion of cells to their ECM is one of the crucial properties for in vitro proliferation and differentiation. Thus, modifying the ECM by using various biomimetic scaffolds may improve the cell adhesion and osteoinduction property. Interestingly, the hBMSCs were found perfectly oriented and acquired the scaffold pores after 3 days of 1% CNC/Alg/Gel scaffolds. This was confirmed by DAPI staining through the scaffold surface after 7 days of incubation. Moreover, the cells occupied the entire space of the scaffold when the incubation time was increased. Furthermore, the cells were found penetrating the scaffold strands after 7 days of culture. Real-time polymerase chain reaction (qRT-PCR) was performed to investigate the osteoblast-specific gene expression during osteoinduction in the presence of 1% CNC/Alg/Gel scaffold. The 1% CNC/Alg/Gel scaffold was taken for gene expression analysis because of its greater mineralization potential. The relative mRNA expression in the presence was evaluated after 7 and 14 days of incubation. There are various genes that randomly ‘turn-on’ and ‘turn-off’ during osteoblast development, such as runt-related transcription factor-2 (Runx2), bone morphogenic protein-2 (BMP-2), alkaline phosphatase (ALP), Osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP), Osterix (OSX), collagen (COL), etc. Interestingly, enhancement of mRNA expression of Runx2, ALP, BMP-2, OPN, and COL1 was noted after 7 days of culture. Although the gene markers were also expressed in the control groups containing hBMSCs, their expression was higher in the presence of 1% CNC/Alg/Gel scaffolds. The in vivo bone regeneration efficiency of 1% CNC/Alg/Gel scaffold was evaluated in the calvaria critical defect model after 3 weeks of transplantation. After anesthesia, a 1-1.5 cm sagittal inclusion was made very carefully on the scalp creating a critical defect (approximately 5 mm) in the central area of the calvaria bone. The defect area was cleaned carefully, and a 5 × 5 mm circular scaffold was transplanted aseptically. Next, the soft tissue was repositioned and sutured. Given that the 1% CNC/Alg/Gel scaffold demonstrated enhanced osteogenic efficacy, confirmed in the cell viability and qPCR results, it was the concentration chosen for the bone regeneration study. Three groups were formed: not treated (negative control), BMP2 treated (positive control) and BMP2 in combination

with 1%Alg/Gel scaffold. BMP2 was implemented due to its potential to regenerate the bone tissue. It was interesting to note that the defect site was diminished by the positive control, however it was significantly reduced in the 1% CNC/Alg/Gel + BMP-2 scaffold-treated groups compared to the positive and negative control. The hematoxylin and eosin (H&E) staining was performed to evaluate the histological development. The 1% CNC/Alg/Gel + BMP-2 scaffold-treated groups exhibited remarkable new bone formation [200].

The current use of hydrogels for musculoskeletal tissue repair is not perfect due to the lack of suitable mechanical properties, as well as the high biomimetic requirement for success. To overcome this limitation, Heo, D.N., et al. developed a novel functionalized hydrogel with bioactive gold nanoparticles (GNPs) To verify cytoskeletal organization, spreading, and proliferation of cells, ADSCs were cultured in the various hydrogel component with the 3D printing PLA structure, and determined by F-actin staining and CCK assay at different time intervals. Cell spreading on the outside and inside of ADSC encapsulated hydrogel was confirmed using optical microscopy and confocal laser scanning microscope (CLSM). Among these specimens, Gel-RGNP showed the highest cytoskeletal spreading, large lamellar morphology and numerous stress fibres. It is well known and recognized that the cRGD peptide is an integrin-binding site, which has beneficial effects on cell adhesion, spreading, and actin-filament formation. Moreover, structurally cRGDs have many advantages such as high affinity interaction with cell surface integrin and low biodegradability than linear RGDs. Therefore, Gel-RGNP provided a greater positive effect on surface cell adhesion when compared to Gel and Gel-GNP groups. The quantitative analyses of cell spreading area to each hydrogel layer and analyses of the cell proliferation rate to was performed using staining by fluorescence labelled phalloidin. In both types of Gel and Gel-GNP groups, encapsulated ADSCs appeared morphologically granular even after 24h but Gel-RGNP seemed to induce better spreading throughout the hydrogel network. In addition, the average cell spreading area in Gel-RGNP was twofold higher than in Gel and Gel-GNP under the same culture condition. Gene expression profiles of ADSCs encapsulated within various hydrogels during osteogenic differentiation were studied by determining mRNA expressions of COL1, RUNX2, BSP and ALP were through real-time PCR. COL1, which is an early-stage osteogenesis marker, is present in the bone extracellular matrix and expressed at high levels during bone development. Its maximum level of mRNA expression was reached on day 7 and decreased at day 14. Other early osteogenic transcription factors, ALP and RUNX2, displayed an increased expression level over time. The mRNA expressions of the early osteogenic specific markers, COL1, ALP and RUNX2, significantly increased in the order of Gel, Gel-GNP and Gel-RGNP during the whole period of osteogenic differentiation. Amongst these specimens, Gel-RGNP showed much higher expression than all other sample groups. The expression level of BSP, which is a late osteogenic specific marker, increased in all specimens over the course of time. It significantly increased in the following order: Gel < Gel-GNP < Gel-RGNP similar to the ALP and RUNX2 results[202].

In this study, Radhakrishnan, S., et al. synthesized silver nanoparticles (AgNps) in polycaprolactone (PCL) solution by in-situ reduction and further extruded into PCL/AgNps filaments. Antibacterial activity of the materials was first characterized by growth inhibition tests to assess the ability of the material to prevent the implanted site from the formation of bacterial biofilm in presence of nutrients since it could lead to bacterial infection. The results after 24h showed that negative control plates (i.e. without bacteria and scaffold) show clear nutritive soft agar plates while positive control plates (i.e. with bacteria and without scaffold) show a uniform distribution of bacterial colonies within the soft agar. With the 3Ag2 scaffold, a clear inhibition zone (mean diameter 20.4 ± 1.7 mm) is observed whereas no clear zone is observed for PCL and 1Ag2 for which biofilms are evidenced on the material surfaces. Bactericidal tests were done to assess the ability of the material to remove bacteria that has already grown on the material implantation zone, by liquid tests in which the material was immersed in a bacterial suspension at about 102 CFU/mL [203].

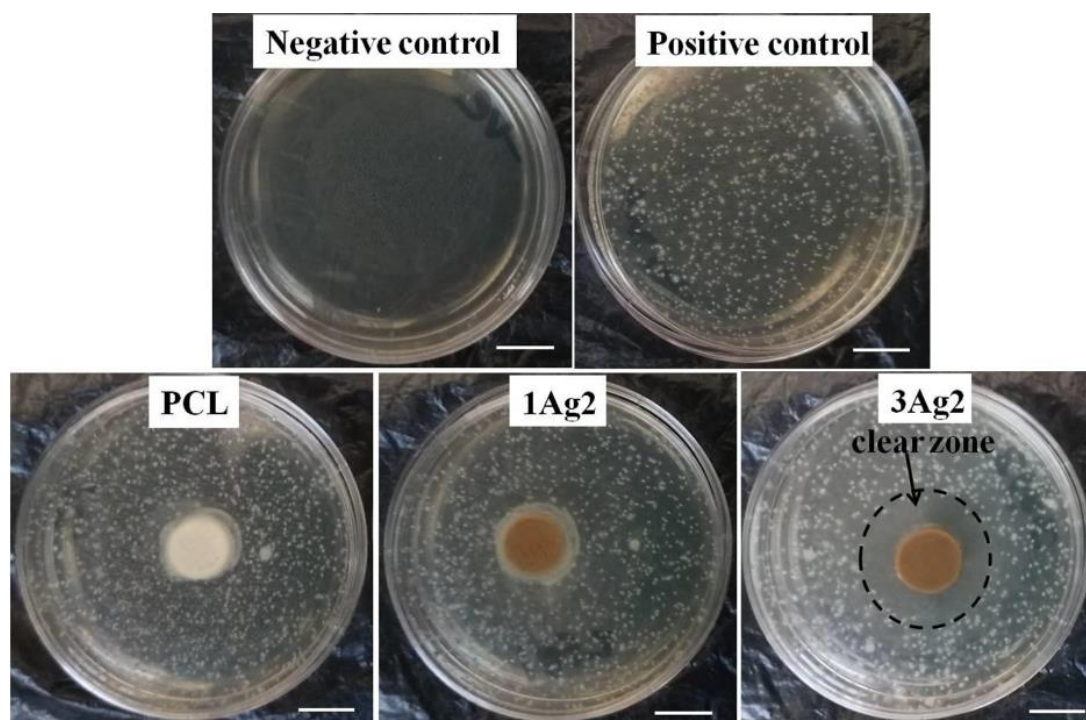


Figure 17. Soft agar plates inoculated with *E. coli* cells and incubated with 3D printed scaffolds in their centre for 24 h (Scale bar denotes 1 cm).

The treatment of tumour-related bone defects should ideally combine bone regeneration with tumour treatment. In this study, Li, Y., et al. prepared novel hydrogel composite scaffolds of polyvinyl alcohol/sodium alginate/hydroxyapatite (PVA/SA/ HA). The magnetothermal conversion ability of the MGO hydrogel composite was studied and it exhibited both a time-dependent heating behaviour with the varying MGO concentrations and an alternating magnetic field (AMF) intensity. When the AMF intensity remains the same, the temperatures increased with the increasing MGO

concentration. To better simulate the human environment, we compared the scaffold heating effects in dry and humid environments. The maximum temperatures of the 5%, 10% and 15% w/w MGO hydrogel composites reached $> 45^{\circ}\text{C}$ in the dry environment. However, in the humid environment, only the 15% w/w MGO hydrogel composite reached 45°C at 250 Gs. In addition, the temperature increased with increasing external AMF intensity at the same MGO content. After 7 days of osteogenic differentiation, there was stronger ALP activity in the PVA/SA/HA and MGO-PVA/SA/HA groups than in the control group. Significantly increased numbers of extracellular matrix mineralisation nodules, which serves as a marker of late osteogenesis, were observed in PVA/SA/HA and MGO-PVA/SA/HA groups compared with the control group, possibly due to the content of HA particles, after 21 days of osteogenic induction. The MGO/PVA/SA/HA had the greatest increase in ALP activity and mineralisation. Infrared thermography with a FLIR system can safely monitor the radiant temperature field of a tumour from its centre to the surroundings. The closer to the centre of the scaffold, the higher the temperature, and the surrounding temperature gradually decreases. Intratumorally implantation with MGO hydrogel composite and treatment with AMF for 14 consecutive days successfully inhibited the tumour growth. In contrast, the MGO hydrogel composite group without AMF exhibited no obvious tumour-inhibition effects [205].

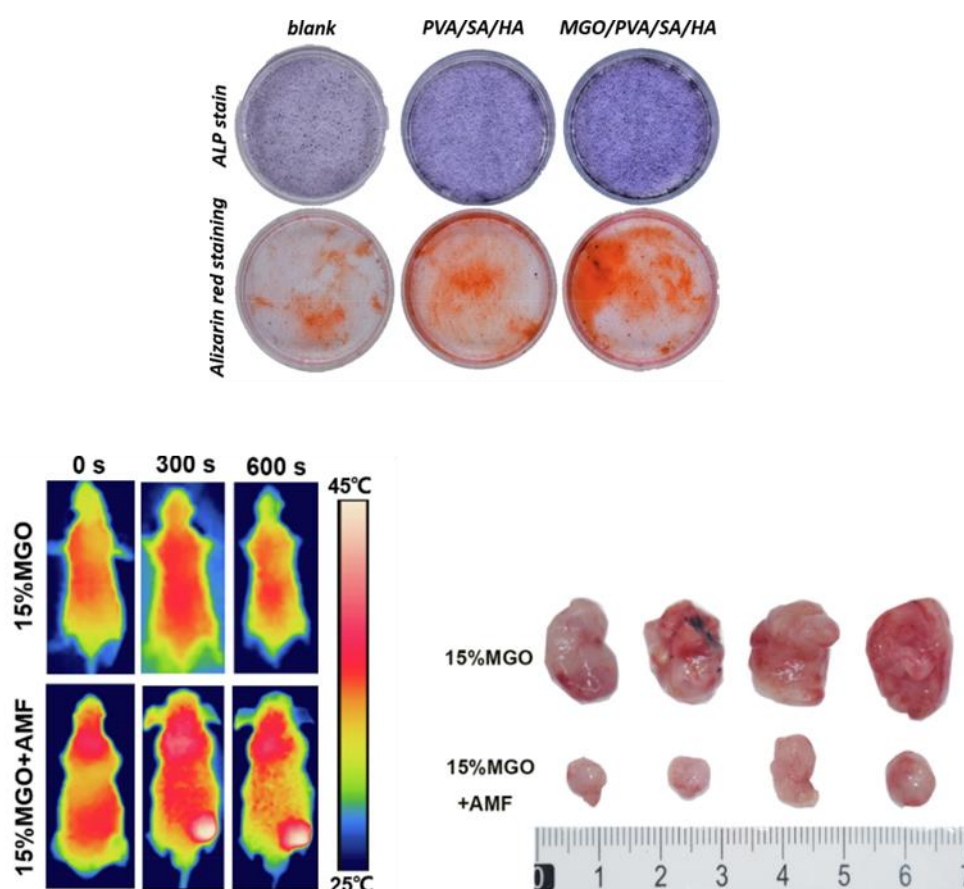


Figure 18. BMSC osteogenesis of the MGO hydrogel composite in vitro. (A) ALP staining and Alizarin Red staining. Inhibition of osteosarcoma tumour growth in vivo. (A) In vivo infrared thermography of 143b-tumour-bearing nude mice after intratumorally implantation with MGO

hydrogel composite under AMF at various time points. (C) Digital photographs of the dissected tumours.

Olate-Moya, F., et al. developed bioconjugated hydrogel nanocomposite inks for 3D printed scaffold fabrication. The hydrogel is based on a photocrosslinkable alginate bioconjugated with both gelatin and chondroitin sulfate (ACG) while the nanofiller is based on graphene oxide (GO). The proliferation of hADMSCs on 3D printed scaffolds was evaluated by alamarBlue assay. For alamarBlue assay, hADMSCs were seeded on the 3D printed scaffolds, utilizing pure ALG scaffold as a control. After one week, the proliferation of hADMSCs on bioconjugated scaffolds was significantly superior as compared with pure ALG control scaffolds, as the cell amount decreases considerably on the latter. Notably, ACG/GO0.1 scaffolds presented higher cell proliferation than ACG and ACG/GO1 scaffolds. The cell proliferation on the scaffolds increases significantly, when ALG bioconjugates with CS and GEL. Notably, in the ACG/GO0.1 scaffolds, the cells proliferate homogeneously on the surface and around the threads of the scaffold without the formation of cell aggregates. By adding more GO, the high proliferation produced not only cells distributed on the scaffold surface but also small cell aggregates. After 14 days, the cultured cells reached a maturation state with a higher cell number compared to day 7. Particularly, the GO-containing bioconjugated scaffolds exhibited significantly higher hADMSC proliferation, showing up to 3.6-fold higher fluorescence intensity as compared with pure ALG scaffolds and as compared with the same samples at day 1 of culture. The F-actin filaments of the cell cytoskeleton and cell nuclei were stained with FITC-phalloidin (green) and DAPI (blue), respectively, to confirm the previous results. All the scaffolds (independent of composition) maintained its original shapes during the formation and maturation of a new tissue. Photocrosslinking of methacrylated biopolymers and ionotropic crosslinking of ALG segments likely help to retain the structural integrity during the time needed for the growth of new tissue. Through TRITC-phalloidin stained microphotography, performed after 28 days of culture in CCM it was determined that the addition of GO considerably increases the directionality on the cell proliferation, likely due to the anisotropic structure of the 3D printed threads. This result offers a novel platform for biomaterial-guided cell organization.

Chondrogenic differentiation of hADMSCs was analyzed by immunofluorescence staining of chondrogenic markers for collagen type II, aggrecan, and SOX 9. Positive expression of collagen type II, aggrecan, and SOX 9 was found in the three scaffolds (with and without GO), suggesting that the biopolymeric matrix based on bioconjugated ALG with both GEL and CS induces chondrogenic differentiation on hADMSCs. High-density cell aggregates in the scaffolds without GO and with high GO concentration (ACG and ACG/GO1 respectively) were observed. Compared with images after 7 days, cells on ACG/GO1 tend to form aggregates after 28 days of culture. In contrast, the presence of GO at low concentration (ACG/GO0.1) improves notably the long-term cell distribution and ECM deposition on the 3D printed scaffold, exhibiting a homogeneous cell proliferation on the scaffold thread, porous, and around the scaffold edge. This behaviour could

be explained by the micro-topography on the thread surface, while the anisotropic grooved surface of ACG/ GO01 helps increase and guides the adhesion of cells. The smoother surface on ACG/GO1 could promote the long-term cell-cell interaction rather than the cell- biomaterial interaction, producing cell aggregation[206].

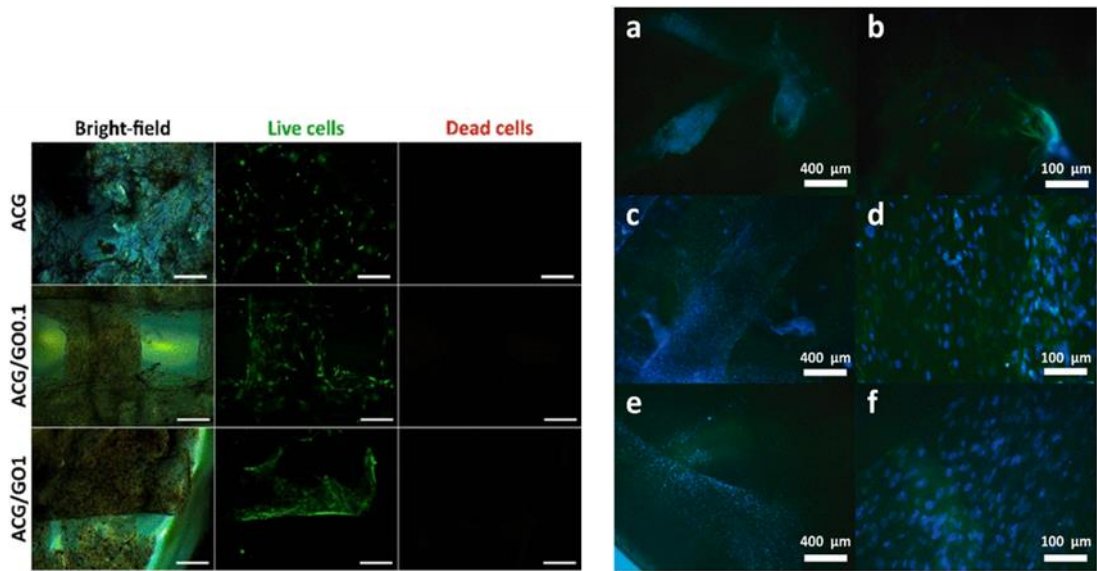


Figure 19. Representative fluorescence microscopy images of Live/Dead assay of hADMSCs seeded on 3D printed scaffolds at day 7 (scale bar: 200 μ m). Fluorescence microscopy images of hADMSCs seeded on 3D printed scaffolds at day 7. Cytoskeleton F-actin (green) and nuclei (blue) of hADMSCs are shown for (a,b) ACG, (c,d) ACG/GO0.1, and (e,f) ACG/GO1 scaffolds.

Table 3. Selected recent studies on carbon nanomaterials colloids for cancer photodynamic therapy nanoparticle-hydrogel systems fabricated by 3DP for tissue engineering, along with the nanoparticles properties, implantation conditions, and biological effect in vitro and in vivo.

Material	Nanoparticles		Implantation	Biological effect			Ref
	production	properties		in vivo		In vivo	
Polymer: Alg/GelMA Nanoparticles: Nap	In situ mineralization	Homogeneous layer of nano minerals coating on both the outer (~80 μ m) and inner surfaces (~10 μ m) of the hollow fibers	Implanting the scaffolds in a lager femur defect model of rats for 4 and 12 weeks	$\uparrow$ higher activity after 7 and 14 days $\uparrow$ cell adhesion and proliferation $\uparrow$ enhance osteogenic differentiation	ALP	$\uparrow$ bone formation: bone defects were almost filled with new bone after 12 weeks $\uparrow$ BMD and BV/TV	[193]
Polymer:		Thickness ~4 nm	Scaffolds have been	$\uparrow$ RUNX2, and	OCN, ALP	$\uparrow$ BV/TV	[198]

GelMA/B-TCP/Alg			implanted into mandibular defects to treat osteomyelitis infection in rats in the presence of 808 nm 1.5 W cm ⁻² NIR irradiation, 5 minutes	expression levels for 7 and 14 days	
Nanoparticles: MXene (Ti ₃ C ₂)		–			
Polymer:		From cotton pulps (10-20 nm width, 50-400 nm length; 12% solid content; crystalline index: >70%)	A 1-1.5cm sagittal inclusion was made on the scalp. A critical defect (approximately 5 mm) was created in the central area of the calvaria bone.	↑osteoblast-specific gene expression	Defect site [200] was significantly reduced, and H&E staining determined remarkable new bone formation
Alg/Gel					
Nanoparticles: CNCs		–			
Polymer:		RGNs have a near-spherical shape with well-dispersion in the range of 20-40 nm.		↑ osteogenic specific markers, COL1, ALP, RUNX2, and BSP especially in the form of RGNP during the whole period of osteogenic differentiation	[202]
PLA	GNPs were conjugated with the RGD peptide				
Nanoparticles: GNPs					
Polymer:		The sizes distribution is in the range of 20 to 150 nm.			[203]
PCL					
Nanoparticles: AgNPs					

			In situ				
			reduction of				
			silver ions				
			using DMF as a				
			reducing agent				
			as well as				
			solvent.				
Polymer:		Fe ₃ O ₄	were	The mice	↑osteogenic	↑	tumour [205]
MGO/PVA/SA		evenly		received a	differentiation at	growth	
Nanoparticles:	–	distributed		subcutaneous	7 days	inhibition	
HA and Fe ₃ O ₄				injection of	↑ extracellular	after	
				metastatic	matrix	treatment	
				human 143B	mineralisation	with AMF for	
				osteosarcoma	nodules	14 days	
				cells (1 × 10 ⁷	↑ ALP activity and		
				cells).	mineralisation		
Polymer:	GO	was	Lateral size of		↑ chondrogenic		[206]
ALG/GEL/CS	synthetized by	GO	sheets of		differentiation		
Nanoparticles:	a modified	~1 μm			markers of		
GO	Hummers'			–	hADMSCs	–	
	method from				↑ long-term cell		
	graphite				distribution and		
	powder				ECM deposition		

Alg, alginate; GelMA, gelatin methacryloyl; NAP, nano apatite; ALP, nonspecific marker of bone formation and osteoblast activity alkaline phosphatase; BMD, bone mineral density; BV/TV, bone volume/total tissue volume; B-TCP, B-tricalcium phosphate; Ti₃C₂, Titanium Carbide; RUNX2, Runt-related transcription factor 2 for osteoblastogenesis; OCN, osteocalcin protein hormone classic marker of bone formation; CNCs, Cellulose Nanocrystals; Gel, gelatin; H&E, hematoxylin and eosin; PLA, Poly(lactic Acid); GNPs, gold nanoparticles; RGD, Arginylglycylaspartic acid most common peptide motif responsible for cell adhesion to the extracellular matrix; RGNPs, gold nanoparticles modified with RGD; COL1, Type I collagen; BSP, Bone sialoprotein component of the bone extracellular matrix; PCL, Polycaprolactone; AgNPs, silver nanoparticles; MGO, magnetic graphene oxide; PVA, polyvinyl alcohol; SA, sodium alginate; HA, hydroxyapatite; Fe₃O₄, iron oxide; AMF, alternative magnetic field; CS, chondroitin sulfate; GO, graphite oxide; hADMSCs, human adipose tissue-derived mesenchymal stem cells; ECM, extracellular matrix.

6. Conclusions and Outlook

This review presented an overview of potential applications of emerging nanoparticles-hydrogels systems in the areas of photothermal therapy, magnetic hyperthermia, and tissue engineering. Hydrogels and bioinks have been widely researched the biomedical field. However, it was only recently that the enticing properties of different nanoparticles enriched the area with their capability of revolutionizing conventional medical treatments. There was a brief discussion regarding the most used polymers and nanoparticles, combining with requirement properties to succeed in biomedical applications. High biocompatibility, desired biodegradability, tailoring physic and chemical properties, and easily manipulated surfaces are some of the superior aspects analysed in the materials mentioned. Regarding photothermal therapy, the photothermal conversion efficiency is the main aspects required for the nanoparticles chosen, whilst in terms of magnetic hyperthermia, the magnetic properties play the central part. For tissue engineering one mandatory quality is the ability to mimic natural tissues properties such as bone's young modulus while inducing new tissue formation. Combining these with already established hydrogels and bioinks, has shown to be highly beneficial. For the most part, the photothermal, magnetic, and mechanical properties and overall performance of the polymeric platforms were further improved, alongside with eliminating side effects characteristic of conventional methods. From the collected data, it's clear an associating between photothermal therapy and controlled drug delivery, improving chemotherapy's safety. The association of wound healing in systems enhancing tissue regeneration was also exposed. The on-demand drug delivery explored in this review is achieved through the photothermal conversion ability of light-stimulated nanoparticles, as the increased temperatures from the photothermal conversion promote desorption from the nanoparticles-containing hydrogel and bioinks systems' surface that enables drug diffusion. Furthermore, the photothermal conversion also generates enough heat to induce tissue hyperthermia. By having the same heat-generating mechanism for both drug delivery and PTT, the composites can be employed in a dual-mode treatment. Another multifunctional approach for the nanoparticles-hydrogel systems was cancer treatment combined with tissue regeneration, mainly in the context of bone tissue engineering. 3DP nanoparticles-containing hydrogels and bioinks demonstrated enhanced osteogenic capacity, facilitating growth and proliferation of both hard and soft tissues. Therefore, a faster healing process can be achieved by incorporating osteogenic nanoparticles in wound dressing hydrogels/scaffolds. One of the main advantages of incorporating nanoparticles in polymeric scaffolds/hydrogels is an efficient and localized treatment. This way, current invasive treatments that inevitably cause the patient discomfort can be avoided without compromising the outcome. The pre-clinical trials using these materials demonstrate their suitability for biomedical applications, despite the lack of in vivo testing in some areas. The potential to revolutionize the current medical approaches is evident, especially in cancer treatment and tissue regeneration.

Chapter 3

Materials and Methods

3.1 GBM Production

During this dissertation work, two graphene-based materials (GBM) were studied, specifically nanographene oxide (GOn) and magnetic reduced nanographene oxide (@rGOn). GOn was produced following the modified Hummers method [32]. Briefly, a mixture of 160 mL of (H_2SO_4 , VWR, Darmstadt, Germany) and 40 mL of phosphoric acid (H_3PO_4 , Chem-Lab, Zedelgem, Belgium), was added to 4 g of graphite powder (size $\leq 20 \mu\text{m}$, Sigma-Aldrich, Sr. Louis, MO, USA), at room temperature. In a Lenz reactor, the dispersion was cooled to 11 °C using a cooling bath (Julabo F25).

Afterwards, 24 g of potassium permanganate (KMnO_4 , JMGS, Odivelas, Portugal) were gradually added to the solution, while it was stirred at 420 rpm. Following, 600 mL of ultrapure water were introduced slowly, under stirring, and the temperature was controlled ($< 40 \text{ }^\circ\text{C}$) using a cooling bath. Finally, 26.5 mL of hydrogen peroxide (H_2O_2 , VWR, Darmstadt, Germany) were added and the dispersion was left to rest overnight. The dispersion was then decanted to separate the solid phase from the acidic solution, centrifuged at 4000 rpm for 5 minutes, and redispersed in ultrapure water. This process was repeated until the pH of the supernatant was the same as the pH of the ultrapure water.

To produce the @rGOn, the GOn was resuspended at 1 mg/mL, placed in a Hergeus reactor and photoreduced under magnetic stirring to obtain p-rGOn, through UV irradiation for 8 hours. The reactor was wrapped in aluminum foil to avoid leaking UV to the laboratory space and the temperature was controlled ($< 40 \text{ }^\circ\text{C}$) using a cooling bath (Julabo F25).

Finally, the p-rGOn was magnetized using a previously established protocol [50]. Briefly, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1g/100 mL) was added to GOn solution and the resulting solution sonicated for 10 minutes

in an ultrasounds bath. Then, under stirring the solution's pH was adjusted to 9 by slowly adding NH_3 solution, after which the temperature of the stirring plate was increased to 180°C and the solutions were left to react for 10 hours. Iron (II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4 \text{H}_2\text{O}$, 99.0 %, Acros Organics) and ammonium hydroxide (NH_4OH , 28.0-30.0 % NH_3 basis) were purchased from ThermoFisher Scientific, USA. The system was allowed to cool and rested overnight. Lastly, the magnetized reduced graphene oxide (@rGOn) was washed in order to remove not magnetized residual material. For this, a permanent neodymium magnet (Supermagnete, Germany) was employed. The magnet allowed the separation of the magnetic graphene of the supernatant. The supernatant would then be discarded and replaced with ultrapure water. The process was repeated until a clear supernatant was achieved. It is of note, that the high temperatures of the magnetization process have been shown to thermally reduce the GOn [51]. Therefore, after magnetization, @GOn will be referred to as magnetic reduced GOn (@rGOn). The washed @rGOn was resuspended at 60 mg/ml and stored at 4°C .

3.2 GBM Characterization

3.2.1 Physical Characterization

3.2.1.1 Transmission electron microscopy (TEM)

To prepare the @rGOn for the TEM analysis it was required an extra step in order to obtain individual platelets enabling their measurement. For that, with the purpose of stabilizing the material in water, SDS powder was added under stirring with a final concentration of 10 mM. After, the solution was ultrasonicated using a probe (Branson Sonifier 250) for 2 hours.

Morphology and particle size of GOn and @rGOn aqueous dispersions were analysed by transmission electron microscopy (TEM) with a JEOL JEM 1400 TEM (Tokyo, Japan) at a concentration of $500 \mu\text{g/mL}$. The dispersions were sonicated and $10 \mu\text{L}$ were deposited on a carbon-coated TEM grid and left to sediment for 1 min. Afterwards, the excess material was removed by capillarity with a filter paper. The lateral dimensions of the nanomaterials were measured using the ImageJ software.

3.2.2 Chemical Characterization

3.2.2.1 Fourier transform infrared spectroscopy (FTIR)

GOn and @rGOn samples were heated at 60°C , during 24 h to dehydrate. To obtain the infrared spectra of dehydrated samples of GOn and @rGOn, Fourier transform infrared (FTIR) spectroscopy was used, resorting to a VERTEX 70 FTIR spectrometer (Bruker, Karlsruhe, Germany) in transmittance mode at room temperature. Samples were measured in attenuated total reflection (ATR) mode with a A225/Q PLATINUM ATR Diamond crystal with a single reflection accessory. Spectra were recorded by 60 scans, at a resolution of 4 cm^{-1} , in a wavenumber range of 4000 to 400 cm^{-1} .

3.2.2.2 Thermogravimetric analysis (TGA)

To evaluate the thermal stability of dehydrated samples of GOn and @rGOn, Thermogravimetric analysis (TGA) using a Netzsh STA 449 F3 Jupiter (Selb, Germany) was conducted. The thermograms were recorded between 30 and 1000 °C, at a heating rate of 10 °C/min, under nitrogen flow. Results are presented as percentage (%) of weight loss.

3.2.2.3 Ultraviolet-visible spectroscopy (UV-Vis)

To acquire the absorption spectra of GOn and @rGOn aqueous dispersion at a concentration of 12 µg/mL, a Lambda 35 UV/vis spectrometer (Perkin-Elmer, Waltham, MA, USA) was used. Samples were placed in a quartz cuvette (Hellma Analytics, Analytica Munich, Germany) with 10 mm light path length and their spectra were acquired between 200-850 nm. The measurements were made in triplicate, at room temperature, using water as a blank control for the baseline correction.

3.3 Hydrogels Production

3.3.1 Pectin purification

Firstly, pectin was purified from LM citrus pectin (Classic CU701) (Herbstreith & Fox, Neuenbürg, Germany) and termed herein as raw pectin (RAWpec). Briefly, the purification consisted in preparing a 1 wt.% solution of RAWpec in ultrapure water (18 MU, Milli-Q UltraPure Water System, Millipore). After the polymer was fully dissolved, the pH of the solution was adjusted to ~6 by adding NaOH 5 M. Sequentially, the pectin solution was filtered using 0.80 µm, 0.45 µm, and finally 0.22 µm filter membranes (mixed cellulose esters, MCE, Millipore). Then, 1 g of activated charcoal (2 wt.%, Norit SX Plus, Norit) was added for every gram of polymer in solution and the polymer solution was stirred for 1 h at room temperature for subsequent centrifugation at 22 000 rpm for 1 h. Afterwards, the solution was filtered using a 0.22 µm PES membrane. The pH was again adjusted to ~6 through NaOH 5 M. Finally, the solution was lyophilized, and stored at -20 °C until further use. The result of this protocol is purified pectin.

3.3.2 Preparation of hydrogels

In order to fabricate the hydrogels, purified pectin was dissolved in NaCl buffer and @rGOn solution in water. The crosslinker chosen was CaCl₂ and the crosslinking method used to form the hydrogels, is based on the internal gelation strategy. To guarantee that the hydrogels would maintain shape and mechanical properties through incubation in culture medium and heating assays, the structure was reinforced by immersing the hydrogel in a 0.5 M CaCl₂ bath of for 10 minutes.

3.3.3.1 Pectin hydrogels

For preparing the hydrogels, the lyophilized purified pectin and NaCl were separately dissolved in ultrapure water, while CaCl_2 was dissolved in Hank's Balanced Salt Solution (HBSS).

Firstly, the desired amount of NaCl was added to the pectin solution and thoroughly mixed, to ensure polymer dissolution. After this, the CaCl_2 was added and thoroughly dispersed in the solution until the formation of a gel-like material. To prepare hydrogel cylinders, the solution was cast into a 3 mL syringe where the crosslinking process is developed. The crosslinking and consequent hydrogel formation is almost immediate upon CaCl_2 addition. The crosslinking reaction was left to occur for 45 minutes. After, a 0.5 M solution of CaCl_2 is applied in enough volume that covers the exposed top of the cylinder hydrogel and left for external crosslinking for 10 mins. The structure is then expelled using the plunger and the 0.5 M CaCl_2 solution is again applied to the top of the hydrogel.

3.3.3.2 Pectin/@rGOn hydrogels

When preparing pectin hydrogels with @rGOn, the pectin and the NaCl were dissolved in the @rGOn dispersion to maximize the amount of graphene integrated in the structures, while the crosslinker was dissolved in HBSS, as it was done in the previous structures. After this, the same steps were followed as in the previous topic.

3.4 Hydrogels Characterization

3.4.1 Physical Characterization

To use the produced hydrogels for *in vitro* testing and for future applications, these must be stable and have enough mechanical resistance to be handled. Thus, the mechanical strength must be tested. In an initial test as the produced hydrogels are meant to be applied to the skin, being constantly subjected to a temperature of 37 °C, the samples were incubated in DMEM at 37 °C for 24 h and their structural integrity was evaluated by the ability to being manipulated with a spatula without causing any damage to the hydrogel structure. As this requirement was met, being the purpose of this structures their application in cancer phototherapy, these would be subjected to light converted into heat, with the goal of reaching 50 °C, temperature that causes the death of cancer cells [207]. For this, the samples were mechanically characterized by a rheological test where the mechanical properties of the structures were measured as a function of temperature. The rheometer evaluates the samples response to being subjected to a temperature range set to 37-50 °C with an increase of 2 °C per minute and an oscillation single frequency of 1 kHz. Deformation while varying the temperature results were presented in the storage modulus (G') and the loss modulus (G''). These measurements enable an overview of the sample's elastic and viscous behaviour, respectively. For this test, 3 cylindrical shapes with 8 mm diameter and 1 mm thickness were produced, containing the 3 concentrations of @rGOn being studied, 0 wt.%, 1.25 wt.%, and 2.5 wt.%. Each sample was placed in the sampler holder and water drops were placed around it to guarantee the hydration of the

hydrogel during the assay. The values for the storage and loss modulus at 37 and 50 °C were obtained and difference percentage of the modulus throughout the increase of temperature was calculated for each condition.

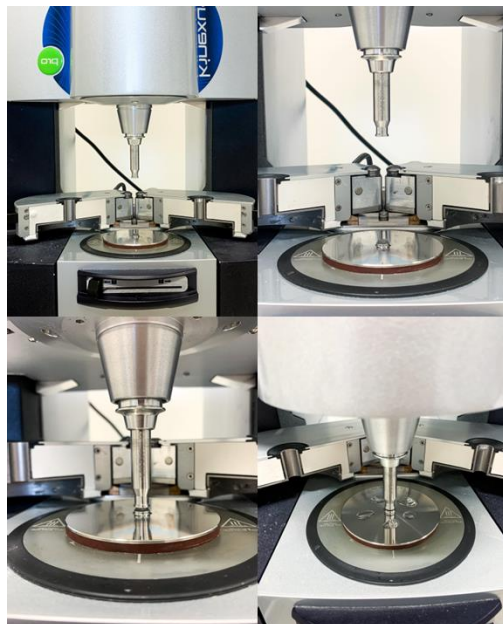


Figure 20. Images from the rheometer analysis exemplifying the preparation for the assay.

3.4.2 Photothermal Properties

To evaluate the photothermal properties of the produced GBM hydrogels, in a 48-well plate, hydrogels with 0, 0.625, 1.25 and 2.5 wt.% @rGOn were placed at the bottom of a well and covered by 600 μ L of DMEM. Samples were irradiated with a custom LED-system, with a peak-emission around 810 nm (NIR region) and 1000 mW/cm² irradiance. The samples were irradiated until 50 °C was reached for the samples containing @rGOn, in order to record the light-induced temperature change. For this, a type K thermocouple was placed in the centre, contacting the DMEM in the well. Four replicates were made per condition, using a control with water only. Before the irradiation, samples were pre-heated to 37 °C in an oven, to mimic biological conditions. Results are presented as mean and standard deviation. The cooling down was also recorded and presented.

3.4.3 Pectin/@rGOn printable ink

To design a printable hydrogel, another step of optimization had to be conducted, in order to modulate viscosity for potential extrusion bioprinting. Crosslinker concentrations of 6 mM, 7.5 mM and 9 mM CaCl₂ were prepared and tested. The pre-crosslinked hydrogels were loaded in 1 mL syringes and extruded through a cylindrical nozzle of 0.25 mm (Nordson EFD).

After determining the concentration of the crosslinker enabling the printing of uniform filaments, a preliminary test was performed towards creating a porous two-layer structure. For this

a Regemat 3D V1 printer (Regemat 3D, Spain) was used, using the same nozzle and the following printing parameters (dimension = $12 \times 12 \text{ mm}^2$, inter fibre spacing = 1.5 mm).

3.5 In vitro biological studies

3.5.1 Cell culture

Biological studies were performed using A-431 human epidermoid carcinoma cells (ATCC, CRL-1555) and HFF-1 human foreskin fibroblasts (ATCC, SCRC-1041). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC) supplemented with 10% (v/v) fetal bovine serum (Alfagene, Carcavelos, Portugal) and 1% (v/v) penicillin/streptomycin (Biowest, Pays De La Loire, France). Cells were maintained in a humidified atmosphere with 5% CO₂ and 95% air at 37 °C. The medium was replaced every 2 days and cells were detached upon reaching 70% confluence. For biological experiments, the effect of pectin, and pectin/@rGOn hydrogels was assessed using both fibroblasts and cancer cells in the presence or absence of NIR irradiation, as described in detail below.

3.5.2 Cytocompatibility of the hydrogels

3.5.2.1 Preparation of hydrogels for in vitro assays

As it was described prior, the graphene oxide used in the hydrogels was reduced using UV light. In this way, sterilizing the hydrogels with UV could influence the materials properties. Because of that, for biologic assays, hydrogels were disinfected to ensure they were as clean as possible before contacting with the cells. For this, the hydrogels were washed by immersion in descending concentrations of ethanol, 96%, 80%, and finally 70%. For the two highest percentages of ethanol the structures were left for 15 minutes, while the last wash had an exposure time of 30 minutes. Finally, the hydrogels were rinsed with Hanks' Balanced Salt Solution (HBSS) three times in order to guarantee that the ethanol that may had been absorbed during the washes was removed. The structures were left in HBSS until further use.

3.5.2.2 Extract exposure assays

To evaluate the cytotoxic effect of the extracts of pectin and pectin/@rGOn hydrogels, cells were seeded in 48-well plates at a density of 1×10^4 cells/well for HFF-1 human foreskin fibroblasts, and at a cell density of 4×10^4 cells/well for A-431 human epidermoid carcinoma cells and incubated at 37 °C and 5% CO₂. Hydrogels (200 mm^3) with different concentrations of @rGOn (0, 1.25, 2.5 wt.%) were produced, disinfected with ethanol and incubated in complete DMEM for either 24 h or 7 days, at 37 °C. Upon cell sub-confluence (24 h), culture medium was replaced by the culture medium in which each hydrogel had been incubated. Cell metabolic activity was quantified using a resazurin assay. Briefly, the hydrogel culture medium was removed, and cells were incubated in 20% (v/v)

resazurin reagent (Sigma-Aldrich, St. Louis, MO, USA) prepared in complete DMEM incubated at 37 °C and 5 % CO₂ for 2 h. Fluorescence ($\lambda_{\text{ex/em}} = 530/590$ nm) of the supernatant was measured using a microplate reader spectrophotometer (Synergy Mx, Bio-Tek Instruments, Winooski, VT, USA). Controls for cell viability were performed by incubating cells with complete DMEM. Data for each sample were normalized to the control and results are presented as mean % of control and standard deviation. Assays were performed in six replicates for each condition, with 3 independent experiments. According to ISO10993-5, the metabolic activity limit was defined at 70%.

3.5.3 Influence of NIR irradiation on the viability of cells embedded within a GelMA 3D hydrogel

3.5.3.1 Production of GelMA

Gelatin (Porcine Type A, Sigma-Aldrich) was dissolved in PBS solution at 1% (w/v) and 55 °C under stirring. The pH was fixed to 9. Protected from light, 0.6 grams of Methacrylic Anhydride (MA) per gram of gelatin was added drop by drop to the solution. The reaction was allowed to occur for 1 hour, at which point pre-heated PBS solution at 40 °C was added to the GelMA solution. The solution was transferred to dialysis membranes and left to dialyze in decreasing salt concentrations (30, 25, 20, 15, 10, 5, 0, 0, 0 g NaCl) for 5 days, in 4 L of deionized water at 55 °C. At the end of the dialysis, the pH was set to 7.4. Then, the solution was filtered into sterile falcon tubes using a 0.20 μm filter and a syringe, and freeze-dried.

3.5.3.2 Preparation of the pectin and pectin/@rGOn hydrogels

For direct contact assays, a new hydrogel structure was produced to mimic the cells contact with the hydrogel once this is applied to the body. Pectin and pectin/@rGOn 1.25 wt% dispersions were produced as prior described, and upon loading in the 3mL syringe a PDMS cylinder cast with 4 mm x 2 mm was carefully placed as centred as possible with tweezers and pushed until only the top surface was exposed. In this way, a centred well was created on the top of the hydrogel structure. The system was left to reticulate for 45 minutes and immersion in the 0.5 M CaCl₂ solution performed.

3.5.3.3 Preparation of the GelMA 3D cell matrix

The produced GelMA matrix consists of 10 wt.% GelMA as the polymer, 2 mM of LAP as the photoinitiator, and PBS as the buffer. The GelMA and LAP were dissolved in 37°C PBS and left in the thermomixer until use. As the polymer was not produced in sterile conditions there was a need for sterilization before cell contact.

Both the components were filtered using a 1 mL syringe filter (PVDF membrane, Millipore, 0.22 μm). The cells (8 wt.% of HFF-1 or A-431) were added into the GelMA-LAP solution performing a snail motion with the pipet in order to homogenise the solution as the mixture increases viscosity quickly with the decrease of temperature. Finally, 20 μL of the cell containing GelMA matrix were placed in the well of each hydrogel structure and photocrosslinked using an UV light (10 mW/cm²) for 2 minutes. After,

the system (pectin and pectin/@rGOn hydrogel containing the GelMA matrix) was incubated in 600 μL at 37 °C and 5% CO_2 for 24 h.

3.5.3.4 NIR irradiation

After 24h of incubation, each plate was irradiated using a custom LED-system, with a peak-emission around 810 nm (NIR region) and 1000 mW/cm^2 irradiance and incubated at 37 °C and 5% CO_2 for 24 h.

3.5.3.5 Resazurin assay

After the pectin and pectin/@rGOn hydrogels combined with the GelMA matrixes NIR irradiation and 24 h incubation at 37 °C and 5% CO_2 , the matrixes were retrieved from the pectin and pectin/@rGOn structures using a spatula. The metabolic activity was accessed by a resazurin assay, like previously. The results are presented as mean % of control and standard deviation. Assays were performed with only one independent experiment, with four replicates for each condition. According to ISO10993-5, the metabolic activity limit was defined at 70%.

3.5.4. Cells' viability assay

The cells' viability assay was performed in parallel with the resazurin assay previously described.

3.5.4.1 Preparation of the GelMA matrix for Live/Dead assays viability assays

To enable the evaluation of the cells' viability, the GelMA matrix was retrieved from the pectin and pectin/@rGOn structure using a spatula. To perform the Live/Dead assay using fluorescence labelling, two dyes were applied: Calcein AM and Propidium Iodide (PI).

The GelMA matrix was first washed through immersion in HBSS, followed by incubation for 5 mins at 37 °C. After, the matrix was washed 3 times using DMEM without FBS and without Phenol Red. The staining is performed through immersion of the samples in DMEM without FBS and without Phenol Red, containing Calcein and PI followed by incubation for 30 minutes at 37 °C. The Calcein and PI were added in order to obtain a final wt.% of 0.10 and 0.125, respectively. Finally, the structures were incubated in HBSS for 5 minutes at 37 °C. To keep hydrated, the samples were maintained in PBS until further use.

3.5.4.2 Live/Dead assays

To perform the cell viability assay, the matrixes were transferred to a 24-well ibidi plate. These well plates are made of optically clear materials, ensuring minimal optical distortion and high image quality for live cell imaging and microscopy. The assay was performed using the Opera Phenix Plus (Perkin Elmer), a high content screening system equipped with a fully automated widefield/confocal fluorescence microscope. The process started with a pre-scan of the wells using

brightfield with a 1.25x Air/0.03 objective to detect the GelMA structures. After the software was able to identify all the gels, a rescan was performed using a 20x Water/1.0 objective. In this scan, 100 z plans were selected with a 5 μm interval, and 10 field of views (fov) were selected in each z plan, randomly by the software. In this way, there was a height evaluation of 500 μm per gel. One field of view corresponds to (1080 x 1080) pixel. As each pixel is equivalent to 0.59 μm , each field of view evaluates an area of 0.41 mm^2 , and the 10 fov enables the evaluation of 4.1 mm^2/GelMa . The images collected were analysed using the software Harmony®. Assays were performed with only one independent experiment, with two replicates for each condition.

3.6 Statistical Analysis

The statistical analysis in this work were performed using GraphPad Prism software (version 8.4.3.471, San Diego, CA, USA). One-way analysis of variance (ANOVA) with Tukey's tests for multiple comparisons were performed. Differences between experimental groups were considered significant with a confidence interval of 95%, whenever $p < 0.05$.

Chapter 4

Results

4.1 GBM Characterization

4.1.1 Morphological Characterization

GOn presented a typical brownish appearance, producing a stable water dispersion, as expected. Partially- reduced graphene oxide (p-rGOn) obtained after 8h UV-C irradiation, presented a darker colour typical of the reduction process, but still great water stability. @rGOn showed an even darker colour, indicative of successful complete reduction, displaying sedimentation at the bottom of the flask due to becoming hydrophobic (as expected), however, easily resuspending after shaking by hand. Successful magnetization was confirmed since all particles headed towards and followed a magnet placed outside the vial. Neither GOn or p-rGOn have lost their water stability nor @rGOn have lost its magnetic properties after 6 months (last observed period), respectively.

TEM imaging of the materials' water dispersions (Figure 21.B) shows that GOn is constituted by individual well exfoliated sheets with an average length of 147.9 ± 82.2 nm, while @rGOn displays an average length of 298.4 ± 67.7 nm. The size increase might be explained by the presence of the iron nanoparticles added to @rGOn surface, and the increase of the hydrophobic nature of the nanomaterial after the simultaneous reduction and functionalization step. In terms of morphology, GOn platelets have a smoother surface with irregular and sharp edges (Figure 21.B1-2), while @rGOn platelets are more wrinkled, presenting usually rounder edges (Figure 21.B3-4). Furthermore, successful functionalization with iron oxide nanoparticles has been demonstrated (Figure 21.B4), with those particles presenting an average size of 12.9 ± 9.3 nm across the surface of the material (Figure 21.C2).

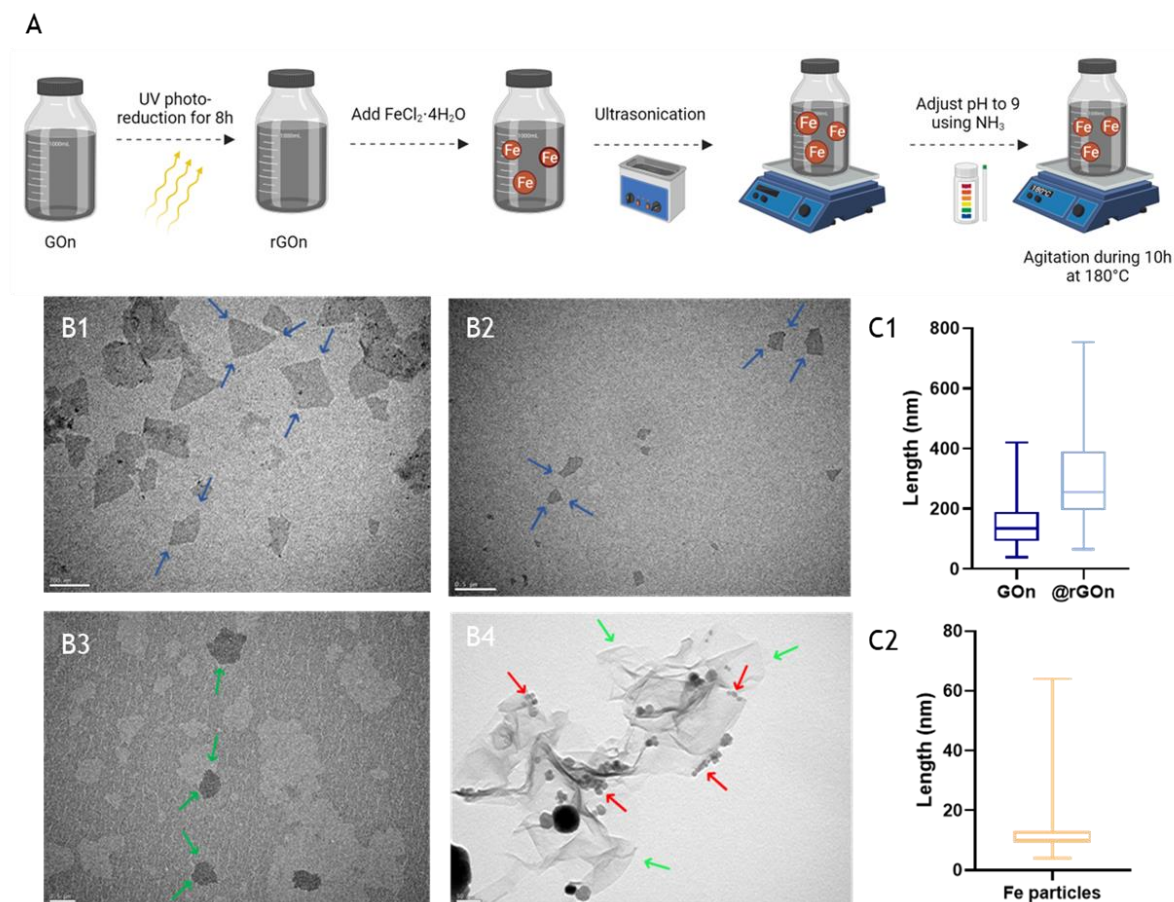


Figure 21. A) Schematic of nanographene oxide (GOn) simultaneous magnetization/reduction process to produce magnetic reduced nanographene oxide (@rGOn); B) Transmission electron microscopy (TEM) images of GOn (B1-2), @rGOn (B3-4); Plot box comparison of GOn and @rGOn lateral particle size (length) (C1), and of iron oxide nanoparticles size (Fe) at @rGOn surface (C2). Sharp edges are indicated by blue arrows (B1-2), while green arrows point out wrinkled edges (B3-4), predominant at @rGOn, and red arrows point out iron nanoparticles at @rGOn surface (4). Scale bars represent 200 nm in image B1, 500 nm in images B2-3, and 50 nm in image B4.

4.1.2 Physicochemical Characterization

Fourier-transform infrared spectroscopy (FTIR) spectra were obtained to confirm the successful oxidation of GOn and its simultaneous reduction and iron functionalization to produce @rGOn (Figure 22.A). GOn displays a typical spectrum, with a broad band between $3000\text{--}3600\text{ cm}^{-1}$ corresponding to O-H stretching vibrations, usually attributed to adsorbed water molecules, hydroxyl, and carboxyl groups. Around 1700 cm^{-1} , C=O stretching vibrations are identified, corresponding to carbonyl and carboxyl groups. Cyclic alkaline (C=C) from unoxidized graphite can be found around 1600 cm^{-1} . The bands around 1163 cm^{-1} and 1037 cm^{-1} , correspond to C-O stretching vibrations from ethers, and epoxides display C-O bending vibrations around 903 cm^{-1} [208, 209]. As expected, @rGOn spectrum is similar to GOn's one, however, a significant reduction of all bands is observed, moreover C=O stretching (1728 cm^{-1}) and C-O bending (1037 cm^{-1}) are completely absent, attesting the success of the reduction process.

Thermogravimetric analysis (TGA) was used to study the thermal stability of the materials produced, as well as the success of the oxidation and reduction processes (Figure 22.B). GOn thermogram reveals two main weight loss steps. The first weight loss (24.84 %) occurred until 160 °C was reached, which could be attributed to evaporation of adsorbed water. The next step, between 160-300, is caused by the thermal decomposition of labile oxygen-containing groups, such as carboxyls, and epoxides, resulting in a weight loss of 31.89 %. Finally, above 600 °C pyrolysis of the carbon skeleton and elimination of more stable oxygen-functionalities (e.g., carbonyls, hydroxyls) takes place, with the final total weight loss for GOn being of 64.43 %. For @rGOn only a small step of weight loss (4%) was detected between 500-640 °C, attributed to the combustion of carbon skeleton and pyrolysis of more stable functionalities that remained after reduction. This confirms that a successful and complete reduction process took place. Furthermore, the simultaneous magnetization and reduction process revealed to improve @rGOn thermal stability, since typical spectra for rGOn materials found in the literature usually show higher weight loss values [210, 211].

The optical properties of both GBM were analysed by ultraviolet-visible-near infrared spectroscopy (UV-Vis-NIR) (Figure 22.C). GOn present a typical spectrum, having a maximum absorbance peak around 230 nm, corresponding to the electronic $\pi \rightarrow \pi^*$ transitions of C-C aromatic bonds. A shoulder peak can be identified at 300 nm, assigned to the $n \rightarrow \pi^*$ transitions of C=O bonds free electron pairs in the oxygen atoms, that exist in the double bonds between carbon and oxygen, from carbonyl and carboxyl groups. However, @rGOn spectrum, reveals almost non distinguishable broad bands and a 7-fold increase in near-infrared radiation (NIR) absorbance (750-850 nm), consistent with almost complete removal of oxygen functionalities from the material and reestablishment of sp^2 bonds, that took place after simultaneous thermal reduction and functionalization with iron nanoparticles [212].

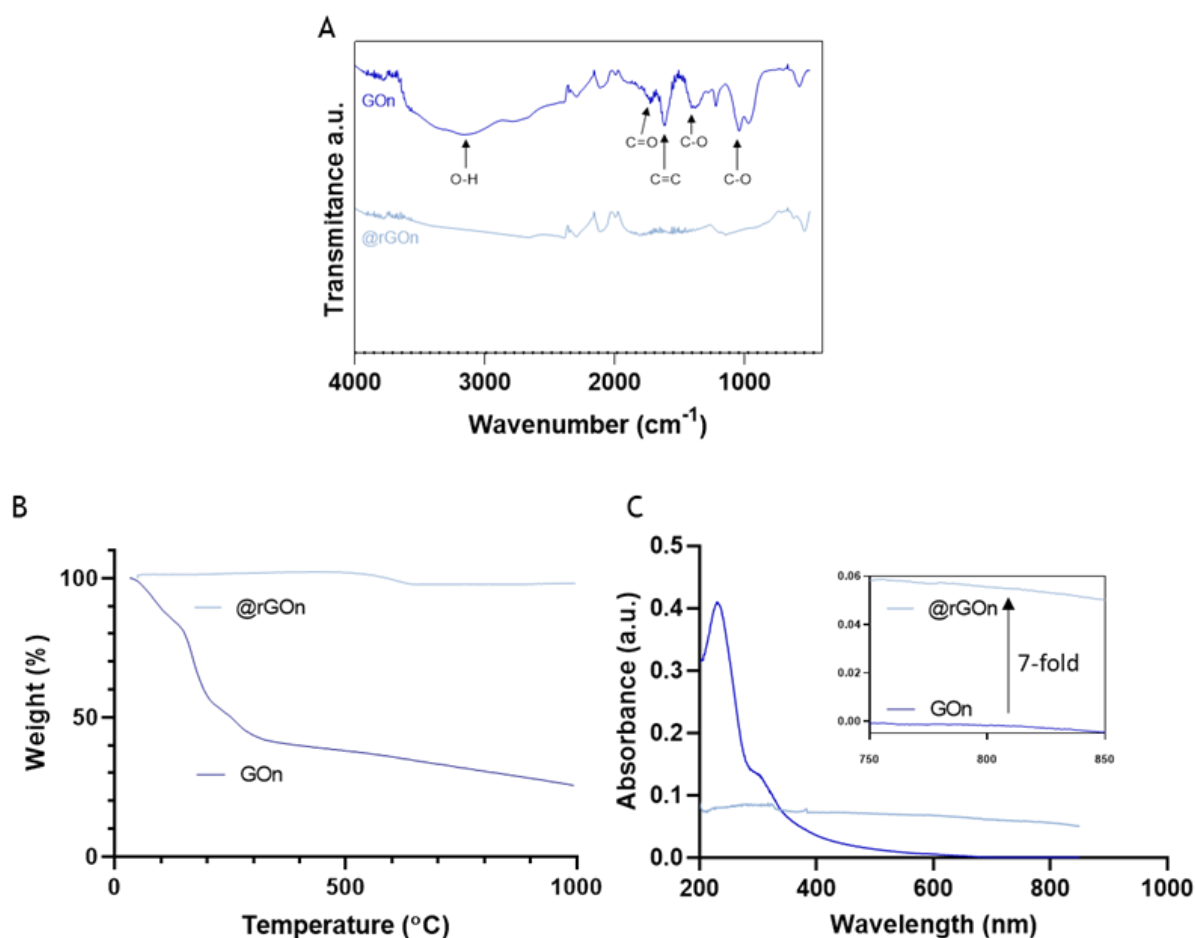


Figure 22. Graphene-based materials physicochemical characterization. A) FTIR spectra of GOn and @rGOn. The arrows indicate the contribution of different surface functionalities. B) Thermogravimetric analysis of GOn and @rGOn, under helium atmosphere. C) UV-Vis-NIR spectroscopy spectra of GOn and @rGOn. The inset shows a zoom-in view of the curves in the NIR range (750-850 nm). GOn curves are represented in dark blue and @rGOn curves in light blue.

4.2 Hydrogels Characterization

@rGOn was incorporated in pectin hydrogels at different loadings (0.625, 1.25, 2.5 wt.%). The initial goal was to develop hydrogels for 3D-printing of scaffolds to be placed after skin cancer surgery, for simultaneous photothermal therapy (destroying any remaining cancer cells and preventing recurrence) and tissue regeneration (to improve aesthetic outcomes). The magnetic properties of @rGOn could eventually be used to mediate the release of active substances/drugs to prevent recurrence or infection, but also to speed up regeneration. The release could be controlled externally using a suitable magnetic field/equipment. Furthermore, magnetic hyperthermia could also be a possibility due to @rGO properties.

Due to time limitations, the work was focused on producing a hydrogel to be applied in the post-surgery defect to deliver photothermal therapy and promote tissue regeneration. Therefore, we defined 50 $^{\circ}\text{C}$ as the temperature that should be achieved to demonstrate that the hydrogels would

have potential to be used for photothermal therapy. Furthermore, those should maintain their structural integrity after being incubated for 24 h at 37 °C in cell culture media (DMEM), and their magnetic properties should be intact. Assays were performed to demonstrate those conditions that are presented next.

4.2.1 Photothermal Properties

The efficiency of composite hydrogels to convert NIR light into heat was evaluated. The pectin only hydrogels were not able to heat until 50 °C, with the maximum temperature they reached being of 42 °C, confirming the key role of @rGOn in the conversion of NIR light into heat. For the hydrogels containing graphene, the heating curves were very similar and all hydrogels were able to increase the temperature of the DMEM in the well until 50 °C. Hydrogels with 0.625 wt.%, 1.25 wt.% and 2.5 wt.% @rGOn concluded the heating process after 12 minutes, 14 minutes and 16 minutes of irradiation, respectively. It is important to note that irradiating the structures until 50 °C exposes them to a temperature above 45 °C for around 20 minutes, taking them more than 30 minutes to return to the physiological body temperature.

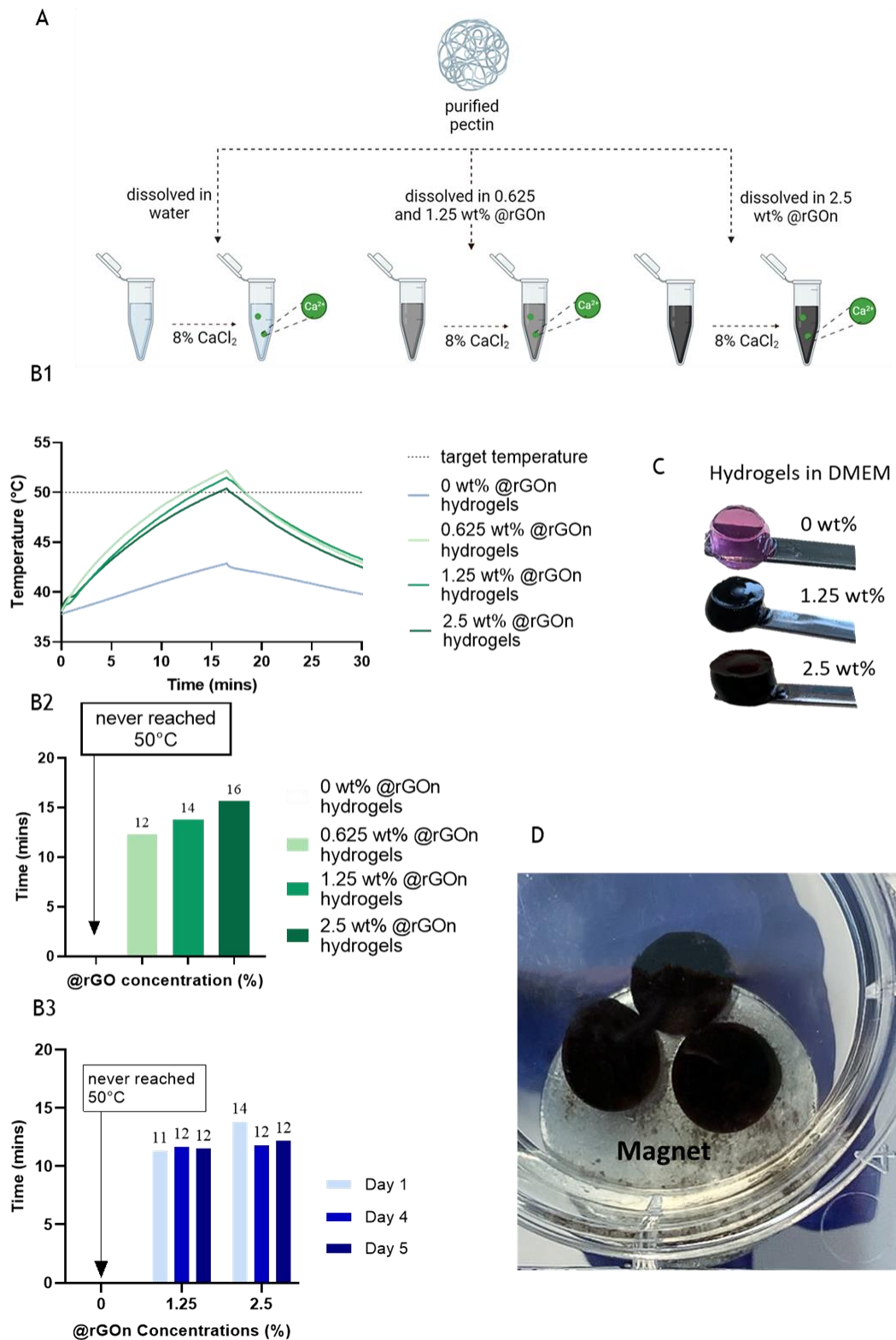


Figure 23. Hydrogels photothermal, magnetic properties and structural integrity characterization. A) Schematic of pectin and pectin/@rGOn hydrogels production. B1) Photothermal properties of the hydrogels under NIR irradiation with our custom-build LED systems (810, 150 mW/cm²). Irradiation for 30 minutes was performed that lead to heating, followed by a cooling period. B2) Time that took as produced hydrogels to reach 50 °C. B3) Time that took hydrogels incubated in cell culture media (DMEM) at 37 °C (1, 4 or 5 days) to reach 50 °C. C) Images of the hydrogels after incubation in complete DMEM for 24h at 37 °C; D) Image of pectin/@rGOn hydrogels (at 1.25 and 2.5 wt%) sustained

by a magnet placed outside a vertically oriented petri dish - the hydrogels stick to the plate due magnetic attraction.

4.2.2 Magnetic Properties

The magnetic properties of the structures with different weight percentages of graphene were assessed using a small magnet under the well plate, while the structures were immersed in Hank's Balanced Salt Solution (HBSS). The HBSS was used to prevent the adherence of the hydrogels to the bottom of the well. The test consisted in evaluating the ability of the structures to follow the magnet as it was moved under the well plate, as it is illustrated by Figure 23.D. Pectin/@rGOn hydrogels at a filler loading of 1.25 and 2.5 wt.% displayed magnetic behaviour, which was similar between them, easily following the magnet movement. However, when the @rGOn loading was just 0.625 wt.%, despite some magnetism being present, the structures did not follow the magnet so easily.

4.2.3 Hydrogel stability in cell culture media

After production, the hydrogels were incubated in complete DMEM at 37 °C for 24 h. The evaluation of the stability was done by the manipulation of the gels with a spatula after incubation. All gels maintained their structural integrity and could be easily manipulated without any damage occurring, as it can be observed in Figure 23.C.

4.2.4 Photothermal Stability

Given that the application of the hydrogels would require the heating of the structures more than once, their ability to perform in multiple cycles of irradiation was evaluated. For this purpose, the pectin/@rGOn hydrogels (1.25 and 2.5 wt%) were incubated in complete DMEM at 37 °C for 5 days and irradiated with NIR until reaching 50 °C, at day 1, 4, and 5. No significant differences were found between all conditions tested and for all time points, just minor variations of 1-2 °C. Therefore, pectin/@rGOn hydrogels (1.25 and 2.5 wt%) maintain their photothermal efficiency after 5 days and 3 cycles of NIR irradiation and heating until 50 °C.

4.2.5 Rheological Properties

In order to understand potential structural alterations of hydrogels as a result of NIR irradiation, a rheological assay was performed. In this test, the rheometer evaluates the mechanical response of the samples to being subjected to deformation while increasing the temperature.

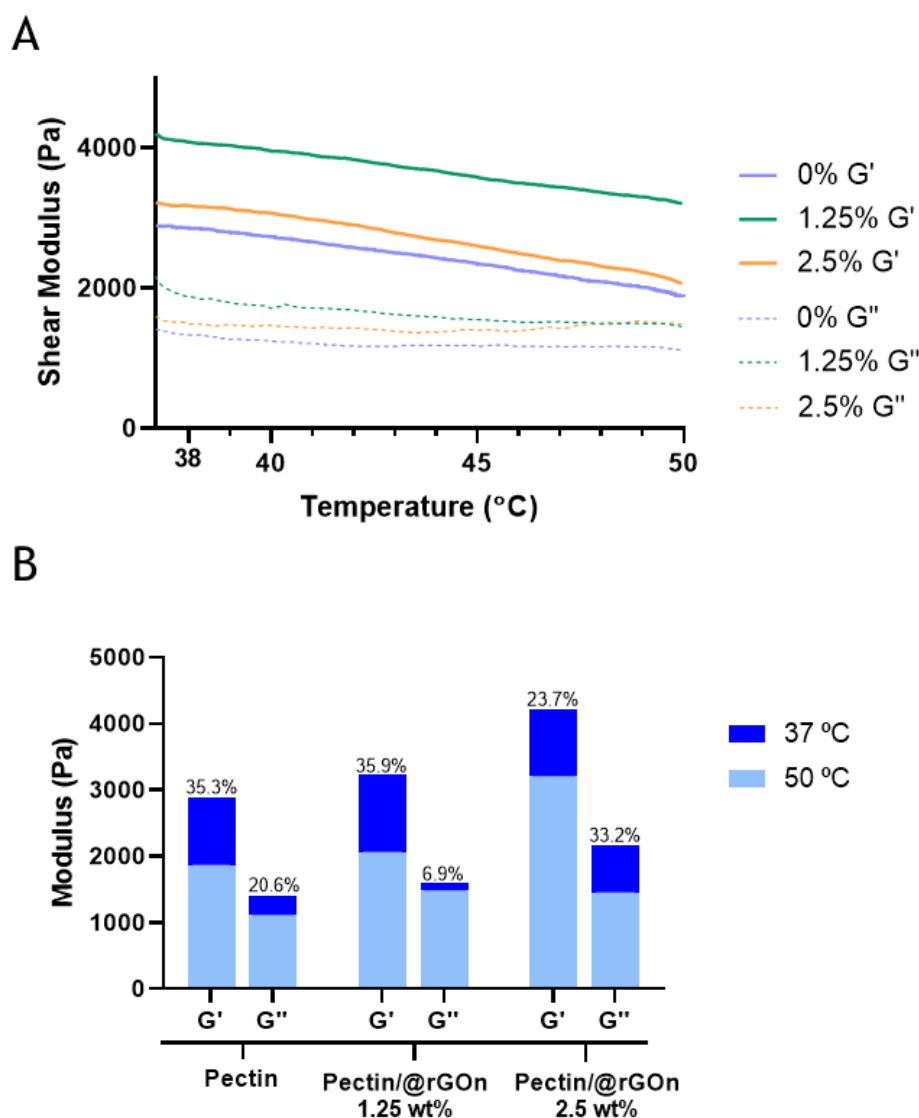


Figure 24. Rheological properties of pectin and pectin/@rGOn hydrogels (1.25 and 2.5 wt.%). A) Storage (G') and Loss (G'') modulus curves with a temperature scanning between 37 and 50 °C; B) Graph bars displaying G' and G'' variation between 37 °C (body temperature) and 50 °C (defined photothermal therapy temperature target) for pectin/@rGOn hydrogels (1.25 and 2.5 wt.%).

The results obtained were presented as the variation of the storage (G') and loss (G'') modulus (Figure 24A and 24B), which are a measure of the elastic and viscous behaviour of hydrogels. The values obtained for the storage modulus (G') at 37 and 50 °C for a 0 wt.% of @rGOn were 2890 and 1870 Pa, for a 1.25 wt.% 3224 and 2066 Pa, and for 2.5 wt.% 4206 and 3210 Pa, meaning a variation of 35.3%, 35.9%, and 23.7% for 0, 1.25, and 2.5 wt.%, respectively. The values obtained for the storage modulus (G') at 37 and 50 °C for a 0 wt.% of @rGOn were 1410 and 1120 Pa, for a 1.25 wt.% 1595 and 1485 Pa, and for 2.5 wt.% 2172 and 1452 Pa, meaning a variation percentage of 20.6, 6.9, and 33.2%. For all @rGOn concentrations, it is clear a decrease in both G' and G'' with the increase of temperature. However, all samples maintain a solid-like state without transitioning to a more liquid-

state, making them all suitable for assays involving the increase of temperature in the range of temperatures considered.

4.2.6 Hydrogels printability

In order to evaluate the potential of the hydrogels to be used to 3D-print a structure that could match defects caused by cancer surgery, their printability was studied.

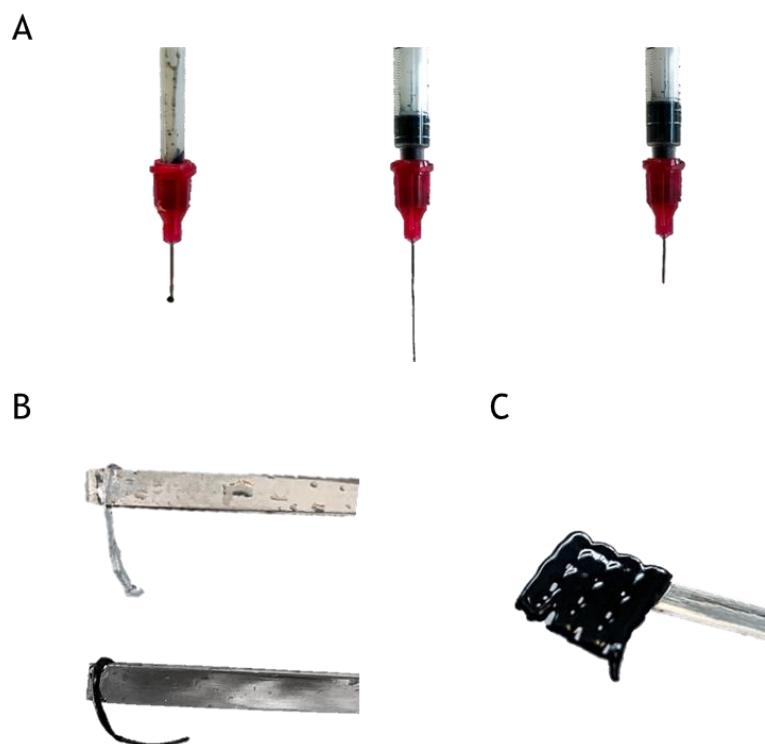


Figure 25. Printing assay for pectin and pectin/@rGOn hydrogels. A) Printing ability of the hydrogels with different concentrations of the crosslinker CaCl₂ (left image = 5 mM; centre image = 7.5 mM; right image = 9 mM) performed with a cylindrical nozzle of 0.25 mm (Nordson EFD); B) Mechanical test of the filaments after the immersion in CaCl₂ 0.5 M solution; Preliminary scaffold printing assay using a Regemat 3D V1 printer (Regemat 3D, Spain).

To create a hydrogel ink that can be processed via extrusion bioprinting, the concentration of the CaCl₂ crosslinker was varied (5, 7.5 and 9mM) and formation of a stable filament at the nozzle was evaluated. As can be observed in Figure 25A, for a concentration of 5 mM CaCl₂ the crosslinking degree polymer is too low, leading to the formation of drops at the nozzle. For a concentration of 7 mM CaCl₂ it is possible to obtain a thin and uniform filament, while for the highest CaCl₂ concentration nozzle clogging was observed, which precludes the filament extrusion. After printing, the filaments would be subjected to external crosslinking by 10-minute incubation in 0.5 M CaCl₂ solution. As can be observed in Figure 25B, the filament exhibits sufficient structural integrity allowing for its manipulation using a spatula without breakage.

After determining the concentration of the crosslinker enabling the printing of uniform filaments, a preliminary printing test was done towards creating a porous two-layer structure. As it can be observed in Figure 25C, the parameters still need to be optimized, as most of the porous appear closed. However, the structure exhibits a uniform appearance and sufficient mechanical and structural properties to be manipulated post-printing.

4.3 In vitro biological studies

To attest the structures cytocompatibility and photothermal efficiency two types of tests were performed. Firstly, the cells were exposed to medium that was in contact with the hydrogels to verify potential release of toxic component that would affect cell viability. Secondly, a new assay was designed to evaluate the photothermal efficiency of the hydrogels when in contact with a cell-laden 3D matrix, simulating the *in vivo* scenario.

4.3.1 Indirect Contact assays

The hydrogel cytotoxicity was assessed through an extract exposure assay, analysing if any GBM particles would leach out of the hydrogel samples and cause alterations in cells metabolic activity or even death. In Figure 26B, the obtained results for HFF-1 fibroblasts are presented and expressed as average and standard deviation. The results were normalized to the values of cells incubated in complete DMEM, in the absence of exposure to hydrogel (negative control).

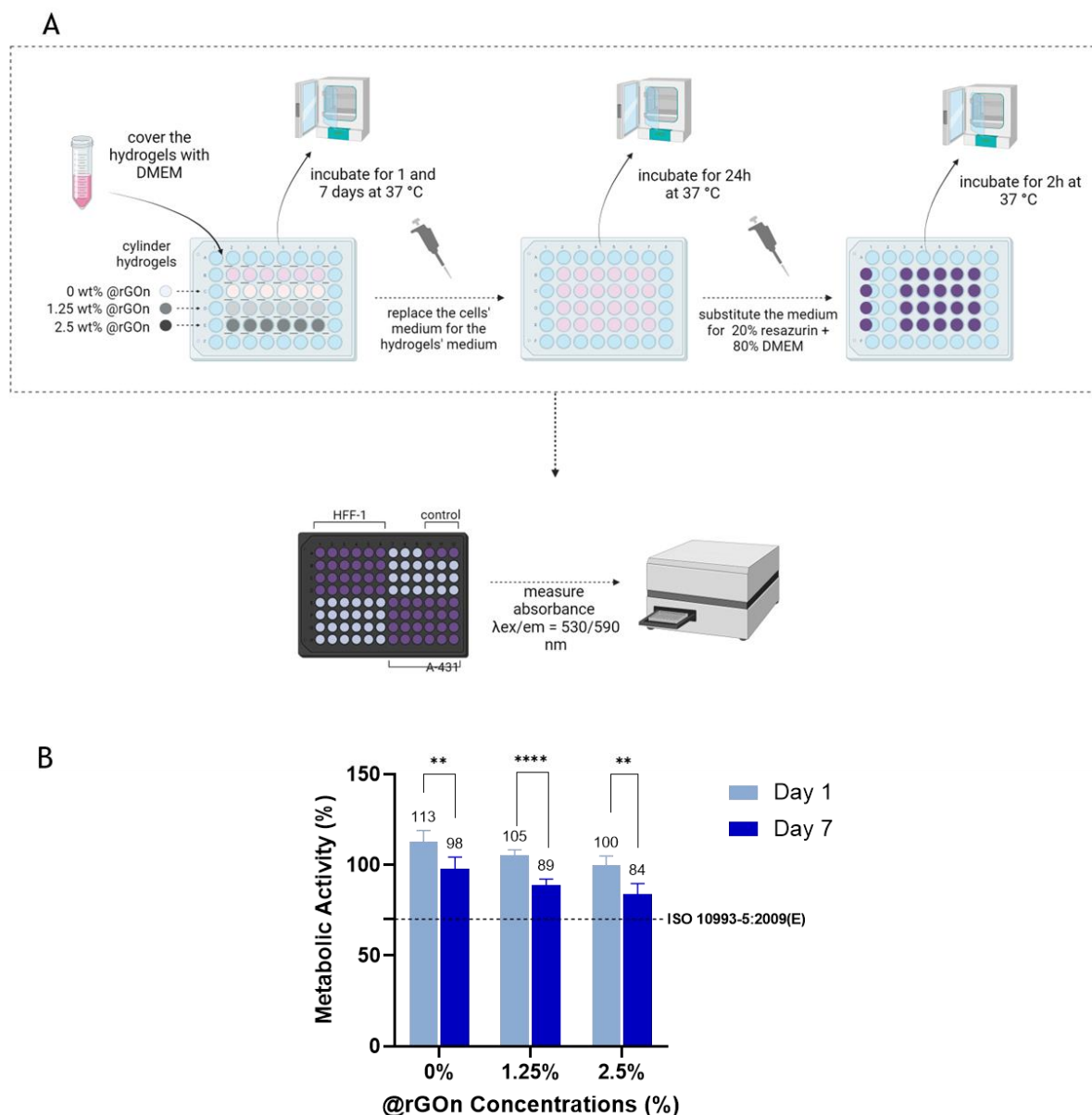


Figure 26. Indirect cytotoxicity tests of pectin and pectin/@rGOn hydrogels (1.25 and 2.5 wt.%). A) Schematic of the procedure used. B) Metabolic activity determined performing the resazurin assay, 24 h after human skin fibroblasts (HFF-1 cells) being incubated with 1- or 7-days extracts from the hydrogels. Results are normalized to values of the control - HFF-1 cells exposed to cell culture medium only (complete DMEM) and presented as average and standard deviation ($n=6$). The dashed line marks the toxicity limit at 70 % defined by ISO10993-5(E). Statistically significant differences are represented as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

For the day 1, the lowest value obtained was equal to the control, showing that the hydrogel presents no cytotoxicity to the cells. At day 7 and for 0, 1.25, and 2.5 wt.% @rGOn, the values of metabolic activity obtained were 98, 89 and 84%, respectively. Despite a significant decrease in metabolic activity for all @rGOn concentrations, the values obtained are above the limit set by the ISO 10993-5:2009(E).

4.3.1 Influence of NIR irradiation on the viability of cells embedded within a GelMA 3D hydrogel- Preliminary results

In order to simulate the final application of these structures, namely to contact with skin normal and cancer cells, and mediate destruction of cancer cells, while keeping normal cells intact and eventually promote tissue regeneration, a new structure was developed to allow the accommodation of a 3D cell matrix during the direct contact assays (Figure 27).

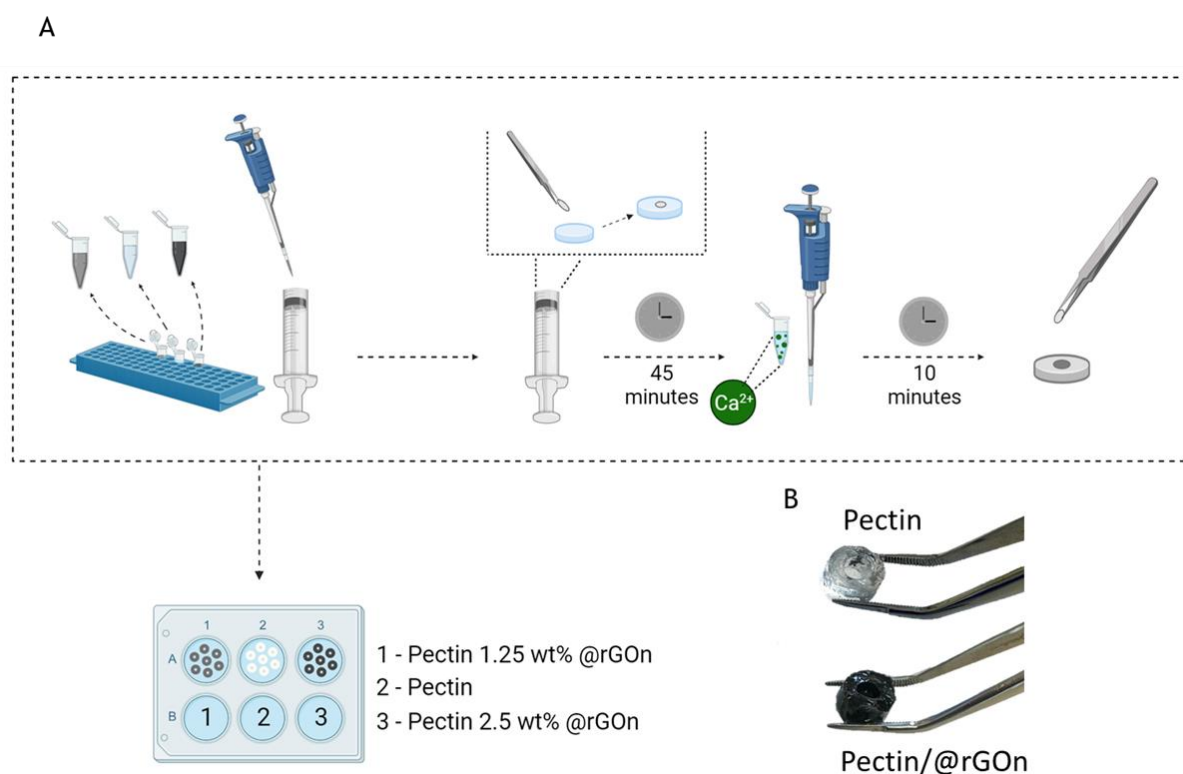
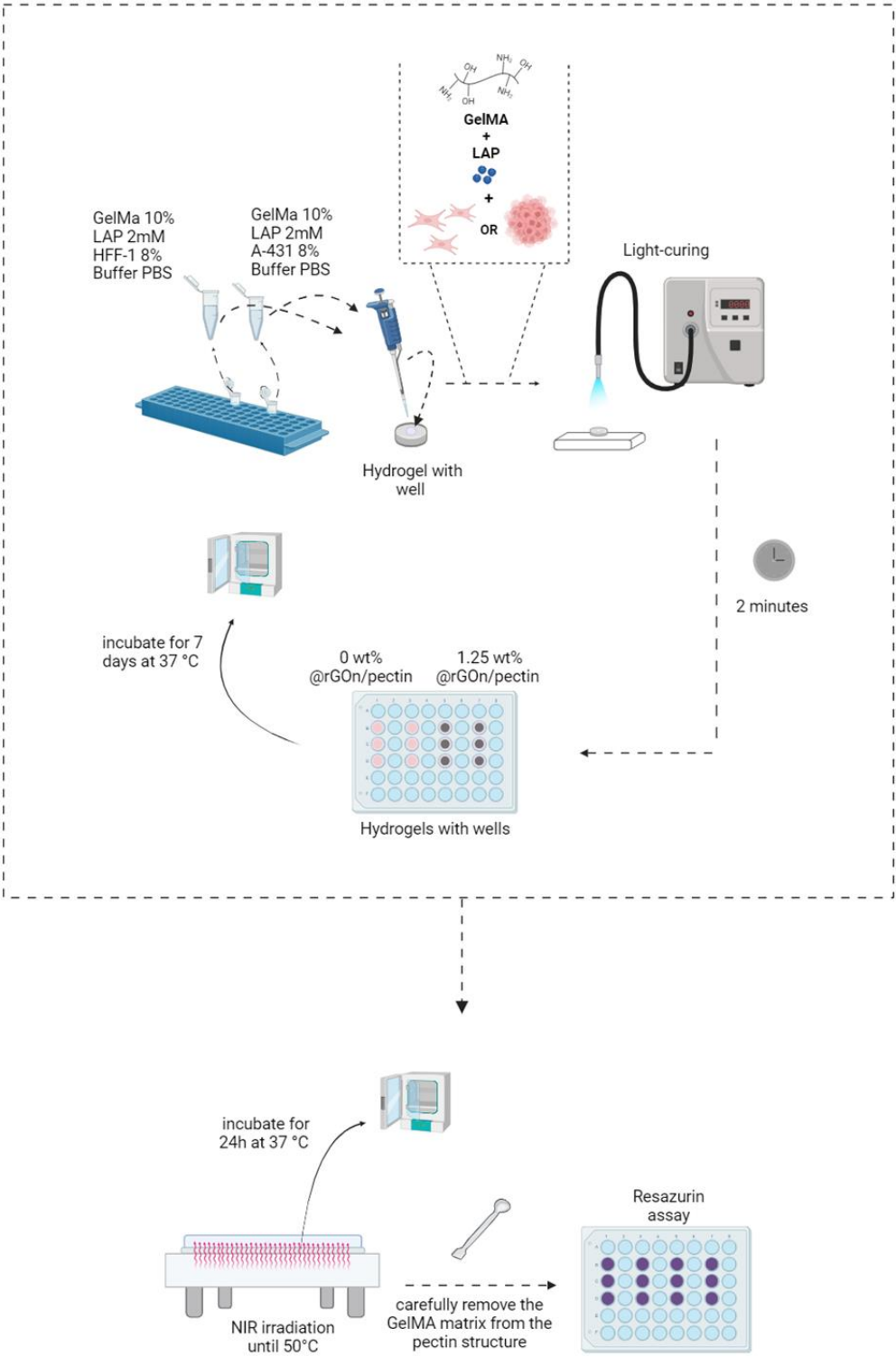


Figure 27. A) Schematic of the production of the novel structures manufactured to nest the GelMA matrixes. Pectin and pectin/@rGOn 1.25 wt.% dispersions were produced as prior described, and upon loading in the 3 mL syringe a PDMS cylinder cast with 4 mm x 2 mm was used to create a depression in the middle of the hydrogel, resulting in a centred well on the top of the structure. The system was left to reticulate for 45 minutes and immersion in the 0.5 M CaCl_2 solution performed. B) Image of pectin and pectin/@rGOn 1.25 wt.% hydrogels after production where the wells produced are visible.

The new pectin and pectin/@rGOn structure to nest the GelMA matrix was successfully produced, as it can be observed in Figure 27B. Due to the fact that the manufacturing process is fully manual, there is a variability when considering different structures.

A



B

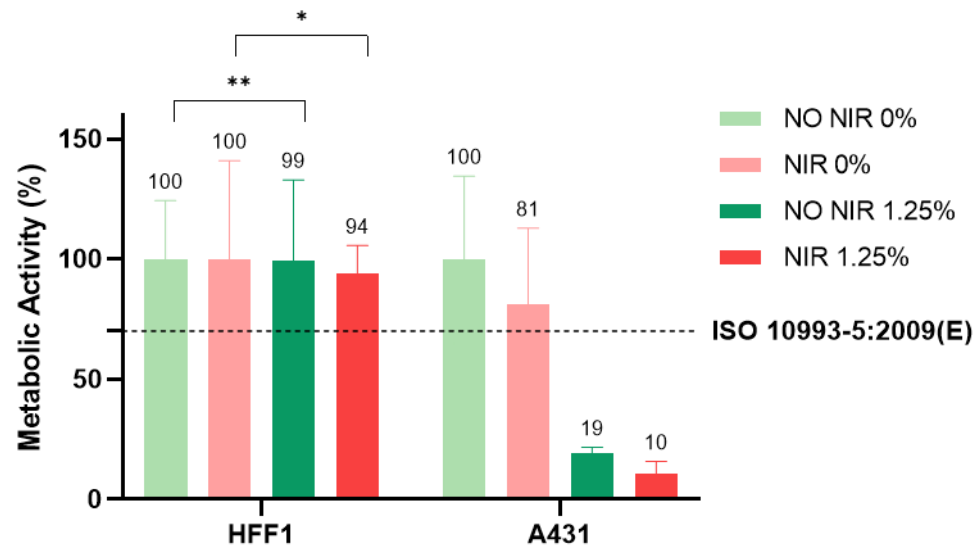


Figure 28. Influence of NIR irradiation on the viability of cells embedded within a GelMA 3D hydrogel. A) Schematic of the production of the 3D GelMA cell matrixes. Two different solutions were prepared for both cell lines. The solutions contained 10 wt.% GelMA and 2 mM LAP. After, HFF-1 and A-431 cells were added to each solution. The cell-containing solution was added to the well created in the pectin-based hydrogel and photocrosslinked using a UV light. The structures were incubated in complete DMEM. After 7 days, the plates were irradiated and a resazurin assay was performed. B) Metabolic activity after 7 days of incubation of the GelMA matrix nested in the pectin or pectin/@rGOn hydrogels followed by irradiation until 45 °C and incubated for 24 h prior to evaluation by resazurin assay for HFF-1 and A-431 cells; Dashed line marks the toxicity limit at 70 % (ISO10993-5(E)). Statistically significant differences are present as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Resazurin assays for HFF-1 cells embedded within a GelMA 3D hydrogel revealed that there was no significant difference between all conditions tested, with cell metabolic activity values being between 94-100 %. For A-431 cells embedded within a GelMA 3D hydrogel in contact with pectin hydrogels only, after NIR irradiation, metabolic activity decreases to 81%. However, this value is still above the toxicity limit of 70% (ISO10993-5(E)), and no statistically significant differences are observed. Interestingly, A-431 sample in contact with pectin/@rGOn 1.25 wt.% hydrogels, reduce their metabolic activity to 19%. This value decreases to 10% when NIR irradiation is performed. However, the test needs to be repeated before any conclusions be withdrawn, since those are just preliminary results.

To further understand the effect of the hydrogels and NIR irradiation on HFF-1 and A-431 cells, a live/dead assay was performed as described in Figure 28.A. Regarding the images obtained, it is possible to conclude that the GelMA matrix creates an environment that enables fibroblast to acquire their characteristic morphology. There is a clear difference between the fibroblasts, where it is possible to see their characteristic elongated structure, and the cancer cells, who present a round morphology. The images also allow the conclusion that the staining process was performed successfully.

Since only 2 replicates were performed, no statistically significant differences have been found between the samples in the quantitative analysis, even considering the clear differences between values observed in Figure 29.B. Therefore, the analysis of the results must be done with extreme caution. Two observations worth to mention are that A-431 cells seem to cause a much higher cell death in general than HFF-1 cells. Hydrogels containing @rGOn (1.25 wt.%) seem to also lead to a lower total number of cells found in the GelMA 3D hydrogel.

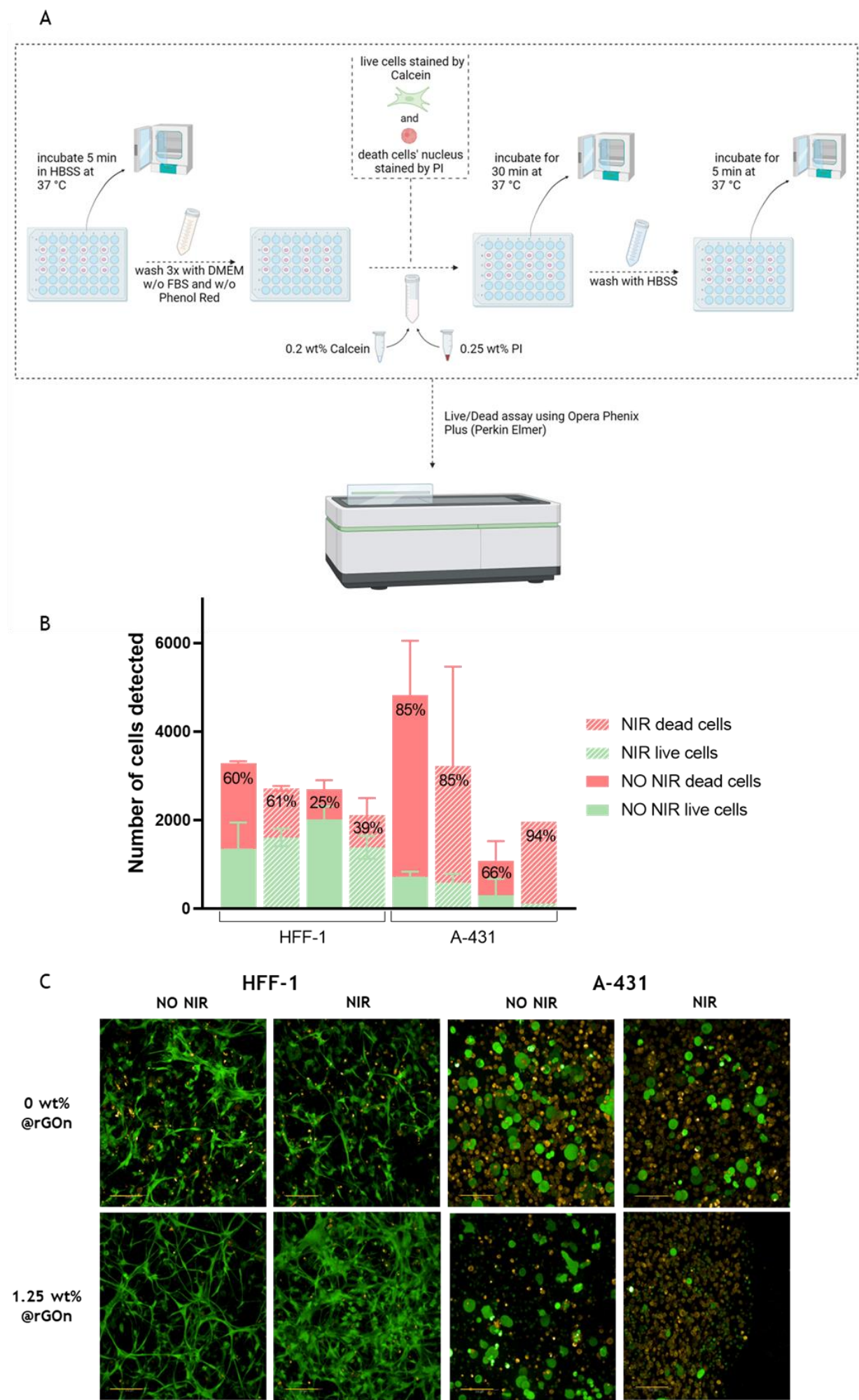


Figure 29. Cell viability assays of human skin fibroblasts (HFF-1) and skin cancer cells (A-431) embedded in GelMA matrix placed in the wells of pectin or pectin/@rGOn 1.25 wt.% hydrogels for 7 days, followed by NIR irradiation until 45 °C and incubation for 24 h. A) Schematic of live/dead staining of cells in GelMA matrixes. B) Total number of cells and live/dead percentage after the treatment mentioned above. C) Representative images of cells in GelMA matrixes after being removed from the wells of pectin or pectin/@rGOn 1.25 wt.%, subjected or not to NIR irradiation. Live cells are stained green with calcein and dead cells' nucleus red with propidium iodide. Scale bar represents 200 μ m.

Chapter 5

Discussion

The primary goal of the presented work was to develop a magnetic graphene-based material (GBM), particularly magnetic reduced nanographene oxide (@rGOn), and include it on a printable hydrogel with the potential to be applied in cancer phototherapy. As cancer therapy demands research to better patients live expectancy and quality, this work was developed with the purpose of fabricating a system through which cancer cells could be eliminated without compromising the surrounding tissues. The work developed could be incorporated into a patch that allows daily irradiation and periodical check-ups upon the progress made.

While graphene has remarkable characteristics regarding mechanical, electronic, and optical properties, which researchers have used to apply in other areas, recently the chemical properties and functionalization of graphene have increased researchers' interest in implementing it to biomedical applications. Graphene oxide (GO) is commonly obtained using modified Hummers method used here and in our group previous work [213]. This method enables the introduction of several oxygen-containing functional groups on the graphene layers, forming nanographene oxide. Although the introduction of functional groups (such as epoxide, hydroxyl, and carboxyl groups) during the oxidation process increases the material's potential to be applied in the biomedical area, it affects pristine graphene properties including electrical conductivity and mechanical properties. GO is typically an insulator, or a poor conductor compared to pristine graphene and the addition of oxygen functional groups disrupts the carbon-carbon bonding in the graphene lattice, leading to a reduction in mechanical strength. In order to combine pristine graphene electric and mechanical properties, while still benefiting from GO functionalization, a wide range of methods has been explored to restore some of the properties of pristine graphene. Over the years different reduction methods have been

explored to produce reduced GOn (rGOn), including chemical, thermal, and electrochemical reduction pathways [214].

Single-step in situ formation of magnetic nanoparticles on reduced graphene sheets has been previously described by Xue et al. using a solution of iron chloride ($\text{FeCl}_2 \cdot 4 \text{H}_2\text{O}$) and oxidized graphene sheets ($\text{pH} = 9$, $T = 180^\circ\text{C}$, $t = 10$ hours). Overall, it was reported the efficient oxidation of Fe^{2+} into Fe_3O_4 nanoparticles and its deposition onto thermally reduced graphene sheets [215]. Following this protocol, in this research, a solution of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and GOn was used to produce magnetic reduced GOn (@rGOn).

Upon GBM production, powders morphology, particle size and stability was assessed.

Through transmission electron microscopy (TEM) the reduction of the GOn was confirmed @rGOn and iron nanoparticles incorporation and distribution across @rGOn surface was observed. In detail, GOn and @rGOn were obtained with an average particle size around 200 and 400 nm, respectively. Concerning the iron nanoparticles, spherical particles with sizes between 4 and 64 nm with an average size around 13 nm were observed.

The optical properties of GBM were analysed by UV-Vis. For GOn it was observed a maximum absorbance peak around 230 nm, corresponding to the electronic $\pi - \pi^*$ transitions of C-C aromatic bonds and a shoulder at 300 nm, assigned to the $n - \pi^*$ transitions of C = O. In the case of @rGOn the peak of the $\pi - \pi^*$ transition shifts to 275 nm, indicating that some groups on the GO surface are removed and the conjugated structure is restored, and the $n - \pi^*$ transitions of C = O are not present, confirming the reduction of the material. These alterations were expected and reported previously by Yang et. al. while studying an easy and green approach of reducing graphene nanosheets with zinc instead of temperature [216].

FTIR spectroscopy revealed the characteristic peak at 1430 cm^{-1} corresponds to un-oxidized graphitic domain's C=C skeletal vibration. Several peaks in the GONs spectrum show oxygen-containing functional groups. The characteristic peaks of O-H (3156 cm^{-1}), C=O (1732 cm^{-1}), C-O (1380 cm^{-1}), C-OH (1404 cm^{-1}), and C-O (1044 cm^{-1}) are thought to be assigned to stretching of hydroxyl, carboxyl groups, carboxylic acid, and carbonyl groups. The presence of oxygen functional groups in FTIR of GOn's spectrum indicates that the flake graphite has been oxidized to GONs. There was no noticeable peak after reducing and converting GONs to @rGONs, indicating that @rGONs was electrochemically reduced. The FTIR confirmed a successful and so far, perfect reduction of the graphene oxide [217].

GBM thermal stability was tested by TGA, where GOn thermogram expressed two main weight loss steps. The material experienced the first weight loss around $100\text{-}210^\circ\text{C}$ due to water evaporation and thermal decomposition of oxygen-containing groups including carboxyl, hydroxyl, epoxy, nitrogen dioxide, and ketone. The second weight loss was present between $210\text{-}600^\circ\text{C}$, corresponding to the combustion of carbon skeleton and pyrolysis of more stable functionalities like carbonyls and residual hydroxyls. The total weight loss of GOn comes up to 72.78%, 38.1% regarding the first weight loss and 34.68% the second. The reduced graphene oxide appears to have only minimal weight loss (4%)

between 500-640 °C, attributed to the combustion of carbon skeleton and pyrolysis of more stable functionalities like carbonyls and residual hydroxyls. The discrepancy of these values may be explained by the presence of Fe nanoparticles, which seemed to act as a thermal stabilizer since. Fe²⁺ ions can promote stronger interactions between adjacent rGO sheets enhancing interlayer bonding resulting in a more stable structure, even at elevated temperatures. Fe²⁺ ions may also be responsible for improving the thermal conductivity of rGO composites. This can help dissipate heat more efficiently, reducing thermal degradation. In another work Saraf et al. have also observed a change in thermal stability when adding Fe nanoparticles to rGO. It was obtained a weight loss of a composite of rGO-Fe₂O₃ of 19% while the rGO presented a weight loss of 90%, when the temperature reached 900 °C [217].

To infer pectin and pectin/@rGOn hydrogels NIR conversion efficiency, a custom LED NIR system (810 nm, 1000 mW/cm²) was used to irradiate the hydrogel structures. The hydrogels in the absence of graphene were not able to reach 50 °C, while the hydrogels containing graphene reached the target temperature in less than twenty minutes. This confirms the ability of @rGOn to convert NIR irradiation into heat. Hydrogels with 0.625 wt.% @rGOn concluded the heating process after 12 minutes, hydrogels with 1.25 wt.% @rGOn concluded the heating process after 14 minutes and, finally, hydrogels with 2.5 wt.% @rGOn concluded the heating process after 16 minutes. The difference can be attributed to the variability of the LED system. However, factors including saturation of absorption and light scattering might explained the results acquired. There is typically an upper limit to how much light a material can absorb efficiently, plus when the material is present at a high concentration light scattering becomes more pronounced. Scattered light does not contribute to the heating of the material, reducing the overall efficiency of light-to-heat conversion. In this way, as much as @rGOn had a clear role in increasing light-to-heat conversion of the pectin hydrogels, the tested concentrations of the GBM appear to be too high to see a concentration-dependent increase of temperature. Photothermal conversion saturated had previously been reported by Kasparis et. al, where using a using an 808 nm laser to evaluate light-to-heat conversion, photothermal conversion saturation was reached at 1 mg_{Fe+Zn} ml⁻¹, for zinc doped iron oxide nanoparticles [218].

To evaluate the hydrogels behaviour during irradiation, their rheological properties were assessed. As expected, all samples experienced a decrease in storage and loss modulus, as the increase of temperature generates an enhanced movement of the particles weakening the connections of the solid hydrogel. Regarding the storage modulus (G') a higher content of @rGOn generated a stiffer gel both at 37 and 50 °C. However, the 1.25 wt.% hydrogel exhibited the highest variation when compared with the other concentrations (35.9 %), compared with 35.3 % for 0 wt.% and 23.7 % for 2.5 wt.%.

The loss modulus values obtained at 37 °C indicated that an increase in @rGOn concentration creates a more viscous hydrogel. In contrast, measurements at 50 °C showed a lower loss modulus for the hydrogel containing the higher @rGO concentration. For a 0 wt.% of @rGOn the variation of the modulus was 20.6%, for a 1.25 wt.% the variation decreased to 6.9%, and for 2.5 wt.% the variation

reached 33.2%. For both modulus it was expected that the graphene would improve the hydrogels rheological properties as it is a material known for its exceptional mechanical properties. However, Bhat et al. obtained similar results where large concentrations of GOn reduced electrostatic interactions due to aggregation of GOn, decreasing the gel strength caused by the weakening of network structure. It is possible that the concentrations chosen generate aggregation of the @rGO compromising the hydrogels rheological properties, in the temperature range observed [219].

The hydrogels were tested for printing ability and showed great potential to be integrated in a scaffold structure in future work. For a 3.5 wt.% of polymer and 7.5 mM of CaCl_2 a thin and uniform filament was printed using a 0.25 mm nozzle. In a preliminary test of the hydrogels behaviour once printed in a scaffold structure, the hydrogel with the same concentrations stated before was printed through a 3D printer into a 2-layer scaffold structure, homogeneously. Although optimization of the printing parameter is still required, the composition studied is printable and shows great potential to be applied in more complex structures than the ones produced during this work.

To attest the hydrogels potential to be integrated into biomedical applications, the cytocompatibility of the structures was evaluated through two assays. The cytocompatibility limit was set at 70 % metabolic activity percentage by the ISO 10993-5:2009(E) norm. The first one consisted of an extract exposure assay where it was observed how the contact of the hydrogel structures with the cells' medium would affect the cells' metabolic activity, after 24h and 7 days of contact. After 1 day of contact, the lowest value of metabolic activity obtained was the same as the one used as a control. In this way, it was concluded that the hydrogels did not release any toxic components after 1 day of incubation. For the day 7, for a 0, 1.25, and 2.5 wt.% @rGOn, the values of metabolic activity obtained were 98, 89 and 84 %. Although these constitute a significant decrease, the values obtained still show cytocompatibility as they are above the set limit. These decrease in metabolic activity may be explained by the hydrogels' absorption of medium components, as it is clear that medium in contact with the hydrogels exhibited a lighter shade. The medium in contact with the hydrogels containing graphene showed a lighter shade than the exposed to the only pectin ones, and the hydrogel with the highest concentration of @rGO resulted in the lightest medium after incubation. Again, the difference in cell medium component absorption might be related to the aggregation properties of graphene where more space is left for absorption of other components, for higher @rGOn concentrations. These results would not impact the final application of these structures as the nutrients used for the cell metabolism are not freely in the body. It is not expected that graphene presents cytotoxicity as cytocompatibility of graphene containing hydrogels has been documented. Dorishetty et al. while producing printable silk/graphene hydrogels. The cell viability presented up to 7 days of L929 mouse fibroblast seeded cells on hydrogels surface was higher than 86% cell viability demonstrating their suitability for biomedical applications [220].

To evaluate the NIR irradiation influence of cells embedded in GelMA, a new pectin and pectin/@rGOn structure was created, similar to the ones fabricated for the extract exposure assays, but adding a small well on the centre top part of the pectin-based structure. In this well, 20 μL of a

GelMA matrix containing fibroblast and cancer cells were placed and photocured using a photoinitiator LAP and UV light. After, the samples were incubated for 7 days in complete DMEM to allow full adaptation and establishment of normal morphology and metabolic activity. After 7 days, the structures were NIR irradiated for 5 minutes (reaching 45 °C in hydrogels containing graphene), to evaluate the ability of the pectin and pectin/@rGOn hydrogels to cause mild hyperthermia. The metabolic activity was evaluated using a resazurin assay and cells' viability was also observed through a Live/Dead assay.

As expected after the extract exposure assays, the metabolic activity of the HFF-1 nested in a pectin only hydrogel and in contact with @rGOn showed no significant difference, as did the irradiated and not irradiated cells. This allows the preliminary conclusion that HFF-1 cell line is resistant to temperatures in the range of mild hyperthermia. In this way, cells nested in a pectin only hydrogel presented a metabolic activity of 100% with or in the absence of NIR irradiation. The cells nested in a pectin/@rGOn hydrogel had a metabolic activity of 99% with no irradiation, and a metabolic activity of 94% after irradiation.

Cancer cells presented very different results as metabolic activity suffered a significant decrease only by contacting with pectin/@rGOn hydrogels. Metabolic activity obtained for cancer cells nested in a pectin only hydrogel was 100 and 81%, with no NIR irradiation and after NIR irradiation, respectively. The cancer cells nested in a hydrogel containing @rGOn showed a metabolic activity of only 19% without NIR irradiation and 10% with NIR irradiation. The NIR irradiation appeared to have no significant effect on the cells' metabolic activity. This allows the preliminary conclusion that A-431 cell line are resistant to temperatures in the range of mild hyperthermia, however @rGOn seems to present significant toxicity to this cell line.

It is important to note that this assay was only performed once and with only four replicates per condition, so further investigation is required to take any conclusions. However, it would be interesting to perform extract exposure assays with A-431 cells to confirm the @rGOn cytocompatibility with this cell line. As NIR irradiation did not seem to impact cells' metabolic activity, higher irradiation periods should be tested to evaluate the NIR influence on cells.

Finally, cell viability was access through a Live/Dead assay. For this the cells were implemented in a 10 wt.% GelMA matrix and left to adapt and proliferate for 7 days. As the presented work is only a preliminary work given that the assay was performed once with only two replicates per condition, optimization is still crucial to obtain Live/Dead results that will agree with the ones obtain in the resazurin assay. However, the viable cells behaviour and morphology acquired after 7 days in the gel was observed, due to Calcein AM staining. The skin fibroblasts presented their normal morphology, being easily distinguished by their characteristic elongation. Skin cancer cells presented round morphology, but quite dispersed. However, it is possible to observe cell-to-cell interaction in the fibroblasts images. The images revealed a 3D network where the cells had established communication and had adapted to the GelMA matrix. Previously, similar results had been reported by Ibañez et al. In this work a 10 wt% GelMA scaffold was manufactured and the fibroblasts metabolic

activity and behaviour were evaluated. After 7 days the cell viability (%) practically doubled when compared to 1 and 3 days, supporting the incubation of the cells on the GelMA matrix for 7 days. Normal dermal fibroblast activity was detected within the 3D-printed GelMA scaffolds while showing that few/no dead cells were present. Additionally, the live cells presented a homogeneous distribution within the 3D-printed structure [221].

In summary, these results demonstrated for the first time the integration of a novel GBM in pectin hydrogels exhibiting great photothermal properties and adequate cytocompatibility enabling its introduction in biomedical applications. The GelMA structure allowed a closer simulation of the hydrogel real application, being the combined pectin or pectin/@rGOn + GelMA matrix system a complete novelty.

Chapter 6

Conclusion and Future work

6.1 Conclusion

The present dissertation focuses on the development of pectin/@rGOn hydrogels for photothermal therapy of skin cancer. Currently, available therapies are not effective, and their safety is not ideal, therefore new strategies are required to offer more selective and effective treatments to patients, such as phototherapy. The state-of-the-art review shows that hydrogels and bioinks are already implemented in the areas of cancer photothermal therapy, magnetic hyperthermia, and tissue engineering, normally conjugated with nanoparticles that enhance desired properties. A variety of polymers and nanoparticles, have been implemented in biomedical applications, including pectin conjugated with CNM, mainly in the area of cancer photothermal therapy. Hydrogels and bioinks have great potential to be used in the 3D printing of biomedical devices. The most common applications of these printed devices are scaffolds for tissue engineering and sensors or biosensors. The constructs can be printed through different techniques, like inkjet-, light- and extrusion-based bioprinting. CNM employed in the inks enable modulation of several properties in the final constructs, like mechanical properties, thermal and electrical conductivity, and biological response. Herein, GOn was produced through the modified Hummers method. GOn was reduced by UV radiation and magnetization occurred by in situ precipitation forming @rGOn. Physical characterization by TEM revealed average sizes of 147.9 ± 82.2 nm for GOn, 298.4 ± 67.7 nm for @rGOn, and 12.9 ± 9.3 nm for Fe nanoparticles.

FTIR analysis showed in GOn, typical oxygen-containing functional groups, and following UV reduction, the strength of their bands decreased or even disappeared. Additionally, TGA results showed an increase in thermal stability when comparing @rGOn with GOn, further demonstrating the effectiveness of the reduction. For GOn the total weight loss was 64.43 %, while @rGOn only suffered a 4% weight loss. UV-Vis spectroscopy showed that @rGOn displayed a 7 times increase in NIR (810 nm) absorption over GOn. This material was chosen for additional testing with the goal of developing the intended phototherapeutic application after identification of the @rGOn enhanced photoproperties. The development of a material combining enhanced photothermal properties while

exhibiting magnetic properties is of great impact. The novel GBM, @rGOn, was further investigated with the goal of evaluating its photothermal efficiency when combined in pectin-based hydrogels. Hydrogels were crosslinked by internal gelation using the CaCl_2 and subjected to a secondary crosslinking by immersion in CaCl_2 . The optimal concentration to produce hydrogel disks was found to be 3.5 wt.% polymer, with 7.5 mM of CaCl_2 concentration. Rheological assay of the effect of the temperature in hydrogel with different concentrations of @rGOn indicated that the GBM increased the strength and viscous behaviour of the material at 37 °C. For all concentrations, the structures were able to endure deformation while the temperature was gradually increased to 50 °C, without a phase-transformation, making them eligible for photothermal therapies.

When the effectiveness of composite hydrogels in converting NIR light into heat was tested, it was concluded that for 1.25 and 2.5 wt.% of @rGOn, the concentrations evaluated were in an area of photothermal saturation, having very similar heating curves, and reaching 50 °C, under 20 minutes of NIR LED irradiation, with a custom-built LED system. Hydrogel printability was achieved using a hydrogel precursor solution with 3.5 wt.% polymer and 7.5 mM CaCl_2 . With in vitro assays, it was seen that there was no drop in HFF-1 metabolic activity when exposed to hydrogel extracts, determining that @rGOn is cytocompatible for the concentrations evaluated. To evaluate NIR irradiation influence on cells embedded in a GelMA gel, a GelMA matrix was created to mimic cells' 3D network, nested in a well created on the centre top of the pectin/@rGOn hydrogels. For this a 10 wt.% GelMA and 2 mM LAP gel was created, where cells with a density of 10×10^6 cells/mL were uniformly dispersed. The solution was placed in the created well and then photocured using an UV light. The cells were incubated for 7 days, based on literature, to allow metabolic activity and cells' behaviour to mimic the normal one. After 7 days the structures were irradiated with a LED custom system until a temperature of mild hyperthermia was reached (45 °C). Preliminary results of metabolic activity and cells viability and behaviour were obtained. The resazurin assay showed that, for this irradiation time, the NIR irradiation had no impact in cells' metabolic activity, both for healthy and cancer cells. The Live/Dead assay didn't allow any conclusions to be retrieved from the cells viability obtained, as the process still needs optimization and more independent assays to be performed. However, calcein staining confirmed that with 7 days incubation fibroblasts are able to acquire normal morphology and implement cell-to-cell interactions.

To sum up, the hydrogels studied showed great potential for implementation in photothermal therapy as excellent light-to-heat conversion was obtained, combined with sufficient rheological and mechanical properties to allow their application with medium contact and temperature increase. The in vitro assays need further optimization to correctly evaluate their effect on cancer cell death.

6.2 Future work

Based on the results, there are several aspects that would require improvement.

The production of the hydrogels should be refined. Since a printable hydrogel was discovered in this experiment, 3D-printed constructs are possible and should be further developed to produce more reproducible structures.

Further investigation of the magnetization properties of the hydrogels should be developed.

The set up for evaluation of NIR irradiation influence on cells nested in GelMA must be optimized.

Chapter 7

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Appendix A

Supplementary Information for Chapter 4

In this Appendix, supplementary images illustrating the process of developing a protocol using the Opera Phenix Plus (Perkin Elmer), a high content screening system equipped with a fully automated widefield/confocal fluorescence microscope, for the Live/Dead assay are presented.

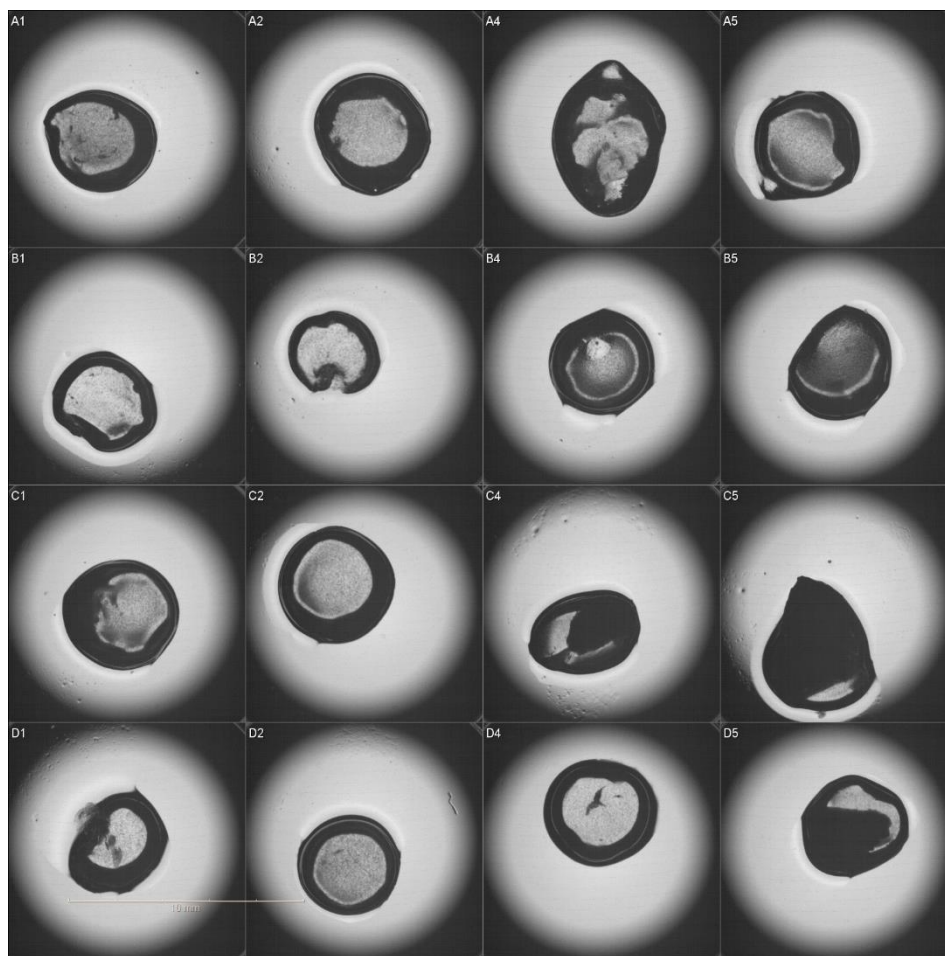


Figure 30. Pre-scan of the wells of interest using brightfield with a 1.25x Air/0.03 objective to detect the GelMA structures using the software Harmony®.

As it is observed in Figure 30., the first step for developing a protocol for utilization of the Opera Phenix Plus (Perkin Elmer) is the pre-scan, which may involve acquiring low-magnification, low-resolution images of the sample, to locate regions of interest and optimize focus. Figure 28. also allows the evaluation of the samples' quality. It is possible to identify some darker figures where light can't penetrate, and imaging will be compromised.

When the pre-scan is complete, a re-scan is performed using a 20x Water/1.0 objective to configure the acquisition parameters for the experiment, such as the number of fields of view (fov), exposure times, and any time-lapse or z-stack settings if required. For this experiment 10 fov were chosen for each plan and 100 z plans capture for each GelMA structure.

After guaranteeing the correct identification of the samples and defining the area of interest, a new optimization step was required to correctly identify the stained structures.

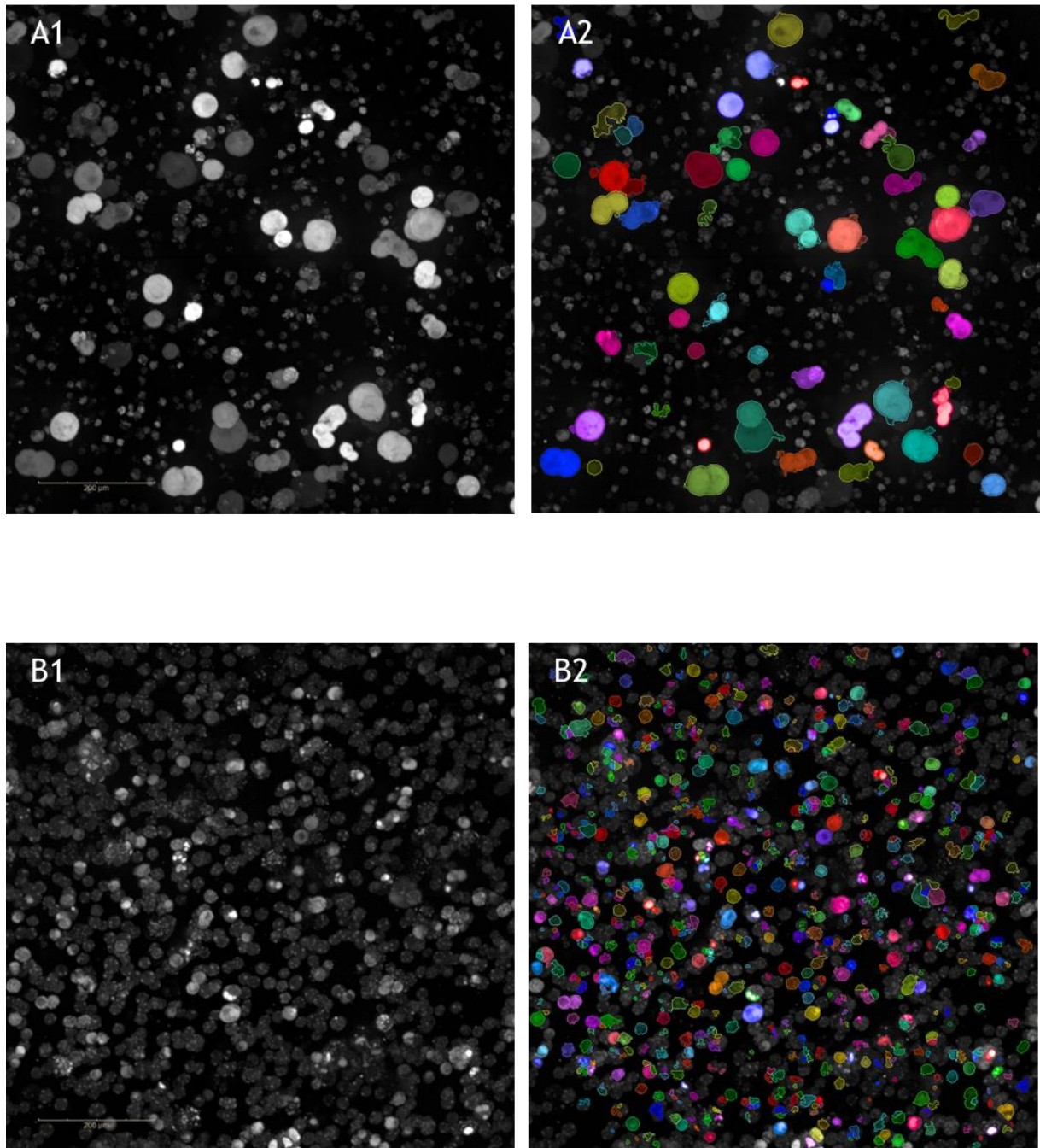


Figure 31. Optimization of the protocol for A-431 cells' identification. A1) Calcein channel of the Harmony@ software; B1) PI channel of the Harmony@ software; A2) Result of the protocol used for the experiment in identifying the cells stained with Calcein; B2) Result of the protocol used for the experiment in identifying the nucleus stained with PI.

In Figure 31, it is possible to observe a before (A1-B1) and after (A2-B2) of the application of the protocol used for the Live/Dead assay. This confirms a correct identification of the structures, as the lighter ones, correspondent to the stained ones in the before, appear identified in the after. However, the protocol can improve in terms of the separation of the structures. In some cases, one cell is identified as multiple ones, and vice-versa.

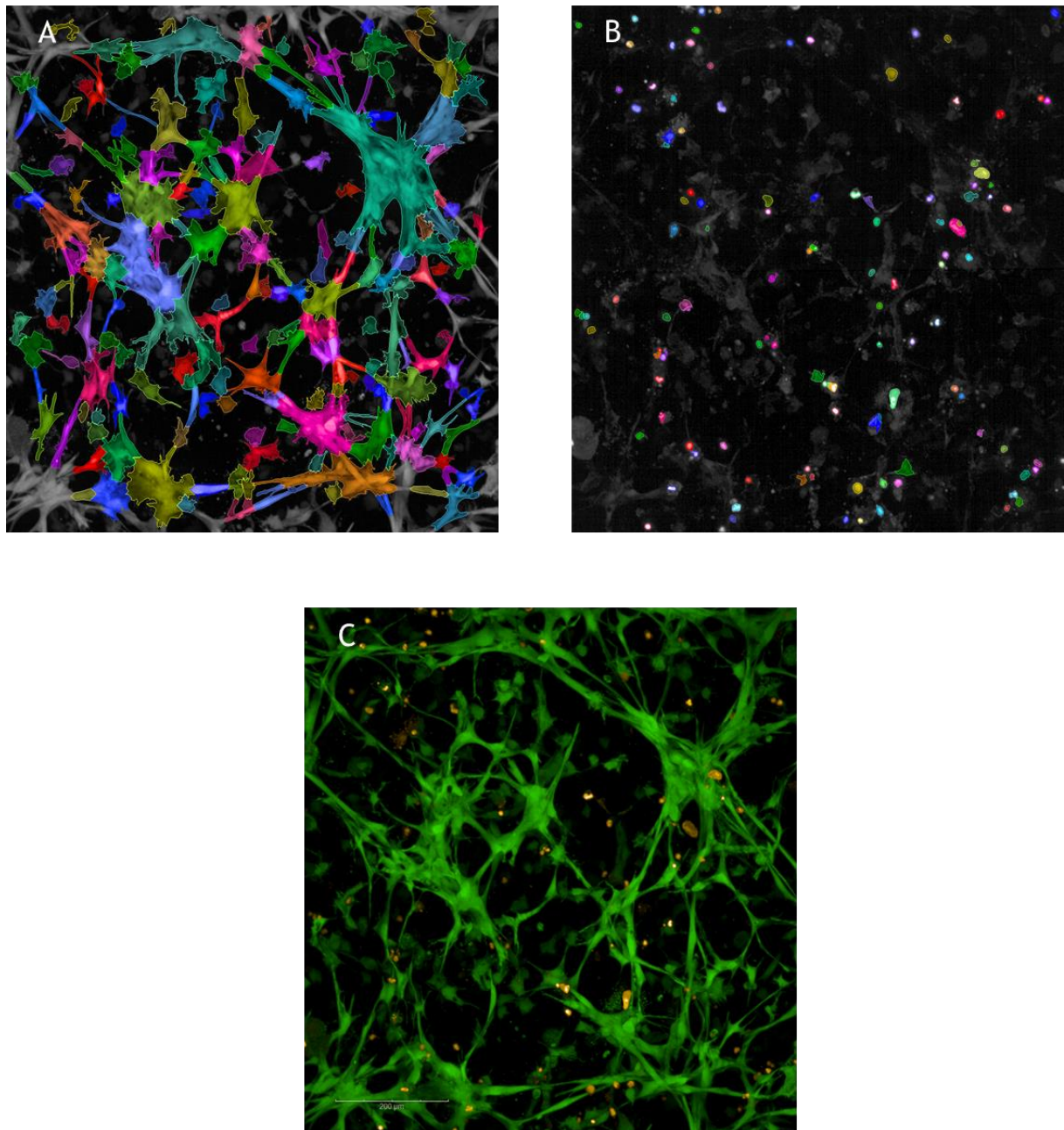


Figure 32. Final result of the application of the created protocol for identification of live and dead cells in an HFF-1 containing GelMA. A) Result of the protocol used for the experiment in identifying the cells stained with Calcein; B) Result of the protocol used for the experiment in identifying the cells stained with PI; C) Final result of the fluorescence image.

Figure 32. confirms the adequate performance of the created protocol. In this way, the following figures correspond to the images of 1 fov for all wells considered.

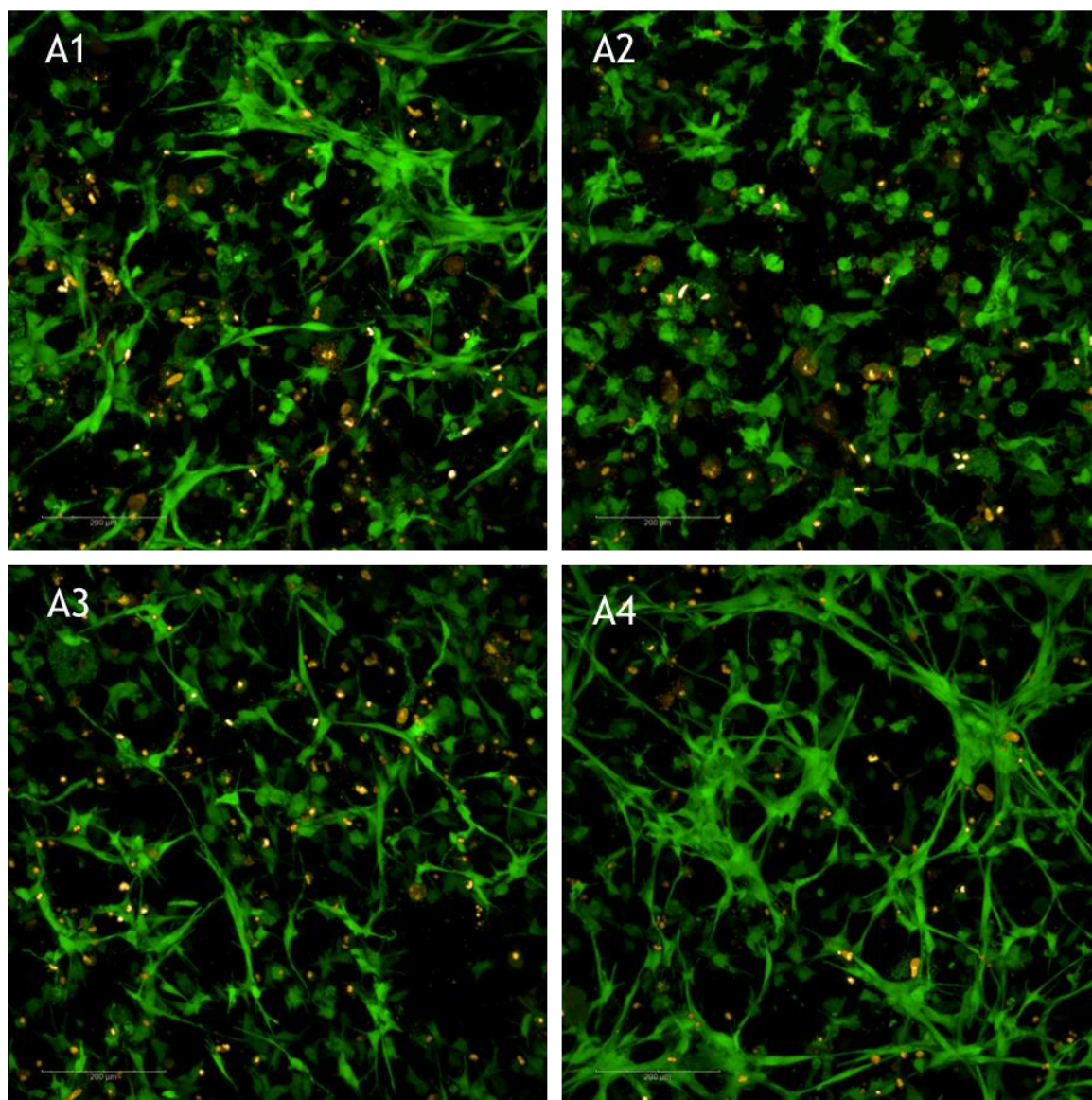


Figure 33. Fluorescence images of HFF-1 with no NIR irradiation. A1, A2) 0 wt.% @rGOn; A3,A4) 1.25 wt.% @rGOn.

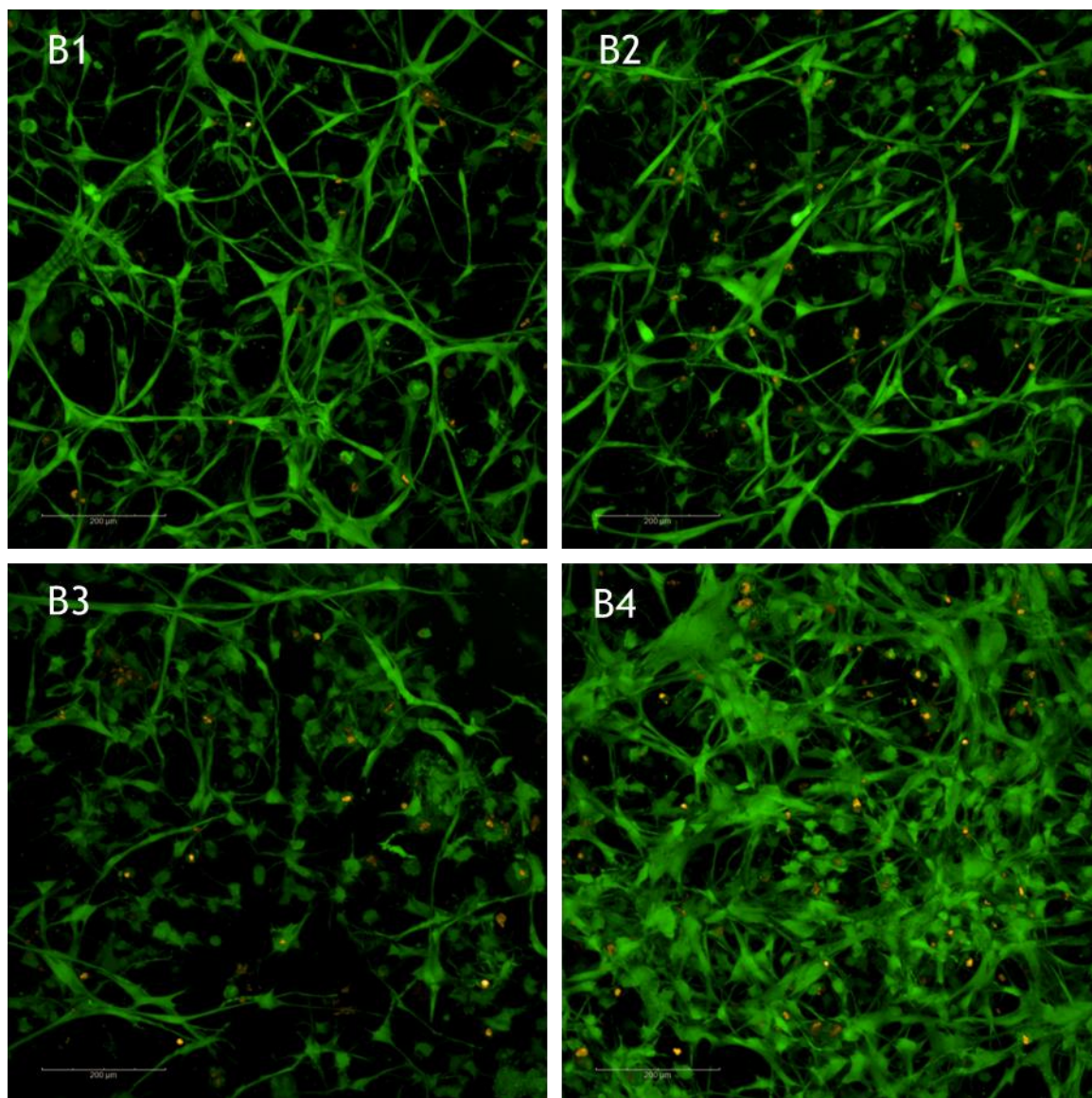


Figure 34. Fluorescence images of HFF-1 with NIR irradiation. B1, B2) 0 wt.% @rGOn; B3,B4) 1.25 wt.% @rGOn.

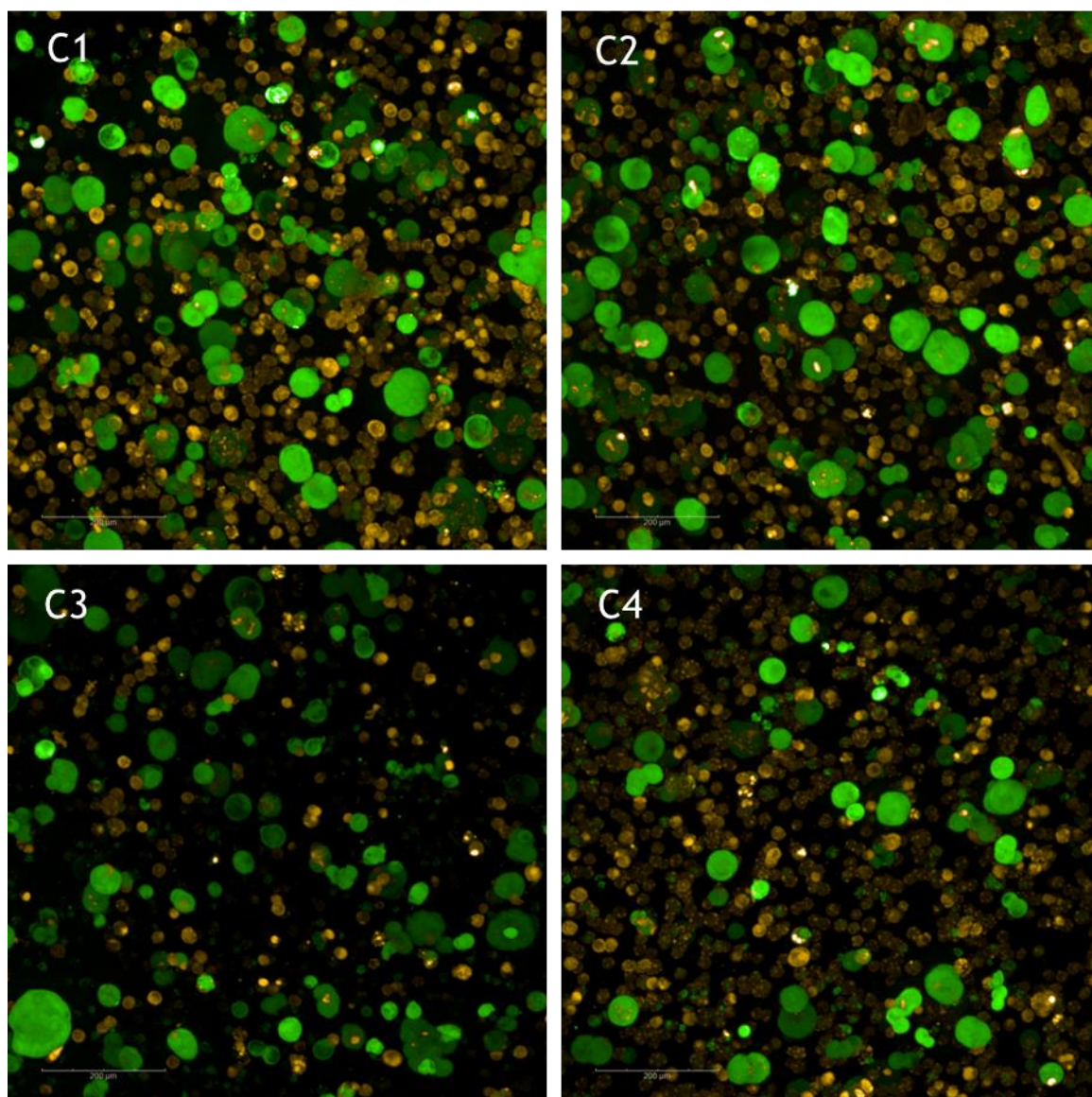


Figure 35. Fluorescence images of A-431 with no NIR irradiation. C1,C2) 0 wt.% @rGOn; C3,C4) 1.25 wt.% @rGOn.

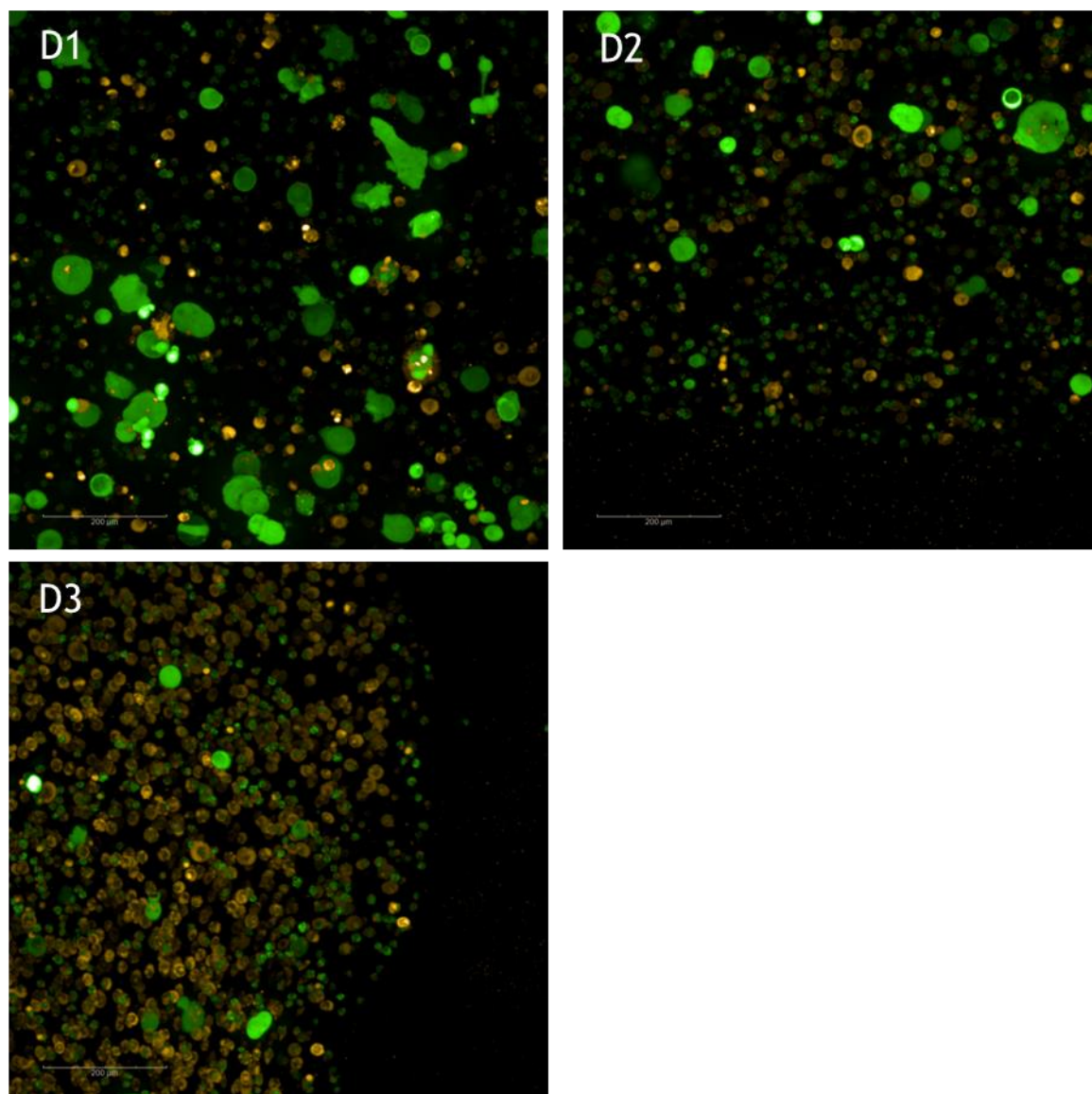


Figure 36. Fluorescence images of A-431 with NIR irradiation. D1,D2) 0 wt.% @rGOn; D3) 1.25 wt.% @rGOn.