M 2021

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FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

BIODEGRADABLE NANOPARTICLES: A STRATEGY TO FIGHT CANCER

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DISSERTAÇÃO DE MESTRADO APRESENTADA
À FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO EM
ENGENHARIA QUÍMICA

Master in Chemical Engineering

Biodegradable nanoparticles: a strategy to fight cancer

Master dissertation

of

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Developed within the course of dissertation

held in

LEPABE / FEUP

lepabe
Laboratory for Process Engineering, Environment, Biotechnology and Energy

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DEPARTAMENTO DE ENGENHARIA QUÍMICA

July 2021

Acknowledgment

I would mostly like to thank Professor Maria do Carmo Pereira and Doctor Maria João Ramalho for their help throughout the semester, for their advice and for making sure that the work was going in the right direction. Thank you for the opportunity as it was a pleasure to work under your supervision. I would also like to thank all my colleagues at the lab for helping with some techniques and for all the fun times.

The people in my personal life cannot be forgotten because they were there every day and a big thank you to my parents for making this journey possible. A big thank to my brother and friends for their support and encouragement.

This work was financially supported by: Base Funding - UIDB/00511/2020 of the Laboratory for Process Engineering, Environment, Biotechnology and Energy – LEPABE - funded by national funds through the FCT/MCTES (PIDDAC); Project 2SMART - engineered Smart materials for Smart citizens, with reference NORTE-01-0145-FEDER-000054, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); and Prize Maratona da Saúde for Cancer Research.

Abstract

Glioblastoma multiforme (GBM) is the most aggressive type of brain cancer with the lowest survival rate. The current treatment for this type of cancer is the invasive surgery for tumor resection followed by a combination of radiotherapy and chemotherapy. The gold standard drug for GBM therapy is temozolomide (TMZ), an alkylating agent with the ability to cross the blood-brain barrier. At physiological pH, TMZ suffers conversion into 5-(3-Methyl-1-triazeno)imidazole-4-carboxamide (MTIC), which is responsible for the cytotoxicity. One of the main limitations associated with the treatment with TMZ is the drug resistance. One of the resistance mechanisms is associated with the DNA repair by the MGMT protein, which is able to repair the TMZ-induced damage. To overcome these resistance, solid lipid nanoparticles (SLN) appear as drug delivery systems for the co-delivery of TMZ and an inhibitor of the repair protein, O6-Benzylguanine (06BG), in order to increase the effectiveness of the treatment. SLN were produced by hot homogenization, high-shear and ultrasound dispersion techniques. For the physicochemical characterization of the developed SLNs, size and polydispersity index were measured by dynamic light scattering and the zeta potential was evaluated by electrophoretic light scattering. The drug-loaded SLNs exhibited mean sizes of about 122 nm and zeta potentials of about -15 mV. The developed SLNs exhibited suitable encapsulation efficiencies, with about 43% for MTIC and about 42% for O6BG. FTIR analysis suggested that both encapsulated drugs were efficiently entrapped within the lipid layers. Stability assays proved that the developed drug-loaded SLNs were stable in storage conditions for at least 7 weeks, and stable for at least 1 week in simulated physiological conditions. Release experiments in different simulated blood fluids showed that the developed SLNs can protect the drugs from the harsh gastrointestinal conditions and are able to maintain a slow and controlled release of both drugs for several days. Thus, the developed drug-loaded SLNs could be a promising approach to be potentially implemented as a strategy to overcome the limitations of the currently available therapies by the co-administration of TMZ and O6BG.

Keywords (theme): Glioblastoma multiforme, temozolomide, O6-Benzylguanine, chemoresistance, MGMT protein, solid lipid nanoparticles, drug delivery systems

Resumo

O glioblastoma multiforme (GBM) é o tipo de cancro do cérebro mais agressivo e com menor taxa de sobrevivência. O tratamento atual para esse tipo de cancro passa por cirurgia invasiva para remoção do tumor seguida de radioterapia combinada com quimioterapia. O fármaco indicado para a terapia do GBM é o temozolomida (TMZ). Este fármaco é um agente alquilante com a capacidade de cruzar a barreira hematoencefálica. A pH fisiológico, o TMZ é convertido na sua forma ativa responsável pelo efeito anti tumoral, o 5- (3-metil-1-triazeno) imidazol-4-carboxamida (MTIC),. Uma das principais limitações associadas ao tratamento com TMZ é a resistência ao medicamento. Um dos principais mecanismos de resistência associados à falha terapêutica é regulado pela proteína MGMT que é capaz de reparar os danos no DNA induzidos pelo TMZ O uso de nanopartículas lipídicas sólidas (SLN) como sistemas de libertação de fármacos para a coadministração de TMZ e um inibidor da proteína de reparo . surgem como uma estratégia promissora para ultrapassar estes mecanismos de resistência e aumentar a eficácia do tratamento. Neste trabalho foram desenvolvidas SLN para co-encapsulação , do TMZ com o inibidor da MGMT - O6-Benzilguanina (06BG). As SLN foram produzidas usando as técnicas de homogeneização a quente e dispersão por ultrassom. Para a caracterização físico-química das SLNs desenvolvidas foi medido o tamanho e o índice de polidispersão por dispersão de luz dinâmica e o potencial zeta foi avaliado por dispersão de luz eletroforética. As SLNs contendo os fármacos encapsulados apresentaram tamanhos médios de cerca de 122 nm e potenciais zeta de cerca de -15 mV. As SLNs desenvolvidas exibiram eficiências de encapsulação adequadas, com cerca de 43% para o MTIC e 42% para o O6BG, respetivamente. A análise de FTIR sugeriu que ambos os fármacos encapsulados ficaram localizados entre as camadas lipídicas. Os ensaios de estabilidade demostraram que as SLNs são estáveis em condições de armazenamento por pelo menos 7 semanas e estáveis por pelo menos 1 semana em condições fisiológicas. Foram realizados ensaios de libertação em diferentes meios para simular diferentes condições fisiológicas, e os resultados obtidos demostraram que as SLNs desenvolvidas são capazes de proteger os fármacos encapsulados das condições gastrointestinais severas, mantendo uma libertação lenta e controlada de ambos os fármacos por vários dias. Este trabalho permitiu concluir que as SLNs desenvolvidas são uma abordagem promissora para poder ser potencialmente implementada como uma estratégia terapêutica para o GBM para ultrapassar as limitações das terapias atualmente disponíveis pela coadministração de TMZ e O6BG.

Palavras-chave: Glioblastoma multiforme, temozolomida, O6-Benzilguanina, quimioresistência, proteína MGMT, nanopartículas lipídicas sólidas, sistemas de libertação controlada

Declaration

I hereby declare, under word of honor, that this work is original and that all non-original contributions are indicated and due reference is given to the author and source

Margarida Fernandes Costa

01/07/2021

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Notation and Glossary

GBM – Glioblastoma Multiforme

TMZ – Temozolomide

O6BG – O^6-Benzylguanine

NPs- Nanoparticles

FDA – Food and Drugs Administration

Da – Dalton

MTIC - Methyl Triazene Imidazole Carboxamide

AIC - 4-Amino-5-Imidazole-Carboxamide

MGMT enzyme - O^6-alkylguanine DNA alkyltransferase

MGMT gene - O^6-methylguanine DNA methyltransferase

BBB – Blood-Brain Barrier

IFN-β – Interferon-β

SLN – Solid lipid nanoparticles

NLC – Nanostructured lipid carriers

HPH – High-pressure homogenization

Tf – Transferrin

MW – Molecular weight

DMSO – Dimethyl sulfoxide

PBS – Phosphate buffered saline

FaSSGF – Fasted state simulated gastric fluid

FaSSIF – Fasted state simulated intestinal fluid

PDI – Polydispersity index

DLS – Dynamic light scattering

ELS – Electrophoretic light scattering

TEM – Transmission electron microscopy

EE – Encapsulation efficiency

DR – Drug released

RT – Room temperature

1 Introduction

1.1 Framing and presentation of the work

According to the World Health Organization (WHO), cancer is the second leading cause of death globally. Cancer is a large group of diseases that can start in almost any organ or tissue of the body when abnormal cells grow uncontrollably. When these cells go beyond their usual boundaries to invade adjoining parts of the body and/or spread to other organs, this process is called metastasizing and is a major cause of death from cancer [1].

Currently the treatment for cancer patients is surgery, chemotherapy and radiotherapy which can be administered in a combined or separate regimen. Among other factors, each treatment varies with the type of tumor and cancer stage. Often these treatments are not effective, leading to serious side effects that condition the patient's well-being or eventual lead to patient's death. Also due to the complexity of some tumors, chemotherapy is often ineffective due to resistance to many drugs. Glioblastoma multiforme (GBM) is the most aggressive type of brain cancer. Patients live up to 15 months, on average, after diagnosis. Chemotherapy in this type of cancer is limited due to the blood-brain barrier, with temozolomide (TMZ) being the most used drug. It is often ineffective due to intrinsic resistance mechanisms to the action of this medication on tumor cells [2].

In this sense, nanosystems are a promising approach to deliver drugs directly into tumors, not affecting healthy cells thus reducing undesirable side effects. These can also allow to overcome drug resistance mechanisms. The aim of this was to produce solid lipid nanoparticles (SLN) capable of delivering drugs for the treatment of GBM.

1.2 Contribution of the author to the work

The novelty of this work is the development of SLN for the co-encapsulation of TMZ with O^6-benzylguanine (O6BG), with the aim of increasing the therapeutic effectiveness of TMZ. O6BG is an inhibitor of an enzyme capable of repairing the effects of TMZ that reverses its therapeutic action. By adding O6BG to the formulation, this work aims reducing or even eliminate the resistance that tumor cells present to TMZ. So far, the only research for the co-delivery of TMZ with this inhibitor used polymeric nanoparticles (NPs). SLN have advantages over polymeric NPs, such as easier and faster production method. Thus, in this work SLNs with suitable characteristics will be developed for TMZ and O6BG co-delivery into the brain.

1.3 Organization of the dissertation

This thesis is divided into four sections. Chapter one presents the motivation of this work, the main objectives and explains how the thesis is structured. In chapter two, the current state-of-the-art is presented. A brief overview about the disease, the drugs for its treatment and the nanosystems in clinical use is presented. Chapter three describes the experimental details for the production and physicochemical characterization the developed SLNs. In the fourth chapter, the obtained results are presented and discussed with the biography data.

2 Context and State of the art

2.1 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most common and aggressive type of brain cancer and is therefore associated with great deterioration of brain capacities and functions. GBM is a tumor in the group of astrocytomas. These tumors originate from astrocytes - cells that support the brain - however, GBM can contain several types of brain cells, even dead cells. Usually, GBM is classified as grade IV astrocytomas due to their rapid growth, since malignant cells multiply rapidly due to excessive vascularization, a characteristic of tumors.

There are two types of GBM with the primary type being the most common and the most aggressive; and the secondary type with the slowest growth, which usually is generated from a less aggressive astrocytoma.

GBM has an annual incidence of 3 in every 100,000 people worldwide and can occur at any age, however 70% of the known cases occur between the age groups of 45 to 70 years [3]. The disease progresses rapidly, causing death in about 15 months after diagnosis in patients who comply with the entire treatment protocol.

2.2 Conventional treatment and its limitations

The current treatment of patients with GBM is invasive surgery for total or partial removal of the tumor, followed by combination of chemotherapy and radiotherapy to eliminate possible tumor cells that remained after surgery. However, GBM treatment is not curative due to the tumor characteristics, such as being a tumor with rapid growth and composed by several types of cells. Conventional treatments may work for certain types of cells and not for others. Also, due to the infiltrative character of GBM, complete surgical resection is often not possible, with a large number of tumor cells still remaining after surgery.

Radiotherapy has several disadvantages such as causing damage to healthy cells. Patients undergoing radiation therapy in the head region are at increased risk of thyroid changes and low levels of hormones that can cause infertility. It can also cause neurological problems such as difficulty in concentrating, remembering and learning. Like radiation therapy, chemotherapy also causes damage to healthy cells. Patients undergoing chemotherapy often experience side effects such as hair loss, loss of appetite, anemia and more frequent infections due to decreased white blood cells. Another limitation of chemotherapy is drug resistance mechanisms that do not allow the therapy to be effective [2].

The current gold-standard drug used for GBM chemotherapy is temozolomide (TMZ), an antitumor drug that belongs to the class of alkylating agents.

2.3 Temozolomide

TMZ is a drug approved by the Food and Drugs Administration (FDA) for use in the first-line treatment of GBM as well as for recurrent anaplastic astrocytoma. It is an alkylating agent deriving from imidazotetrazine with a low molecular weight (194 Da) and lyophilic nature. This drug has the ability to cross the blood-brain barrier (BBB) and reach brain tumors. TMZ can be administered intravenously or orally [4].

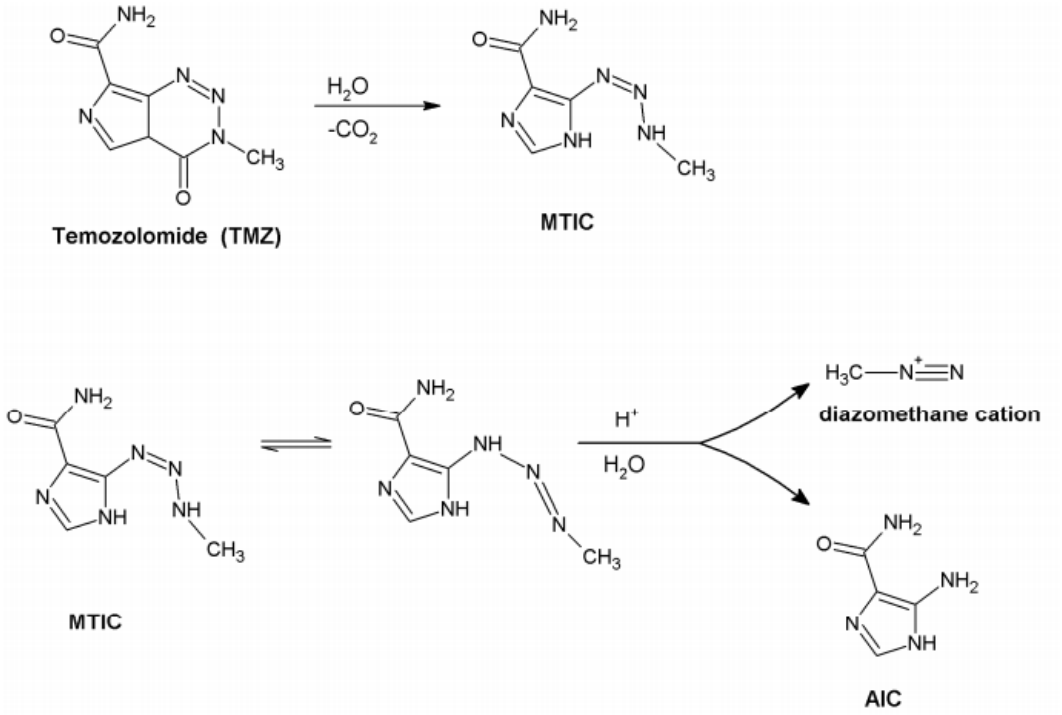

Figure 1 – TMZ conversion [5]

As it is a precursor substance, TMZ only presents pharmacological activity when hydrolyzed to 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC). The conversion of TMZ to MTIC and the further breakdown of MTIC to 4-amino-5-imidazole-carboxamide (AIC) and a methyl-diazonium cation (Figure 1) is irreversible and pH-dependent. In aqueous buffers, TMZ is stable at pH 4, but rapidly decomposes to MTIC at pH 7. In contrast, MTIC is stable at alkaline pH, but rapidly breaks down to AIC at pH 7 [5].

MTIC is the active form of TMZ. Its cytotoxicity occurs due to the methylation of DNA. MTIC methylates DNA in positions N^7 and O^6 of guanine and N^3 of adenine, the most severe being O^6-methyl-G which causes toxicity and mutagenicity and results in the formation of DNA damage, leading to cell apoptosis.

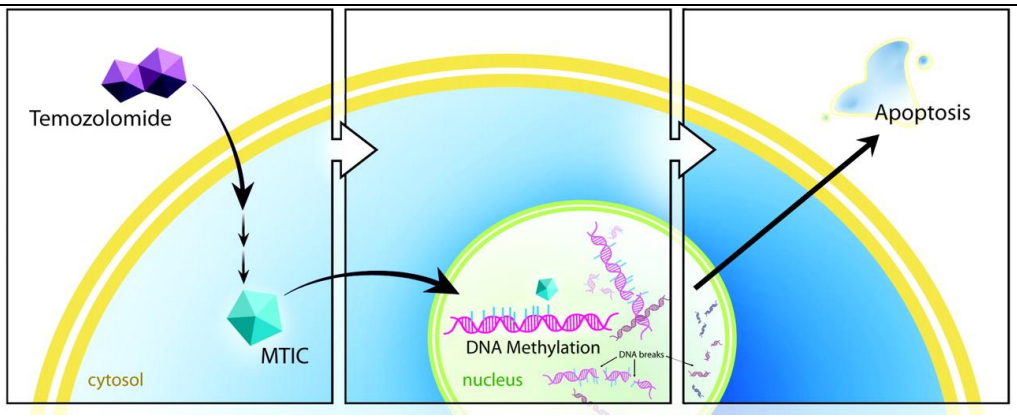

Figure 2 - Mechanism of action of temozolomide in cells [3]

2.3.1 Limitations in the use of TMZ for GBM treatment

GBM treatment with TMZ has several limitations, such as its toxicity to healthy tissues. The most common side-effects reported by patients are gastrointestinal problems such as nausea, vomiting and anorexia. Another problem associated with this therapy is the low bioavailability of MTIC due to its low affinity for biological membranes or barriers, which means that it is not taken up by cells limiting the therapy. Unlike TMZ, MTIC does not have the ability to cross the BBB effectively and *in vivo* studies show that there is a low accumulation of MTIC in brain tumor tissue due to its poor penetration through the BBB. The BBB, which protects the brain, is composed of tightly packed endothelial cells, astrocytes, smooth muscle cells and pericytes. Endothelial cells form tight junctions and therefore prevent penetration of 98% of small-molecule drugs and 100% of large-molecule (> 500 Da). In addition, endothelial cells express efflux pumps, which remove potential neurotoxic molecules [6].

Another major limitation of GBM treatment with TMZ is the repair mechanism mediated by the O^6-alkylguanine DNA alkyltransferase (MGMT) enzyme. This enzyme is encoded by the MGMT gene (O^6-methylguanine DNA methyltransferase) and is able to repair the TMZ-induced O^6-methyl-G lesion. The MGMT enzyme is one of the most important DNA repair enzymes and it is responsible for chemoresistance to alkylating agents. It repairs damaged guanine nucleotides by transferring the methyl at O^6 site of guanine to its cysteine residues (Figure 3), thus avoiding gene mutation, cell death and tumorigenesis caused by alkylating agents [7].

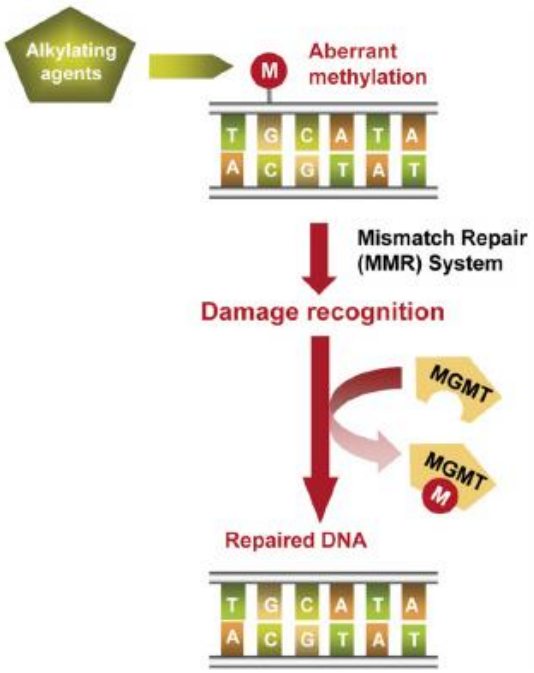

Figure 3 - MGMT repair mechanism [8]

A high expression of MGMT counteracts TMZ-induced cytotoxicity being therefore linked to a poor prognosis. When the promoter of MGMT gene is methylated, no or reduced levels of the protein are produced. Hypermethylation of the promoter region of the MGMT gene has been known for several years as a possible cause to explain the lower expression of the MGMT protein in neoplastic cells. Although being essential in preventing mutations in normal tissues, MGMT is one of the major limiting factors for GBM therapy. This hypermethylation occurs in 30 to 60% GBM patients and the median overall survival of patients receiving TMZ chemotherapy and radiotherapy with a silenced MGMT promoter was 21.7 months compared to 12.7 months with active or unmethylated MGMT [9]. In this way, MGMT gene promoter methylation has been investigated as a potential biomarker of sensitivity to chemotherapy.

2.4 Strategies to overcome MGMT resistance

Since one of the factors associated with resistance to therapy is the MGMT enzyme, strategies such as gene therapy and the use of inhibitors have been most recently studied as promising approaches to overcome this resistance.

With gene therapy, genes are replaced, inactivated or introduced into cells to treat a disease. In gene therapy to overcome TMZ resistance, human interferon-β (IFN-β) plays an important role. In addition to direct effects on tumor cells (eg, growth inhibition and apoptosis), IFN-β also has immunomodulatory and anti-angiogenetic activities [10]. A recent study demonstrated a synergistic activity between TMZ and INF-β at a clinically relevant concentration in TMZ-resistant GBM, possibly through attenuation of MGMT expression [11].

In addition to the study of IFN-ß, the use of replicating adenoviruses (CRAds) has also been studied as antitumor therapeutics since CRAds replicates in tumor cells and can eventually lead to cell lysis. The adenovirus E14 gene binds with p300, a protein with an important role in cell proliferation, to inhibit the transcription of MGMT for removal of methyl group from O6-methylguanine leading to silencing of MGMT promoter.

Besides gene therapy to modulate gene expression, also inhibitors for MGMT protein can be used. The choice of using inhibitors comes from their advantage over gene therapy because in the latter the aim is to normalize defective cells and organ function, and the use of inhibitors works to minimize symptoms in addition to treating the disease by inactivating the protein.

There are at least three MGMT protein inhibitors being study recently. The least studied is diethylamine NONOate sodium salt that did not prove to be effective in causing cell death. Another widely studied inhibitor that showed positive results in inactivating the MGMT gene is Lomeguatrib. However, the disadvantage is that when applied with TMZ in therapy it can cause myelosuppression, by reducing the activity of bone marrow [12]. The MGMT protein inhibitor that currently has showed the most promising results is the O6-Benzylguanine (O6BG) that among the MGMT inhibitors, exhibits the lowest toxicity.

2.4.1 O6-Benzylguanine

O6BG is a synthetic derivative of guanine with ability to inhibit the MGMT protein. The MGMT protein removes alkyl groups located on the O^6 position of guanine from DNA restoring DNA in a single step as shown in figure 4. However, O6BG is able to transfer a benzyl moiety to the active site cysteine of the protein that leads to irreversible inactivation with 40% reduction in alkyltransferase activity (figure 4). The interruption of DNA repair increases the chemotherapeutic effectiveness of alkylating agents [13].

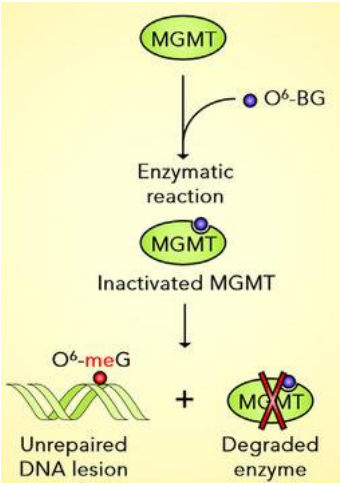

Figure 4 – Mechanism of action of O^6-Benzylguanine [14]

The combination of TMZ and O6BG has shown promising results in *in vitro* studies using human GBM cells. These results suggested that O6BG decreased cell resistance to TMZ's activity by inhibiting the MGMT protein [15]. The concomitant therapy with both drugs was also studied in GBM patients. In a phase II clinical trial, it was studied the role of O6BG in restoring TMZ sensitivity in patients with recurrent or progressive TMZ-resistant glioma and to assess the safety of co-administering the two drugs. The study was conducted with patients with GBM and anaplastic glioma and the two drugs were administered on day 1 of the 28-day evaluation cycle. However, the combination of TMZ with O6BG was able to reverse the resistance in only some patients [15]. The limited response seen in GBM can be explained by the administration of free drugs and propels the research for alternative dosing regiments or alternative administration routes for chemotherapy such as the use of nanotechnology. Generally, systemic administration of the two drugs is not as effective in terms of pharmacological activity when the two drugs have a different pharmacokinetics.

In addition, this form of therapy has other disadvantages/limitations. The inhibitor is non-toxic when administered in the treatment of neoplasms, but it is not intended to affect healthy tissues so as not to inhibit the repair protein, as this protein is essential for normal cells to prevent mutations. Thus, to achieve an effective therapy, nanomedicine appears as an answer.

2.5 Nanomedicine

Nanomedicine is the medical application of nanotechnology for the treatment, diagnosis, monitoring and control of biological systems. It combines medicine with nanotechnology to develop new therapies and improve existing ones and a number of nanomedical applications have been already developed. So far, research has focused on the development of biosensors to aid in diagnosis and vehicles to administer vaccines, medications and genetic therapy, including the development of nanosystems to aid in cancer treatment, best known as drug delivery systems.

2.5.1 Drug Delivery Systems

The current therapies for the treatment of cancer are the same used for decades and have limited efficacy in addition to toxicity and side effects. On the other hand, given the nature of the disease, if all tumor cells are not eliminated, the chances of recurrence are high and are generally more aggressive and treatment-resistant tumors.

Over the years, different alternatives to standard treatment have been presented, which act through the induction of apoptosis, cell cycle dysfunction or inhibition of the angiogenesis process. Although these new methods are limited due to the toxicity of the drugs and little selectivity, among others. Nanoparticles (NPs) can offer several advantages such as, new drug formulations and new forms of administration and ability to pass to BBB due to their

small size. NPs are colloidal carriers with dimensions on the nano scale (10^{-9} m) with unique physicochemical properties as stability, varied composition, biocompatibility and biodegradability.

One of the ways that NPs can enter cells is by passive targeting, that is, by penetration into cells. Thus, the physicochemical properties of the NPs play an important role in the biological fate and success of the NPs. In particular, the NP size is one of the most important factors. Depending on the intended application, different ranges of sizes can be considered acceptable. Generally, NPs must have a size below 200 nm to be considered for clinical application. The size of the NPs also regulates their toxicity. Smaller NPs have a relatively large surface area as compared to larger ones and this increases the interaction with biological elements and consequently may trigger more toxicity. Particles with 200 nm or larger tend to activate the lymphatic system and are removed from circulation quicker [16].

The surface charge of the NPs is also a key feature. The NPs can have a positive, negative or neutral surface charge. Cationic NPs can easily enter cells, because of cells negative surface charge, however neutral and low concentration of anionic NPs were found to have no effect on BBB integrity whereas high concentration of anionic NPs and cationic NPs can have a deleterious effect on the BBB. It also reported that the brain uptake rates of negatively charged NPs are superior to neutral and positively charged ones [17].

Another advantage of using NPs for cancer therapy is the enhanced permeability and retention effect (EPR effect), a concept in which molecules of small dimensions (eg. NPs) tend to accumulate in tumor tissue much more than they do in normal tissues. The EPR is based on the hyper-permeability of the tumor vessels that allows NPs to enter the tumor interstitial space as well as on the absence of functional lymphatics in the tumor interior that enables them to stay there for a long time. The hyper-permeability of tumor vasculature is mainly due to the large intercellular openings between endothelial cells that comprise the neoplastic tumor vessel wall. These openings might be hundreds of nanometers in size, contrary to the openings of the normal vessel wall, whose size is less than 10 nm. Therefore, the reason for taking advantage of the EPR effect to treat cancer is that, in passive targeting, particles with sizes larger than 10 nm will not be able to extravasate into normal tissues and are able to act more effectively in tumor tissues [18].

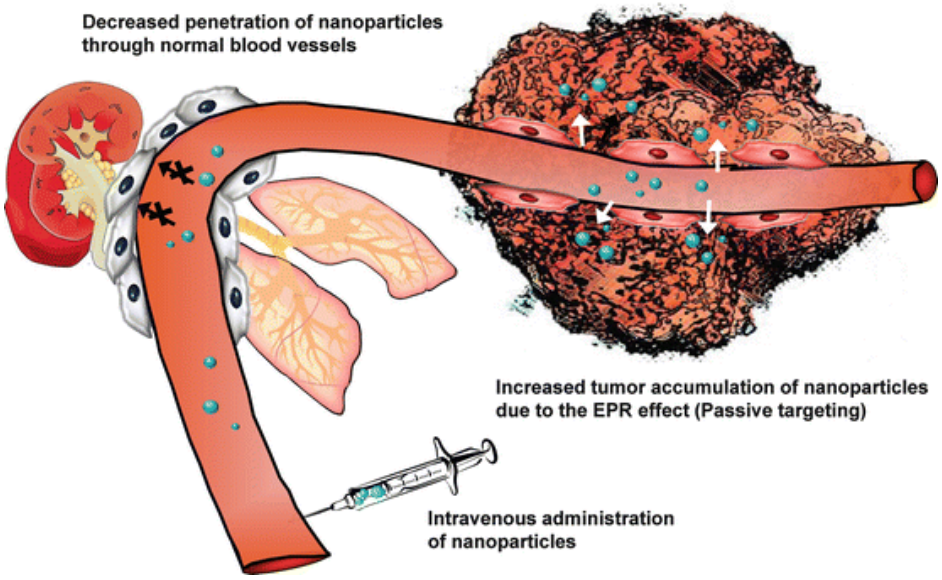

Figure 5 - Illustration of the EPR effect on normal cells and tumor cells [19]

Active targeting is another suitable strategy to enhance NPs accumulation in the target tissues. By modifying the surface of the NPs with ligands with affinity for cell receptors, it is possible to ensure selective and successful transport to target cells. These cell receptors should be overexpressed in the target cells. There are at least three types of active targeting (figure 6) [20]:

i) Antibody-based targeting is based on attaching an antibody to the NPs surface and this antibody targets a specific antigen only found on target cells.

ii) In aptamer-based targeting, aptamers, single-stranded DNA or RNA, can specifically bind to target molecules.

iii) In ligand-based targeting, ligands such as small molecules, peptides, proteins, etc., bind to receptors in target tissues.

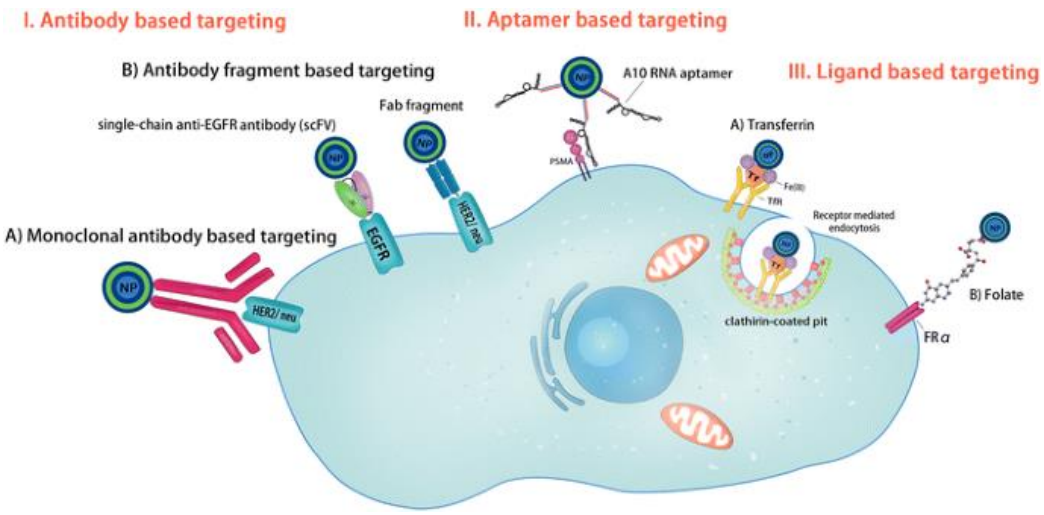

Figure 6 - Illustration of the different types of active targeting [20]

In this way, the use of NPs for drug delivery for cancer treatment allows for the direct application of chemotherapy to tumors, reaching only the affected area and not subjecting the rest of the body to treatments that destroy the patient's immune system.

There are several types of nanosystems that can be used for drug delivery. The best-known and most studied are liposomes, polymeric NPs, dendrimers, micelles, metallic and magnetic NPs and lipid NPs. Figure 7 shows the illustration of the various nanosystems presented.

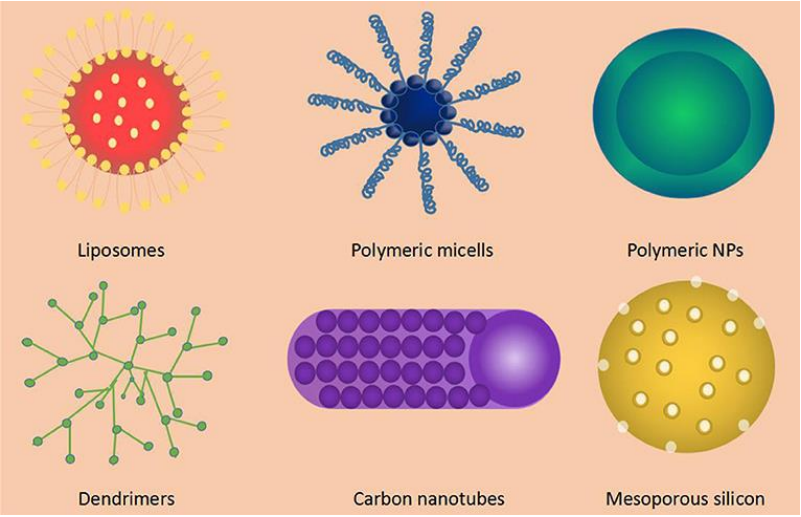

Figure 7 - Illustration of the various nanosystems [21]

2.5.1.1 Drug Delivery Systems approved for clinical use

In 1995, FDA approved the first nanosystem for clinical use, a doxorubicin-loaded liposome for the treatment of Kaposi's sarcoma. In 2005, it was approved a nanoformulation of paclitaxel loaded-albumin NP, better known as Abraxane, for the treatment of breast, lung and pancreatic cancer. With the advancement of nanomedicine, other formulations such as Myocet (doxorubicin-loaded liposomes), Genexol-PM (paclitaxel-loaded polymeric micelles), among others, have appeared.

Figure 8 shows a representation of a chronological line of the various nanosystems approved until this date for clinical use. Others nanoformulation still in study are also presented in figure 8. The polymeric PEG-PLGA NPs (BIND-014) and the liposomes with anti-epidermal growth factor receptor (EGFR) completed the phase II clinical trials for advanced cancers and breast cancer, respectively [22].

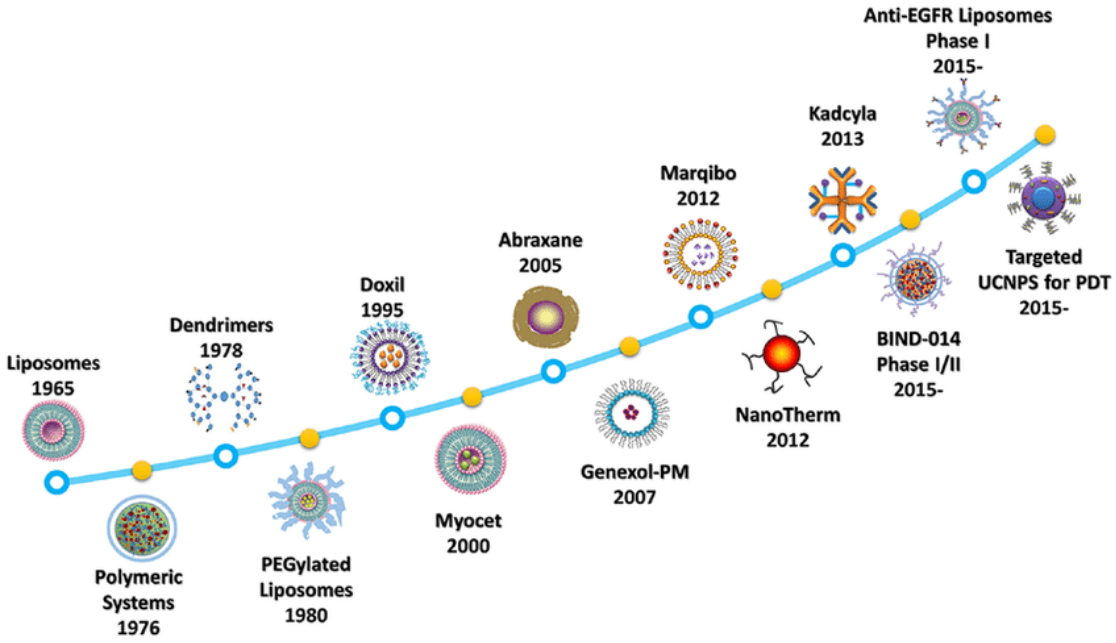

Figure 8 - Timeline of the various nanosystems approved for clinical use and in the testing phase [22]

2.5.2 Solid Lipid Nanoparticles

Lipid NPs are widely studied for drug delivery due to their advantageous properties, such as biodegradability and biocompatibility. There are two types of lipid NPs, the first type are the solid lipid nanoparticles (SLN) and the second type are the nanostructured lipid vectors (NLC). SLNs are composed of solid lipids at room temperature and NLCs are made up of a mixture of liquid and solid lipids [23].

Solid lipid nanoparticles (SLN) are aqueous dispersions of solid NPs at room temperature and at body temperature that are stabilized by one or more surfactant agents. The materials used to produce SLN are biodegradable, biocompatible and safe for human use. Within the solid lipids, the most used are pure triglycerides(e.g., tripalmitin and tristearin); fatty acids (e.g., stearic acid and palmitic acid); waxes (e.g., cetyl palmitate); mixtures of triglycerides (e.g., Witepsol® and Softisan®) and fats (e.g., glyceryl monostearate). Surfactants are substances that reduce the surface tension between the two phases and influence the contact surface between two liquids, thus helping to disperse the liquid in the aqueous phase and stabilize the SLNs after cooling. The most used are polysorbate 80 and poloxamer 188 and 407.

The incorporation of drugs in the SLNs depends on the type of lipid, surfactant and production technique so there are three theoretical models that describe this incorporation as illustrated in figure 9. In type I SLN, or homogeneous matrix model, the drugs are dispersed in the core. In type II SLN, or model of the active substance wall, the core is covered by a wall

containing the drugs and in type III SLN the drugs are in the core which is covered by a lipid wall [24].

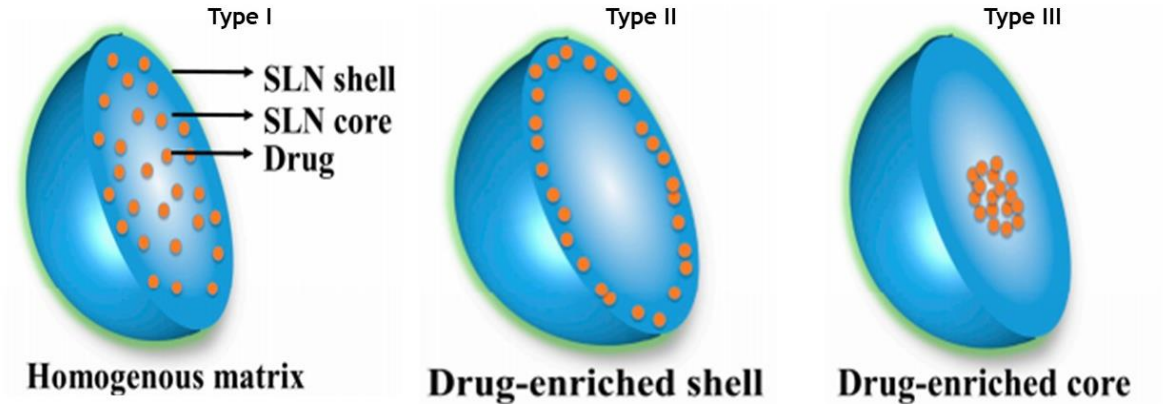

Figure 9 - Illustration of the three theoretical models for SLN incorporation [24]

There are several methods for producing SLN:

- **Hot and cold high-pressure homogenization (HPH) method** - the drugs are dissolved in a previously melted lipid. In hot HPH, the hot surfactant is added and homogenized at high speed. The pre-emulsion is processed by HPH which generates reduced size particles. In cold HPH the difference is that the mixture of lipid and drug is cooled with dry ice obtaining microparticles that are suspended by a cold solution of surfactant.
- **High-speed sonication and homogenization method** - the lipid and aqueous phases are heated to the same temperature and emulsified by mechanical agitation and sonication. Subsequently, to obtain the SLNs, the sample is cooled to room temperature.
- **Microemulsion method** - the aqueous and lipid phases are heated to the same temperature and then homogenized. It is then dispersed on ice to break the microemulsion into a nanoemulsion.
- **Solvent emulsification-evaporation method** – It is ideal for non-lipid soluble drugs. The water-immiscible organic solvent solubilizes the dispersed lipid in an aqueous phase to produce the emulsion. Solvent is evaporated during high-speed homogenization.
- **Double emulsion method** – novel method based on the above except that the drugs are encapsulated with a stabilizer to prevent drug partitioning to external water phase during solvent evaporation in the external water phase of w/o/w double emulsion.
- **Solvent displacement method** – this method was initially used as a production method for polymeric NPs and was then adapted to lipids. This method uses water-miscible semi-polar organic solvents that solubilize the lipid. The drug and surfactant are then added by injection under magnetic stirring. SLNs are formed after complete removal of the solvent.

- **Coacervation method** - consists of the interaction of an acid solution (coacervation solution) in the presence of an amphipathic polymer as a stabilizing agent. As the pH drops, SLNs precipitate. This method allows for the incorporation of various drugs.

2.5.2.1 Applications of Solid Lipid Nanoparticles

SLNs have been widely studied for the treatment of cancers and other diseases. Currently, no treatment with SLN has been approved for use in patients, however there are several studies for the development of SLNs for the delivery of anticancer agents.

Since TMZ has a wide application and interest for the treatment of different types of cancers, even the most aggressive ones, there are several studies for the delivery of TMZ using SLN for cancer therapy. A summary of the various works with TMZ incorporated in SLNs is presented in Table 1.

Table 1 - Summary of the bibliography data on SLNs loaded with temozolomide and their application

Formulation	Funcionlization	Drugs	Size (nm)	Surface charge	Aplication	Ref.
1. Stearic acid + Soya Lecithin	None	TMZ	66	negative	Glioma	[25]
2. Triolein + cholesterol	transferrin	TMZ	249	negative	Brain cancer	[26]
3. Tripalmitin	None	TMZ	147	negative	Brain cancer	[27]
4. Stearic acid + Soya Lecithin	None	TMZ + Vincristine	179	positive	Glioma	[28]
5. Stearic acid + Soya Lecithin	None	TMZ	95	positive	Glioma	[29]
6. Gelucire 44/14 + Vitamin E	None	TMZ	148	negative	Glioma	[30]

7. Behenic acid	None	TMZ	279	negative	Melanoma	[31]

Huang and colleagues (2008) developed SLNs to be applied to gliomas. Their study showed that the use of SLN allows for a reduction in the required TMZ dosage and a decrease in the systemic toxicity of the drug.

Jain and colleagues (2013) developed transferrin (Tf) conjugated SLN for TMZ encapsulation. Studies have shown lower cytotoxicity in unconjugated SLNs than in Tf-conjugated ones, which may be due to slower drug release over a longer period of time and also showed significant tumor inhibition superior to free TMZ.

Gastaldi and coworkers (2014) developed SLN to deliver TMZ to the brain. Animal studies in mice models indicated enhanced potential of the developed system in the effective treatment of GBM. Wu and coworkers (2016) also developed cationic SLN for GBM therapy. However, in this work SLN were developed for the co-delivery of TMZ and other anticancer drug – vincristine. The antitumor activity was evaluated *in vitro* using U87MG cells and the tumor inhibition rate was 55.7%. Tapeiros and colleagues (2017) developed cationic SLN for TMZ encapsulation. The efficacy of the developed SLNs were evaluated *in vitro* on human GBM cells (U87MG) and *in vivo* in mice-bearing malignant glioma model showing antitumor activity.

Khan and colleagues (2018) studied the possibility of applying TMZ-SLN in a hydrogel for intranasal application. The formulation was developed using chitosan as a gelling agent. The cytotoxicity of the formulation was evaluated against C6 rat glioma cell line and expressed in terms of IC50 (dose required to kill 50% of the alive cells). The encapsulation of TMZ in SLN enhanced the cytotoxicity of the drug by almost 50-fold. Clement and colleagues (2018) studied a formulation of SLN containing TMZ for melanomas. Cytotoxicity studies were performed on different human and mouse melanoma cell lines (A2058, JR8 and B16-F10). SLN-TMZ showed more toxicity than free TMZ in all cell lines. In B16-F10 cells, SLN-TMZ induced 70% tumor inhibition while free TMZ only 34%. This is a concentration dependent factor because with lower concentration SLN-TMZ showed only 35% tumor inhibition and free TMZ was ineffective.

Although the various formulations shown above presented good results in cytotoxicity and tumor growth inhibition, none of them addresses the drug resistance mechanisms associated with therapy failure. Also, some of the developed SLNs (table 1) exhibited a positive surface charge and which, as mentioned earlier, is associated with higher toxicity and lower brain uptake. The main novelty of my work is to incorporate a MGMT inhibitor (O6BG) in the formulation for the combined therapy with TMZ aiming to overcome this resistance to therapy.

3 Materials and Methods

3.1 Materials

TMZ (purity ≥99%; MW: 194 Da) was obtained from Selleck chemicals (Houston, USA), O6BG (purity ≥98%; MW: 241 Da) was obtained from Abcam (UK). Apifil was obtained from Gattefossé SAS (France). Dimethylsulfoxide (DMSO) (MW: 78 Da) was obtained from VWR Chemicals (Ohio, USA). Pluronic F-127 (Poloxamer 407, MW: 12,6kDa), sucrose (MW: 342 Da) and phosphate buffer saline (PBS) were obtained from Sigma Aldrich (St. Louis, MO). Fasted State Simulated Gastric Fluid (FaSSGF) and Fasted State Simulated Intestinal Fluid (FaSSIF) dissolution media were acquired from Biorelevant (London, UK). Uranyl Acetate (MW: 424 Da) was obtained from Electron Microscopy Sciences (Hatfield, UK). All aqueous solutions were prepared using ultra-pure water. Pluronic aqueous solutions used were prepared at 10% (w/v).

3.2 Drugs Stability tests

For drug stability tests, 0.10 mg/mL solutions of each drug were prepared in water. The solutions were incubated at 70 ºC for 2, 5 and 10 minutes. At the end of each time, the samples were analyzed by UV–Vis absorption spectroscopy (BioTek synergy 2, USA). Then the solutions were subjected to a 30 minutes sonication step (UP400S ultrasonic processor, Hielscher, Berlin, Germany) at a 70% amplitude and ultrasonic frequency of 24 kHz. At the end of the sonication process, the samples were analyzed by UV–Vis absorption spectroscopy. The UV-Vis absorbance of each drug was also measured before the beginning of the stability tests.

3.3 Preparation of SLN

SLN were prepared using hot homogenization that consists in heating the lipid and the aqueous phase to 5 ºC above the lipid melting point for 5 minutes until lipid melting. An aqueous solution of Pluronic F-127 10% (w/v) was used, and different lipids were tested as the solid lipid Cetyl palmitate, compritol and Apifil. The two phases were then mixed, vortexed for 30 seconds and placed in the Ultraturrax DI-25 basic to high-shear (IKA, Germany) for 2 minutes at 13,500 rpm. High-shear technique allows to create the emulsion. The rotation produces suction, which pulls the medium into the rotor and then pushes it to the outside [32]. This process results in the sample's dispersion and during the process the temperature was maintained to guarantee that the lipid does not solidify. Then the sample was subjected to ultrasound dispersing technique in a probe sonicator at an ultrasonic frequency of 24 kHz (UP400S ultrasonic processor, Hielscher, Berlin, Germany). Ultrasonication leads to particle size reduction and improves size uniformity through cavitation forces and ultrasonic waves. The tip rapidly vibrates, causing bubbles in the surrounding solution to rapidly form and collapse [33].

Different experimental parameters were tested to produce the most suitable SLN formulation. Parameters such as the type of lipid, lipid/surfactant ratio and the sonication time and intensity were varied. The different experimental parameters evaluated are summarized in the table below.

Table 2 - Experimental parameters tested for the preparation of formulations for blank SLN

Formulation	Lipid	Amount of lipid (mg)	Surfactant	Amount of surfactant (mL)	Sonication	
					Time (min)	Intensity (%)
F1	Cetyl palmitate	100	Pluronic F-127	4.35	30	70
F2	Cetyl palmitate	100	Pluronic F-127	1	30	70
F3	Cetyl palmitate	100	Pluronic F-127	1.5	30	70
F4	Cetyl palmitate	100	Pluronic F-127	2	30	70
F5	Compritol	160	Pluronic F-127	4	30	70
F6	Apifil	2000	Pluronic F-127	17.4	15	70
F7	Apifil	50	Pluronic F-127	4.35	15	70
F8	Apifil	500	Pluronic F-127	4.35	15	70

3.4 Preparation of TMZ-loaded SLN

After the selection of the most suitable experimental parameters for SLN preparation, TMZ and O6BG were encapsulated. Drug-loaded SLN were produced using the same methodologies used for blank SLN (hot homogenization, high-shear and ultrasound dispersion). Apifil and Pluronic F-127 were used as the solid lipid and the surfactant, respectively. Dimethyl sulfoxide (DMSO) was used to solubilize the drugs, since these were not soluble in the lipid. The organic solution of drugs in DMSO was added to the lipid during the melting step. Blank SLN (without drug) were produced using DMSO in the lipid phase as control to assess if it did not significantly alter SLN properties.

Different experimental conditions were tested to produce the most suitable drug-loaded SLN formulation. Parameters such as lipid/surfactant ratio, amount of drug and DMSO were tested. The sonication intensity and duration were also varied. SLN without drug using the same experimental conditions were prepared as control.

Table 3 – Experimental parameters tested for the preparation of TMZ-loaded SLN

Formulation	Amount of lipid (mg)	DMSO	Amount of DMSO (µL)	Amount of TMZ	Amount of O6BG	Sonication	
						Time (min)	Intensity (%)
DF1	50	No	-	1	1	15	70
DF2	200	Yes	400	0.5	0.5	15	70
DF3	300	Yes	400	0.5	0.5	15	70
DF4	400	Yes	400	0.5	0.5	15	70
DF5	200	Yes	500	0.5	0.5	15	70
DF6	200	Yes	400	0.5	0.5	15	90
DF7	200	Yes	200	0.5	0.5	15	100
DF8	200	Yes	300	0.5	0.5	30	100

3.5 Physicochemical caracterization of SLN

3.5.1 Size and polydispersity index determination by dynamic light scattering

Dynamic light scattering (DLS) is the most used method for particle size analysis in the nanometer range. It can be used to determine the size distribution profile. DLS is based on the Brownian motion of dispersed particles since when particles are dispersed in a liquid they move randomly in all directions. Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler shift when the light hits the moving particle, changing the wavelength of the scattered light. The rate of change in the scattered light is directly proportional to the movement of the particles and can be related to their diffusion coefficient. Then it is possible to apply the Stoke-Einstein equation and hence correlate the diffusion constant with the particles' size: [34]

$$RH = \frac{kT}{6\pi\eta D}$$

Where RH is the hydrodynamic radius, k is the Boltzmann constant, T is the temperature, η is the viscosity of the solvent and D is the diffusion coefficient.

The polydispersity index (PDI) is a measure of the heterogeneity of a sample based on size. The tendency of lipidic NPs to accumulate in the target tissues depends on their physicochemical properties including particle size distribution thus, a successful, safe and stable formulation requires a homogenous or monodisperse sample of a certain size. Polydispersity can occur due to size distribution in a sample, agglomeration or aggregation of the sample during analysis. The value of PDI ranges from 0.0 (perfectly monodisperse sample) to 1.0 (highly polydisperse sample). International standard organizations (ISO) have established that PI values < 0.05 are more common to monodisperse samples, while values > 0.7 are common to a polydisperse distribution of particles. In drug delivery applications, a PDI of 0.3 or bellow is considered acceptable [35].

Size and PDI measurements were performed using a DTS0012 cell in a Nano ZS equipment (Malvern Instruments, UK). Ultrapure water was used as the dispersant and samples were diluted (1:100) to a final SLNs concentration of 0.46 mg/mL. The samples were analyzed with a 633 nm red laser at 25 °C with a backscattering angle of 173°. The refractive index (1.330) and viscosity (0.8872 cP) of water as the dispersant were used. Lipid refractive index and absorption were fixed at 1.4 and 0.0 respectively. The obtained results were given in intensity distribution.

3.5.2 Zeta potential determination by Laser doppler velocimetry method

The zeta potential values of the prepared SLN were determined by laser doppler velocimetry method. Zeta potential is a measure of the magnitude of the electrostatic repulsion/attraction between particles and is one of the fundamental parameters known to affect stability [36]. Zeta potential cannot be measured directly and is from the electrophoretic mobility of charged particles under an applied electric field. The electrophoretic mobility (μe) of the particles is first calculated:

$$\mu e = \frac{V}{E}$$

Where V is the particle velocity (μm/s) and E is the electric field strength (Volt/cm).

The zeta potential is then calculated from the obtained μe by the Henry's equation:

$$\mu e = \frac{2\varepsilon_r \varepsilon_0 \xi f(Ka)}{3\eta}$$

Where ε_r is the relative dielectric constant, ε_0 is the permittivity of vacuum, ξ is the zeta potential, f(Ka) is the Henry's function and η is the viscosity at experimental temperature [36].

Zeta potential measurements were also performed using the ZetaSizer Nano ZS (Malvern Instruments, UK), in a DTS1060 cell. Ultrapure water was used as dispersant and samples were diluted (1:100) to a final SLNs concentration of 0.46 mg/mL. Zeta potential values were obtained by the Smoluchowski model using the dielectric constant (78.5), refractive index (1.330) and viscosity (0.8872 cP) of the dispersant at RT.

3.6 Fourier-transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption or emission from a solid, liquid or gas. For an FTIR analysis the sample is subjected to infrared radiation. The basic theory says that the bonds between the elements absorb light at different frequencies and the FTIR measures the range of wavelengths in the infrared region that are absorbed by the material [37].

The spectra of blank SLN, loaded-SLN, the two drugs, the lipid and the surfactant, Pluronic F-127, were read. For FTIR analysis, the samples were placed in the crystal, and the water was removed, because it has a spectrum with wide bands that can interfere with the sample's spectra. To increase signal-to-noise ratio, each spectrum was taken as an average of 64 scans. All spectra were registered in transmittance mode using an Alpha-P (Bruker,USA) in a spectral range of 350 to 4000 cm^{-1}.

3.7 Encapsulation efficiency

The encapsulation efficiency (EE%) allows to determine the amount of drug that remains inside the SLN after the production process and therefore it is an indicator of quality. The EE of the drugs in the prepared SLN was determined by an indirect method, using the following equation:

$$EE(\%) = \frac{total\ amount\ of\ drug - total\ amount\ of\ drug\ free}{total\ amount\ of\ drug} 100$$

To separate the non-encapsulated drugs from the prepared SLN, the samples were centrifuged using Amicon Ultra-0.5 centrifugal filter devices (Molecular cut-of 3kDa, Milipore, Country Corkk, Ireland) for 15 minutes at 14,500 rpm. Before centrifugation, the samples were diluted (1:10) to a final SLN concentration of 4.6 mg/mL.

Non-encapsulated drugs were quantified by UV–Vis absorption spectroscopy (BioTek synergy 2, USA). By the Lambert-Beer law, the absorbance is proportional to the concentration of the absorbing chemical species, thus the intensity of radiation can be related to the concentration present in the sample. In this way, it is possible to draw a calibration curve for a compound and from there, by reading the absorbance, determine the concentration in the samples. [32]

The obtained absorbance spectra were correlated to calibration curves for each drug (curves and respective data in appendix A, figure 3).

3.8 Storage stability assays

The stability of drug-loaded SLNs under different storage conditions was studied. It is important that these SLNs remain stable for a long time, when thinking about a clinical application it is important that the particles do not degrade quickly. Ideally, SLNs should be stable in various forms of storage which would make them more suitable for long-term storage. Then, stability tests were carried out for SLN at different storage conditions: freeze-dried; and in aqueous suspension at room temperature (RT) and 4 ºC, respectively.

3.8.1 Stability at room temperature and 4 ºC

The stability of the prepared drug-loaded SLNs was assessed for 7 weeks. To assess the influence of different storage conditions, SLNs were divided in 10 aliquots and stored at RT or 4 ºC. The average size, size distribution and zeta potential were monitored over time. At predetermined timepoints, each aliquot was centrifuged (amicon 3K, 15 minutes, 14,500 rpm). Size, PDI and zeta potential were measured by DLS and ELS as described in sections 3.5.1 and 3.5.2, respectively.

3.8.2 Freeze-Drying

Freeze-drying is commonly used to prolong the stability of NPs, this because it avoids the instability to which the particles are subjected in an aqueous suspension. This technique consists of dehydrating the NPs by applying pressure (vacuum pressure) at -80 ºC freezing the material, then reducing the pressure and adding heat forcing a sublimation of the water present in the sample. As this process can cause the destabilization of the particles, cryoprotectants are usually added to help reduce possible damage during freezing and drying. Generally, the most used cryoprotectants are dextran, glycine, mannitol, sorbitol, trehalose and sucrose with concentrations between 5 and 20% (w/v) [38].

In this work, sucrose was used as cryoprotectant at different concentrations ranging from 5 to 20% (w/v). Before freeze-drying, non-encapsulated drug was removed using amicon filters, and the drug loaded-SLN were freeze at -20 ºC about 15 minutes, and then at -80 ºC.

Freeze-drying was carried out in a BenchTop^{TM} K series freeze-drier from VirTis® (NY, USA) at $5x10^{-5}$ Bar and – 95 ºC for 24 hours. The freeze-dried samples were sealed and stored at RT until further analysis. Prior physicochemical characterization, the dried samples were reconstituted with ultrapure water. Size, PDI and zeta potential were measured by DLS and ELS as described in sections 3.5.1 and 3.5.2, respectively.

3.9 Stability assays under physiological conditions

Since the developed drug-loaded SLNs could be potentially used in different routes of administration, the stability of the developed drug-loaded SLNs was studied in different simulated biological conditions. The stability of SLNs was evaluated in terms of changes in the mean size, PDI and zeta potential. DLS and ELS measurements were performed using the ZetaSizer Nano ZS (Malvern Instruments, UK) as described in sections 3.5.1 and 3.5.2, respectively.

3.9.1 Simulated intravenous administration

Since drug-loaded SLN could be administered intravenously, the blood circulation conditions were simulated. To simulate the blood circulation, PBS (0.01 M, pH 7.4) was used, since it closely mimics the pH, osmolarity, and ion concentrations of the blood [39]. The stability of SLNs was evaluated in terms of changes in the mean size, PDI and zeta potential over time using DLS and ELS as described in sections 3.5.1 and 3.5.2, respectively.

3.9.2 Simulated oral administration

Drug loaded-SLN could also be administered orally, thus it is necessary to create an environment similar to that of the digestive system. In this work, fasted state simulated gastric fluid (FaSSGF, pH 1.6) and fasted state simulated intestinal fluid (FaSSIF, pH 6.5) were used to simulate the gastric and intestinal conditions, respectively. These solutions contain physiological surfactants present in the gastrointestinal tract to simulate these fluids. Generally, contains taurocholate (bile acid), phospholipids, sodium, chloride (present in the gastrointestinal tract) and phosphate but in different concentrations, depending on the medium [40].

For the stability studies, SLNs were dispersed in FaSSGF for 2 hours and in FaSSIF for 3 hours and at the end of this time their mean size, PDI and zeta potential were measured using DLS and ELS as described in sections 3.5.1 and 3.5.2 respectively.

3.10 Release studies *in vitro*

The *in vitro* release study consists of an experiment to determine the efficacy and quality of the developed SLNs. It also allows to trace the drug release profile over time as well as to determine if the amount of drug released (DR%) is within the limits of the therapeutic amount. The drug release profile is a mechanistic model that predicts the behavior of a drug.

Drug released was determined by quantifying the amount of drug in the release media by UV-Vis spectroscopy measurements. The percentage of drug released was obtained through the following equation:

$$DR\ (\%) = \frac{drug\ released}{encapsulated\ drug} 100$$

Since the developed drug-loaded SLNs could be potentially used for different types of administration, different release media were used to simulated different biological conditions. All release studies were performed using the dialysis membrane method, that rely on a semi-permeable membrane to achieve physical separation of the SLNs and the free drug.

3.10.1 Simulated intravenous administration

To simulate the blood stream, the experiments were carried out at 37 ºC under mild agitation (250 rpm) in PBS (0.01 M, pH 7.4). Before the beginning of the release studies, non-encapsulated drugs were removed using amicon filters (Molecular cut-of 3kDa, Milipore, Country Corkk, Ireland) as described in section 3.8. Then, drug-loaded SLNs were diluted in PBS at a final volume of 2,0 mL and then placed on the dialysis membrane (Float-A-Lyzer G2 Dialysis Device, 8-10 kD). 5.5 mL of PBS was placed on the outside space of dialysis device to ensure sink conditions. At predetermined timepoints, samples were collected from the outside buffer and, after measurement by UV-Vis spectrophotometry, returned to the release medium. To ensure that drugs do not have membrane affinity and are not retained by the membrane, a solution containing MTIC and O6BG in PBS was used as control. This allows us to trace the release profile of the free drug across the membrane.

3.10.2 Simulated oral administration

To simulate the SLNs path after oral administration, simulated gastrointestinal fluids were used. First, drug-loaded SLNs were centrifuged using an amicon filter to remove the free drug and were resuspended in 2,0 mL of PBS. SLNs were then placed in the inside space of a dialysis membrane (Float-A-Lyzer G2 Dialysis Device, 3.5 -5 kD). To simulate the oral path, the dialysis device containing the drug-loaded SLNs were first placed in 5.5 mL of FaSSGF to mimic the gastric conditions. 2 hours later, the medium outside the membrane was replaced with 5.5 mL of FaSSIF to mimic the intestinal environment. After 3 hours, the SLNs were moved to 5.5 mL of PBS. Released drugs were quantified by UV-Vis measurements every half hour. A solution containing free TMZ and O6BG was used as control.

3.11 Statistical analysis

All measurements were performed for at least three independent samples. All obtained results are expressed as the mean and standard deviation (SD). Statistical analysis was performed using the t-student test, and p-values below 0.05 were considered significant.

4 Results and discussion

4.1 Drugs stability tests

Initially, drug stability tests were performed to assess if the drugs would remain stable after being subjected to the different steps involved in the production of the SLNs. So, drugs were subject to high temperature (70 ºC) and to a sonication process to understand if degradation occurred. O6BG remained stable during the stability experiments, proving that this drug does not suffer degradation during SLNs preparation (results in image 1 in appendix A).

However, as shown in figure 10, TMZ is slowly converting to MTIC at 70 ºC as shown by the decrease in absorbance at the peak of the TMZ (320 nm) and a slight increase in the peak of the MTIC (259 nm). pH and temperature regulate the conversion of TMZ into MTIC. At physiological pH, TMZ undergoes non-enzymatic hydrolysis to MTIC and it is known that temperature accelerates the degradation process [41]. The experiments were performed in ultra-pure water, with a pH of 7 which explains the conversion to MTIC. As, the drug solution was subjected at 70 ºC accelerating the conversion process. The results also shown that after 30 minutes of sonication, there is a total conversion to MTIC. Ultrasound energy is known by increasing the mass transfer and shortening the reaction time [42].

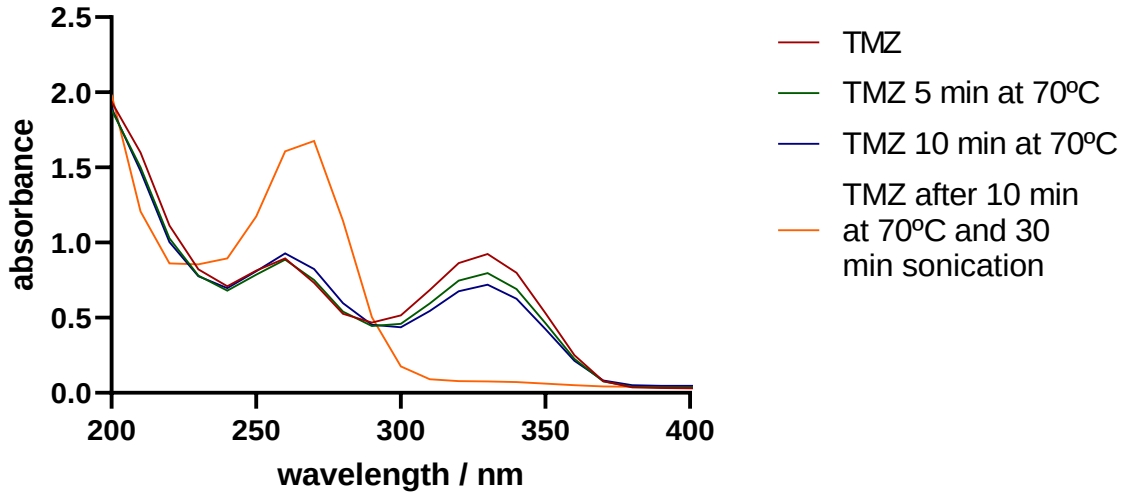

Figure 10 - Stability tests for TMZ

Since during the NPs production process there is a complete conversion from TMZ to MTIC, it was necessary to test the MTIC under these conditions to see if it remained stable (figure 2 in appendix A). After the MTIC stability test, it was verified that it does not degrade during the production process as the absorption spectra before and after overlap. Since TMZ

fully converts to MTIC, henceforth the designation used to refer to this drug will always be MTIC.

4.2 Physicochemical characterization of the developed SLNs

4.2.1 Blank SLNs

Blank SLNs, without drugs, were prepared using the hot homogenization, high-shear and ultrasound dispersing methodologies. The effect of different experimental parameters on the physicochemical properties of the prepared SLNs were evaluated and the obtained results are shown in table 4.

Table 4 - Physicochemical features of unloaded SLNs

Formulation	Size (nm)	PDI	Zeta potential (mV)
F1	320	0.419	-13
F2	1000	0.239	-10
F3	400	0.236	-12
F4	480	0.189	-12
F5	325	0.246	-15
F6	250	0.481	-17
F7	220	0.217	-14
F8	153	0.261	-15

SLNs size is a critical factor for brain drug delivery, and ideally size should be below 200 nm. NPs intended for drug delivery in the brain must have a size between 50 and 200 nm to be effective, because the BBB is very selective, and the small size of NPs is what makes them reach target tissues in the brain easily [16]. Therefore, SLNs exhibiting sizes above 200 nm were excluded. Formulations that had a PDI greater than 0.3 were also excluded because these were considered to be polydisperse. PDI values are also a determining factor for delivery of drugs to the brain, because SLNs tend to accumulate more in tumor tissues than in healthy ones, so if they present similar sizes they will be more effective [35].

The zeta potential values only suffered minor changes along the different formulations. Although it was not a decisive factor in choosing the most suitable formulation, this is an indicator of colloidal stability. Pluronic F-127 was used in all formulations as a non-ionic

emulsion stabilizer forming a uniform layer on the SLNs surface, conferring stability against aggregation.

As can be seen from table 4, the nanoformulations prepared with cetyl palmitate (F1 to F4) and Compritol (F5) exhibited higher dimensions. On the other hand, the formulations prepared with Apifil (F6 to F8) exhibited lower sizes and closer with desired range (sizes below 200 nm). From F6 to F9, different ratios of lipid (Apifil) and aqueous phase were evaluated, and formulation F8 showed the best results, being the one with both sizes and PDI values within the desired range (size < 200 mm and PDI < 0.3). These SLNs exhibited suitable physicochemical properties for brain delivery such high absolute zeta potential, lower PDI and sizes. Since the lipid used, Apifil, is composed essentially of fatty acids and most of these have a negative charge, and as a non-ionic surfactant is used, these SLNs were expected to have a negative charge. As previously mentioned in section 2.5.1, anionic NPs do not show any effect on the BBB integrity, that is, they are not toxic to it [17].

Thus, the experimental conditions used for formulation F8 (500 mg of Apifil, 4.35 mL of Pluronic F-127, and 15 minutes of sonication at 70% amplitude) were selected to proceed with the preparation of drug-loaded SLNs.

4.2.2 Drug-loaded SLNs

After selecting the most suitable experimental conditions, drug-loaded SLNs were also prepared using the hot homogenization, high-shear and ultrasound dispersing techniques using the selected experimental parameters (500 mg of Apifil, 4.35 mL of Pluronic F-127, and 15 minutes of sonication at 70% amplitude). The obtained results for the formulations (DF1) are shown in table 5. Drug-loaded SLNs produced with the selected experimental conditions (DF1) did not presented suitable dimensions for brain delivery (size of 465 nm). TMZ and O6BG were not soluble in the liquid lipid, therefore causing the formation of aggregates and SLNs with bigger dimensions. Thus, DMSO was further used to solubilize the drugs. The experimental conditions were then varied again to find the most suitable formulation.

Table 5 – Physicochemical features of drug-loaded SLNs

Formulation	Size (nm)	PDI	Zeta potential (mV)	MTIC EE %	O6BG EE%
DF1	465	0.413	-15	-	-
DF2	275	0.433	-5	-	-
DF3	253	0.430	-18	-	-

DF4	280	0.458	-14	-	-
DF5	330	0.437	-12	-	-
DF6	110	0.289	-17	29.5	22.8
DF7	172	0.443	-13	27.9	33.3
DF8	157	0.319	-14	49.4	46.9

The results of the different prepared drug-loaded SLN formulations are shown in table 5. A formulation was made without drugs to confirm that DMSO did not change SLNs properties, and it was found that the addition of an organic solvent does not influence the size or zeta potential (data not shown). In formulations DF2 to DF4, the volume of DMSO (400 μL) that showed good results in the blank formulation (without drug) was maintained, and the amount of lipid was varied between 200 and 400 mg. As the obtained SLNs showed dimensions higher than 200 nm, it was decided to vary the amount of DMSO (formulations DF5 to DF8) to understand its effect on the properties of NPs. However, by changing the amount of DMSO to 500 μL, the sizes obtained were greater than 200 nm, so the next step was to change the sonication intensity.

In the DF6 formulation, the sonication intensity was increased to 90% and in the DF7 and DF8 it was increased to 100%, because probe sonication can be used reduce the size of SLNs. Although the intensity causes changes, when the sonication time is increased, few effects are observed [42]. In formulations DF6, DF7 and DF8 the size was within the expected range (<200 nm), however the high PDI of DF7 led this formulation to being excluded.

Thus, the chosen formulation was DF8 because exhibited higher encapsulation efficiency. DF8 (200 mg of Apifil, 4.35 ml of Pluronic, 0.5 mg of each drug and 200 μL of DMSO, 15 minutes sonication at 100%) exhibited an average size of 157 nm, which is in the ideal range of 50 to 200 nm, a PDI of 0.3 that is at the heterogeneity limit of the solution. The zeta potential is negative, which is associated with less toxicity to the BBB. DF8 also exhibited the higher obtained EE values (49% for MTIC and 47% for O6BG). The higher EE values verified in all formulations for O6BG can be explained by its greater affinity for the organic phase, comparatively with MTIC that is more hydrophilic and has higher affinity for the aqueous phase.

The encapsulation efficiency (EE) values were only determined for the formulations with suitable physicochemical properties, i.e. with dimensions below 200 nm.

FTIR allows the identification of the functional groups present in the materials and the observation of the spectrum of the developed drug-loaded SLNs may allow to evaluate the type of interaction that occurs between the drug and the matrix material.

By observing figure 11, it can be seen that the transmittance spectra of Apifil and Pluronic overlap in some ranges with the blank SLN spectrum. The blank SLN spectrum shows several characteristic peaks of the Apifil spectrum shows. In fact, between 3000 to 2700 cm^{-} the blank SLN and Apifil spectra overlap, corresponding to the aliphatic C-H bonds of methyl and methylene groups (two bonds were distinguished in the spectrum at about 2916 cm^{-1} and 2848 cm^{-1} that correspond to the CH_2 functional group). The black SLN spectrum also presents several peaks that coincide with the Pluronic F-127 spectrum, highlighting the peak in the wave number region of 1111 cm^{-1}, characteristic peak of the C-O bond [43]. The presence of characteristic peaks of Pluronic F-127 on the SLNs spectra are in accordance with the TEM analysis were a Pluronic F-127 layer was observed in the surface of the SLNs.

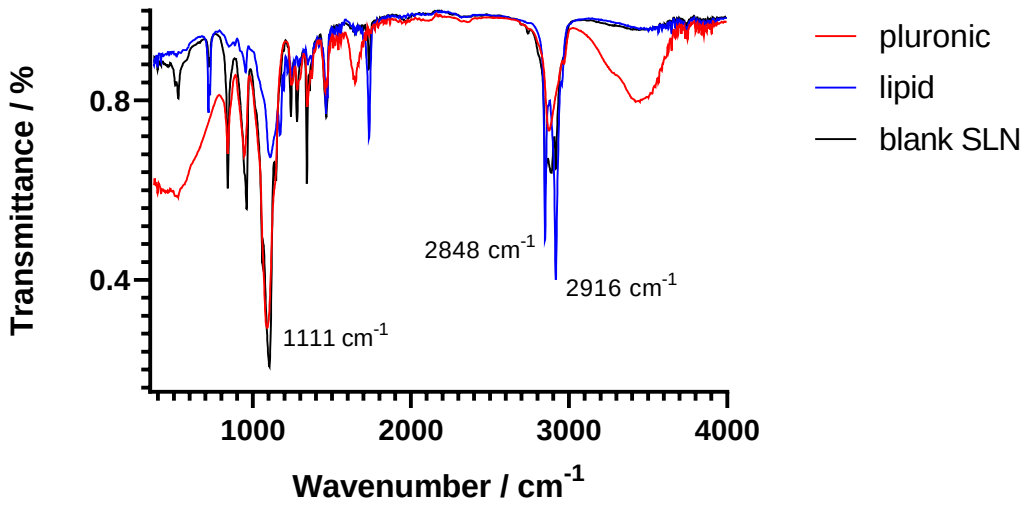

Figure 11 - Transmittance spectrum of blank SLN, Pluronic F-127 and Apifil

As seen in figure 12, in the drug loaded-SLN spectrum, the characteristic peaks of Apifil and Pluronic also appear. The transmittance spectra of blank SLN and drug loaded-SLN overlap, without the presence of characteristic peaks of MTIC and O6BG (figure 13), suggesting that there is complete encapsulation of the drugs within the layers of Apifil. This also indicates that the encapsulated drug is not found adsorbed on the surface of the SLNs. Other authors have also reported the absence of drug characteristics peaks in the FTIR analysis when the drugs were encapsulated between the lipid layers [43].

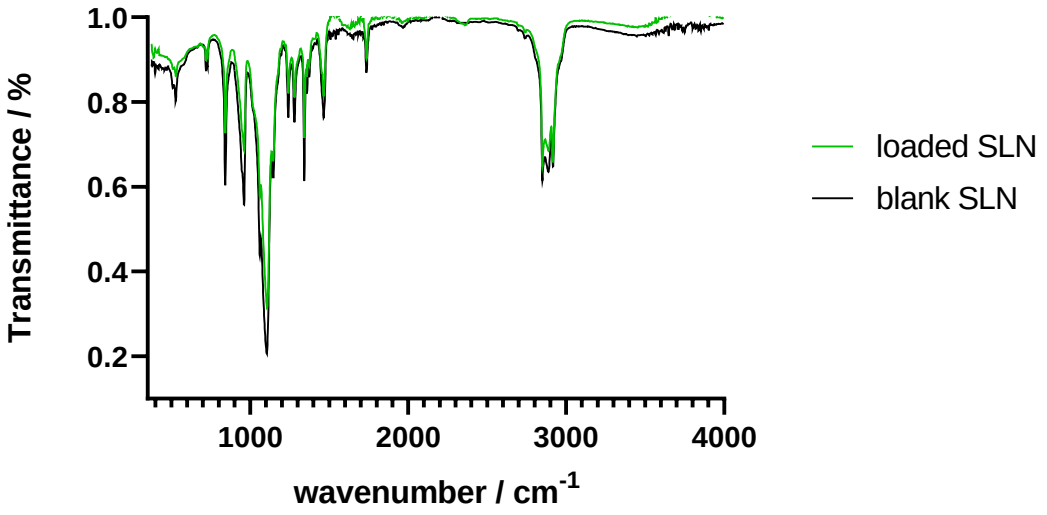

Figure 12 - Transmittance spectrum of blank SLN and loaded-SLN

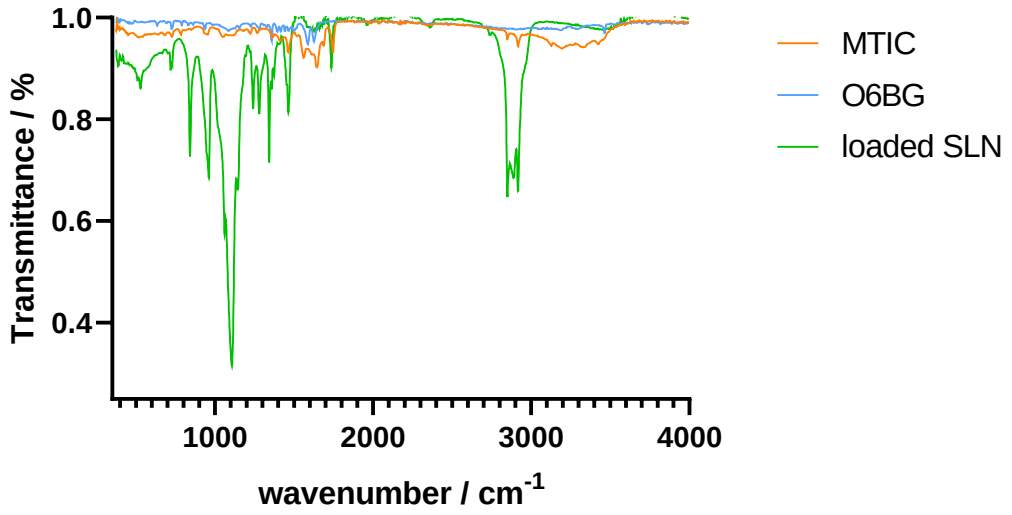

Figure 13 - Transmittance spectrum of loaded-SLN and the two drugs: MTIC and O6BG

4.2.3 Blank SLNs vs Drug-loaded SLNs

After selecting the most suitable experimental conditions for the co-encapsulation of MTIC and O6BG on SLNs, drug-loaded SLNs were produced in triplicates to verify the reproducibility of the protocol. Blank SLNs were also produced in triplicates as control. The obtained results are presented in table 6.

Table 6 - Physicochemical features of unloaded SLNs and drug-loaded SLNs. Results are presented as mean ± SD (n=3).

	Size (nm)	PDI	Zeta potential (mV)	EE (%)	
				MTIC	O6BG

blank SLNs	122 ± 8	0.29 ± 0.01	-14 ± 3	-	-
TMZ+O6BG-SLNs	124 ± 10	0.32 ± 0.05	-15 ± 1	43 ± 3	42 ± 1

The physicochemical properties of the SLNs did not significantly change with drug encapsulation, since no significant changes were verified in the sizes, PDI and zeta potential values for blank and drug loaded SLNs ($p>0.05$). Also, the standard deviation of the samples for the size, zeta potential and for the PDI are low, it can be said that the production method is reproducible. As the standard deviation of encapsulation efficiency is also low for both drugs, it can be said that the method is reproducible.

Higher encapsulation efficiencies are generally obtained for SLNs, however several factors can decrease encapsulation such as the partition coefficient of the target molecule in the solvents in the formulation, the method used in production and also the fact that trying to encapsulate two drugs can decrease the efficiency of encapsulation [25] [28].

4.3 Storage stability of the drug-loaded SLNs

The storage stability of the developed drug-loaded SLNs was evaluated by monitoring changes in size, PDI and zeta potential over time. Drug-loaded SLNs in an aqueous suspension were stored at different temperatures – room temperature (RT) and 4 ªC - to assess the impact of storage temperature on SLNs stability. The physicochemical properties of drug-loaded SLNs stored at RT and at 4ºC were monitored for 7 weeks and the results are presented in table 7.

Table 7 - Mean diameter and zeta potential values for drug-loaded SLNs over 7 weeks at RT and at 4ºC. Mean size variation is expressed in terms of ratio St/Si, where St is mean diameter after t days of storage and Si is the SLNs initial mean size. Data represented as mean ± SD (n=3).

Time (days)	Mean size (nm)	Ratio Sf/Si	PDI	Zeta potential (mV)
Drug-loaded SLNs at RT				
0	88 ± 0	1.00	0.31 ± 0.00	-11 ± 0
2	79 ± 1	0.90	0.25 ± 0.00	-11 ± 1
6	101 ± 13	1.15	0.38 ± 0.00	-11 ± 3
10	77 ± 4	0.87	0.25 ± 0.00	-7 ± 4
14	73 ± 10	0.83	0.23 ± 0.00	-6 ± 1
21	88 ± 14	1.00	0.32 ± 0.10	-4 ± 2
28	78 ± 4	0.89	0.28 ± 0.00	-9 ± 2
35	77 ± 1	0.87	0.24 ± 0.00	-14 ± 2
42	79 ± 4	0.89	0.28 ± 0.00	-8 ± 1
49	84 ± 0	0,96	0.31 ± 0.00	-15 ± 2
Drug-loaded SLNs at 4ºC				

0	114 ± 39	1.00	0.39 ± 0.08	-8 ± 0
2	87 ± 12	0.77	0.27 ± 0.01	-6 ± 5
6	88 ± 7	0.78	0.27 ± 0.02	-8 ± 4
10	121 ± 14	1.07	0.44 ± 0.00	-7 ± 4
14	88 ± 11	0.78	0.35 ± 0.00	-9 ± 1
21	86 ± 6	0.76	0.30 ± 0.02	-9 ± 1
28	110 ± 33	0.97	0.47 ± 0.01	-11 ± 1
35	83 ± 10	0.73	0.29 ± 0.01	-8 ± 0
42	101 ± 4	0.89	0.38 ± 0.01	-12 ± 3
49	97 ± 13	0.85	0.35 ± 0.00	-9 ± 1

Regardless of the storage conditions, no significant changes were verified in the size, PDI and zeta values of the drug-loaded SLNs over time (p-value >0.05). Thus, these results proved that drug-loaded SLN stored at RT and 4 ºC are stable for at least 7 weeks after production. The results obtained are consistent with the results presented by some authors indicating that SLNs are stable under storage conditions at 25ºC [44].Some authors report that SLNs stored at 4°C are not as stable as those stored at RT, however the majority state that, at 4 ºC, SLNs are stable with size values below 200 nm until the second month of storage, and then tendency to precipitate, making them unfeasible for therapeutic use. However, the stability of SLNs at 4 ºC may vary depending on the type of lipid and formulation [45]. After a few days at 4 ºC, some type of flocculation has occurred, probably due to loss of water through evaporation.

It is important that NPs are stable for a long term so that they can be stored for the time needed until application without losing therapeutic properties. After analyzing the stability results of SLNs at both RT and at 4 ºC, there is no preferential storage conditions

4.3.1 Freeze-Drying

Freeze-drying was applied to SLNs to enhance stability, since this method allows to store the NPs for longer periods of time. In lyophilization it is usually necessary to use a cryoprotectant to protect the NPs from damage caused in the process. One of the most used cryoprotectant is sucrose, which was used in this work. Several authors reported that sucrose it is able to maintain the compact structure of SLNs, compared to other cryoprotectants [46].

The percentages of sucrose most commonly used in the literature were evaluated in this experiment, being 5, 10 and 20%. The intention was to select the most adequate. SLNs without cryoprotectant were also lyophilized as control, and the obtained results are presented in table 8.

Table 8 - Average size and PDI for loaded-SLNs before and after lyophilization with different percentages of sucrose. Data represented as mean ± SD (n=3).

	Average size (nm)	PDI
Before freeze-drying	126 ± 6	0.28 ± 0.01
0 % sucrose	994 ± 59	0.63 ± 0.09
5% sucrose	457 ± 79	0.45 ± 0.03
10% sucrose	399 ± 91	0.47 ± 0.02
20% sucrose	304 ± 89	0.41 ± 0.04

The obtained results showed that drug-loaded SLNs that were freeze-dried in the presence of sucrose (5 to 20%) exhibited smaller sizes and PDI values that the ones freeze-dried without cryoprotectant ($p<0.05$) However, none of the used sucrose concentrations prove to be adequate for the lyophilization of the SLNs developed in this work. As it can be seen from table 11, the average size of the SLNs significantly increased after lyophilization ($p<0.05$) and were always above the desired range (<200 nm). That said, SLNs loaded with MTIC and O6BG cannot be lyophilized under these conditions. However, in future, it is possible to try to lyophilize the SLNs with different percentages of sucrose or even try different cryoprotectants, such as trehalose which has already shown good results in lyophilization of SLNs [47].

4.4 Stability of drug-loaded SLNs in simulated physiological conditions

4.4.1 Stability in simulated blood circulation

The properties of NPs were evaluated in a simulation of bloodstream conditions, to evaluate their stability. For that the drug-loaded SLNs were resuspended in PBS (0.01 M, pH 7.4), since it closely mimics the pH, osmolarity, and ion concentrations of the blood [39]. The stability of SLNs was evaluated in terms of changes in the mean size, PDI and zeta potential and the results are presented in table 9.

Table 9 - Properties of loaded-SLN for stability assessment in simulated blood fluid. Results are presented as mean ± SD (n=3).

Time	Mean size (nm)	Ratio Sf/Si	PdI	Zeta potential (mV)
0 h	133 ± 17	1.00	0.38 ± 0.07	- 9± 3
1 h	108 ± 11	0.81	0.30 ± 0.01	-2 ± 0
2 h	116 ± 13	0.87	0.34 ± 0.05	-2 ± 0
4 h	109 ± 14	0.81	0.33 ± 0.03	-2 ± 0
24 h	110 ± 6	0.83	0.28 ± 0.01	-1 ± 2
48 h	121 ± 27	0.91	0.35 ± 0.09	-1 ± 0
96 h	126 ± 26	0.95	0.31 ± 0.04	-2 ± 0
7 days	143 ± 33	1.07	0.38 ± 0.02	-1 ± 0
10 days	123 ± 6	0.92	0.34 ± 0.09	-2 ± 0

The zeta potential values of the drug-loaded SLNs significantly decreased ($p<0.05$) after 1 hour in PBS. The decrease in zeta potential values (becoming less negative) may be due to the high concentration of salts in PBS that can mask the surface charge of the SLNs. Nevertheless, the SLNs remained stable during the studied 24 hours and as the table shows, the average size and the PDI did not change significantly ($p>0.05$).

In the literature it is reported that SLNs dispersed in simulated blood fluid have a smaller diameter that the standard particles, suggesting a degree of deflocculation. Zeta potential values of the SLNs dispersed in this fluid are usually reported as being lower (less negative) than the standard NPs [48].

4.4.2 Stability in simulated gastrointestinal conditions

To evaluate the stability of the drug-loaded SLNs under simulated gastrointestinal fluid conditions, the physicochemical properties of the developed SLNs were evaluated using a well-described standardized static *in vitro* digestion model that sequentially simulate the different steps of human digestion (stomach and small intestinal) [49]. The drug loaded-SLNs were first incubated in FaSSGF (pH 1.2) and then placed in FaSSIF (pH 6.5). The oral phase simulating the saliva fluid was not included since drugs are not usually masticated. Initially, at time 0 SLNs were measured in ultrapure water. The stability of drug-loaded SLNs was evaluated in terms of changes in the mean size, PDI and zeta potential and the results are presented in table 10.

Table 10 - Properties of loaded-SLN for stability assessment in simulated gastrointestinal media. Results are presented as mean ± SD (n=2).

Time (hours)	Media	Mean size (nm)	Ratio Sf/Si	PdI	Zeta potential (mV)
0	UP water	126 ± 35	1.00	0.30 ± 0.03	-12± 2
2	FaSSGF	206 ± 5	1.64	0.47 ± 0.03	-1 ± 0
3	FaSSIF	152 ± 38	1.21	0.44 ± 0.04	-2 ± 0

When dispersed in both simulated gastrointestinal media (FaSSGF and FaSSIF) the size of the drug-loaded SLNs increased although not significantly ($p>0.05$). As for the PDI, when SLNs were dispersed in FaSSGF and in FaSSIF it undergoes significant changes ($p<0.05$). Although not significantly, the zeta potential values slightly decreased when the SLNs were incubated in both gastrointestinal media ($p>0.05$). The decrease in zeta potential when SLNs are dispersed in the gastrointestinal fluids may be due to the fact that, at acidic pH, the SLNs have acquired an almost neutral net charge due to the protonation of the surface groups of the SLNs.

In the literature, SLNs in simulated intestinal fluid show similar results to particles in simulated blood fluid. However, some authors reported that SLNs dispersed in gastric fluid aggregate as they present around twice the size of the standard SLNs [49].

4.5 Release profile of the drug-loaded SLNs

The prepared drug loaded-SLNs were evaluated to assess their ability in maintaining a controlled and sustained release of both MTIC and O6BG. Different simulated physiological conditions were used since the developed SLNs could be used for different administration routes.

4.5.1 MTIC and O6BG release profile in simulated blood-circulation

To simulate the biological conditions in the case of an intravenous administration, the *in vitro* release profile of MTIC and O6BG from SLNs was evaluated at 37 °C in PBS (pH 7.4, 0.01 M). The obtained results are shown in figure 14.

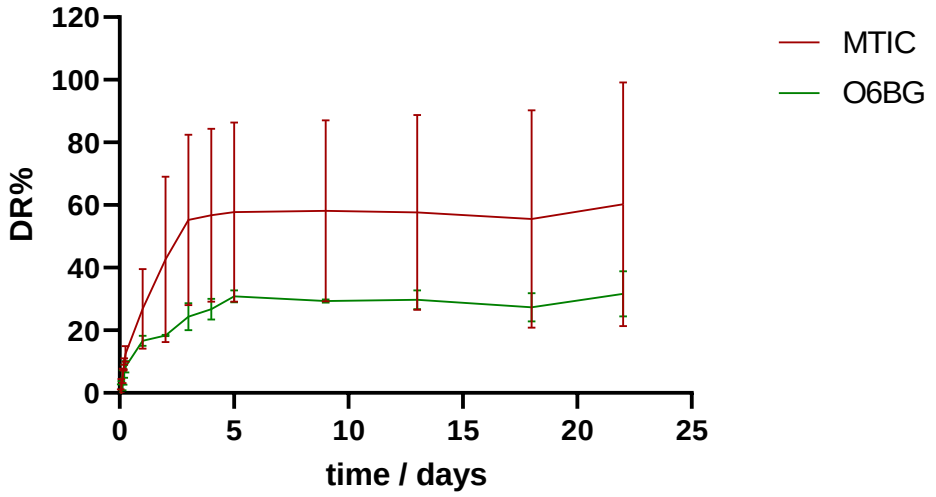

Figure 14 - Drug release profile for intravenous release studies

As can be seen in figure 14, in the first 24 hours there is faster release. After 24 hours, about 27±13% of MTIC and about 17%±2% of O6BG had been released. After two days, MTIC released was about 42 ± 27% while O6BG was about 18± 0%. On the fifth day, 58 ± 28% of the encapsulated MTIC was released and the O6BG changed slightly to 31 ± 2%. At the end of the release, after 22 days, the amount of MTIC released was about 60 ± 39% and the amount of O6BG did not change significantly, being about 32 ± 7%. From figure 14 it is possible to see that after the fifth day the release profile remains practically constant.

The biphasic release profile observed, with a burst release in the first hours followed by a slower release in the next days, can be explained by the drug location within the lipid matrix.

Other authors have reported that, during SLNs production by the hot homogenization technique, drugs suffers partition from the liquid oil phase to the aqueous water phase and during the cooling of the produced O/W nanoemulsion the solubility of the drug in the water phase decreases continuously with the decreasing temperature of the water phase, that means a re-partitioning of the drug into the lipid phase. When reaching the recrystallization temperature of the lipid, a solid lipid core starts forming including the drug which is present at this temperature in this lipid phase. Reducing the temperature of the dispersion further increases the pressure on the drug because of its reduced solubility in water to further re-partition into the lipid phase. The already crystallized core is not accessible anymore for the drug, consequently the drug concentrates in the still liquid outer shell of the SLN and/or on the surface of the particles. The amount of drug in the outer shell of the SLNs is released in the form of a burst, the drug incorporated into the matrix core is released in a prolonged way [50].

It was also observed that the release of MTIC was significantly higher (p-value < 0.05) than the release of O6BG. This may happen because MTIC is more hydrophilic than O6BG and therefore has more affinity for the aqueous phase. Free MTIC and O6BG were used as control and confirmed that the drugs have no affinity for the membrane (data not shown), and therefore the dialysis method is suitable for the release studies.

Thus, it can be said that the loaded-SLN allows a controlled release for 22 days, proving to be suitable for the continuous therapy with both drugs.

4.5.2 MTIC and O6BG release profile in simulated oral path

Despite oral administration being the most common and with better patient's compliance route of administration of pharmaceuticals, this pathway has some disadvantages. The intestinal epithelium can constitute a barrier to the absorption of drugs administered orally, and NPs have been extensively studied in order to overcome this problem, among others. Thus, it is essential to study the behavior of SLNs in the digestive tract.

As these developed SLN could be used for oral administration, their path after oral intake was simulated using biorelevant media in a well-described standardized static *in vitro* digestion model. The obtained release profiles are shown in figure 15.

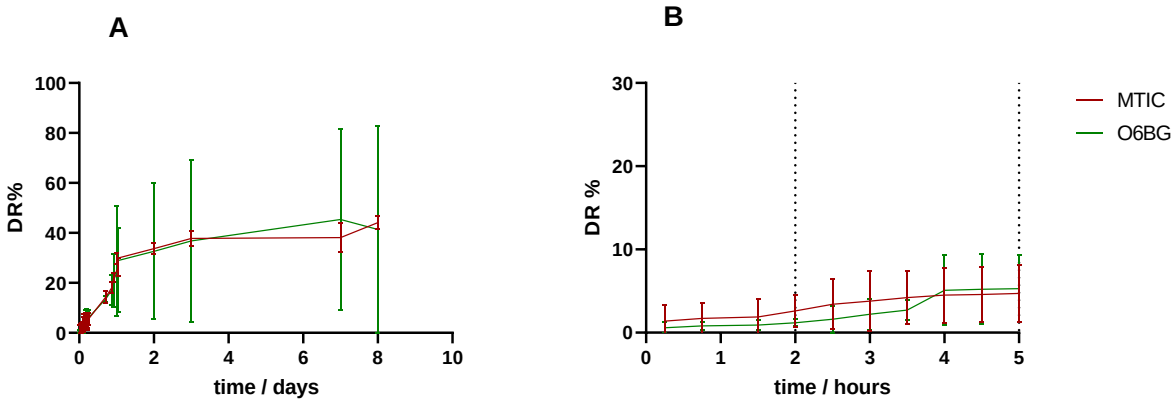

Figure 15 - (A) Drug release profile for oral administration studies; (B) approximation of the chart at the first 5 hours of release (in the first two hours SLNs in FaSSGF and in the two to five hours SLNs in FaSSIF)

Gastrointestinal media were used in this experiment to consider the gastrointestinal dissolution of drugs that very often is overestimated. In addition to pH, other major physiological factors affect the solubility and dissolution rate of the compounds, such as bile and pancreatic secretion, surface tension, osmolality, and the volume of gastrointestinal contents. These factors usually increase considerably the solubility of lipophilic compounds. Thus, these media contain natural micelles composed of bile salts and phospholipids (lecithin) that enhance the dissolution rate of these compounds in physiological conditions in the fasted state [51] [52].

It was verified that the percentage of both drugs released after 2 h in FaSSGF was very low (2.6 ± 1.9 for MTIC and 1.2 ± 0.7 for O6BG), showing that the SLNs are able to protect the drug from the gastric environment. After 3 hours in FaSSIF, both MTIC and O6BG exhibited a slightly higher release (2.1 ± 1.5 for MTIC and 4.1 ± 3.6 for O6BG). These results showed that SLNs prevented the release of both MTIC and O6BG in the simulated gastrointestinal tract, with only a total of 4.7 ± 3.4% of MTIC and 5.3 ± 4.0% of O6BG released after the 5 hours passage in the digestive system. The obtained results suggest that these SLNs are suitable to protect the encapsulated drugs in their path through the gastrointestinal tract, conferring protection through the harsh gastrointestinal environments. Thus, when the SLNs finally reach the bloodstream, more drugs will be potentially bioavailable, therefore potentially increasing their therapeutic properties.

For the rest of the experiment, drug-loaded SLNs were placed in PBS to mimic the blood circulation. The obtained results showed an increased release for both drugs. After 2 hours in PBS, only about 3 ± 2 % for MTIC and 0.8 ± 0.9 % for O6BG. After 24 hours, the percentage of drug released increase slightly (10.5 ± 1.2 for MTIC and 9 ± 2 % for O6BG). At the end of the

release experiments, 22 days, the total drug released, from simulated gastric fluid medium to simulated blood fluid medium, is 32 % for MTIC and 22 % for O6BG.

Although MTIC release values are usually higher than O6BG, the difference was not significant ($p>0.05$). As in simulated blood fluid release experiments, free MTIC and O6BG were used as control and confirmed that the drugs have no affinity for the membrane (data not shown).

5 Conclusion

TMZ is the drug currently used in the treatment of GBM. This treatment has several side effects and is limited by intrinsic drug resistance mechanism, which leads to the treatment being ineffective. The MGMT protein is involved in one of the repair mechanisms that decreases TMZ efficacy as it is able to repair the drug-induced DNA damage. In physiological conditions, TMZ is converted into its active form, MTIC, which is responsible for the therapeutic effect. However, this poses another problem because, unlike TMZ, MTIC does not have the same ability to cross the BBB, which leads to only a small percentage reaching the target tissue. To overcome of the low bioavailability of MTIC and the drug resistance mechanisms, SLN for the co-delivery of TMZ and an inhibitor of the MGMT protein, O6BG, have been proposed in this work. SLNs have numerous advantages such as ability to transport drugs to the target tissue, protect the drug from external physiological factors and also allow the possibility of coupling NPs with ligands with affinity for cell receptors, among others.

Drug-loaded SLNs were produced by hot homogenization, high-shear and ultrasound dispersing technique. The obtained SLNS presented suitable properties for brain delivery, such as mean sizes below 200 nm and negative surface charge. The BBB is a very selective biological barrier and NPs with small dimensions reach target in the brain tissues easily. Anionic NPs are considered safer for the BBB as they are not considered toxic. Stability tests under different storage conditions have shown that SLNs can maintain stability for at least 7 weeks. It is important that SLNs remain stable for a long period of time in storage conditions, because when thinking about a clinical application it is important that they do not significantly alter their properties so that they do not lose therapeutic effectiveness. Release experiments were performed in different simulated body fluids, such as PBS and in simulated gastrointestinal media. Experiments in PBS mimicking the blood circulation showed a faster release in the first hours and that after about five days the maximum release is reached, remaining stable up to 22 days. Release experiments in simulated gastrointestinal conditions showed that the developed drug-loaded SLNs are able to protect the entrapped drugs from the harsh gastric environment. SLNs proved to be stable when dispersed in simulated physiological conditions.

The obtained results show that SLN loaded with TMZ and O6BG could be a promising strategy to overcome the limitations of current therapy. To prove that they can really be effective in the treatment of GBM and to improve the NPs, *in vivo* and pharmacokinetic tests must be carried out in the future. Also, in an attempt to improve the accumulation of NPs in target tissues and increase selectivity, it is possible to modify the SLNs' surface with ligands with affinity for receptors on cells. It might be something to try in a future work in continuation of this one.

6 Assessment of the work done

6.1 Objectives Achieved

The aim of this work was to produce SLNs for the delivery of TMZ and O6BG to the brain for the treatment of GBM. The addition of the O6BG is to increase therapeutic efficacy, as the defense mechanism is decreased/inhibited. It was also intended to evaluate their physical-chemical properties, to evaluate their ability to maintain a controlled and sustained release overtime in different simulated biological conditions and even to evaluate their stability and what is the best storage method.

As for the production of SLNs, the objective was fulfilled because, despite several attempts to find the most suitable formulation, an adequate formulation was obtained. From the best candidate it was possible to encapsulated TMZ and O6BG. Drug release experiments were performed to assess the behavior of the entrapped drugs and the developed SLNs. Simulated intravenous and digestive release tests were performed. Stability tests were performed to assess their stability for 7 weeks.

6.2 Final Assessment

I believe that nanotechnology is the future, especially in the health area, allowing to treat various diseases that kill every day or that leave people incapacitated and prevent them from enjoying life to the fullest. I believe that my work is a valuable step in discovering the effective treatment of patients with GBM. As a suggestion for future work, to further increase therapeutic efficacy it would be a good idea to conjugate the developed SLNs with different receptor recognizable ligands such as transferrin, monoclonal antibody or anti-epithelial growth factor receptor (AEGFR), among others.

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Appendix A

1. Drug stability tests

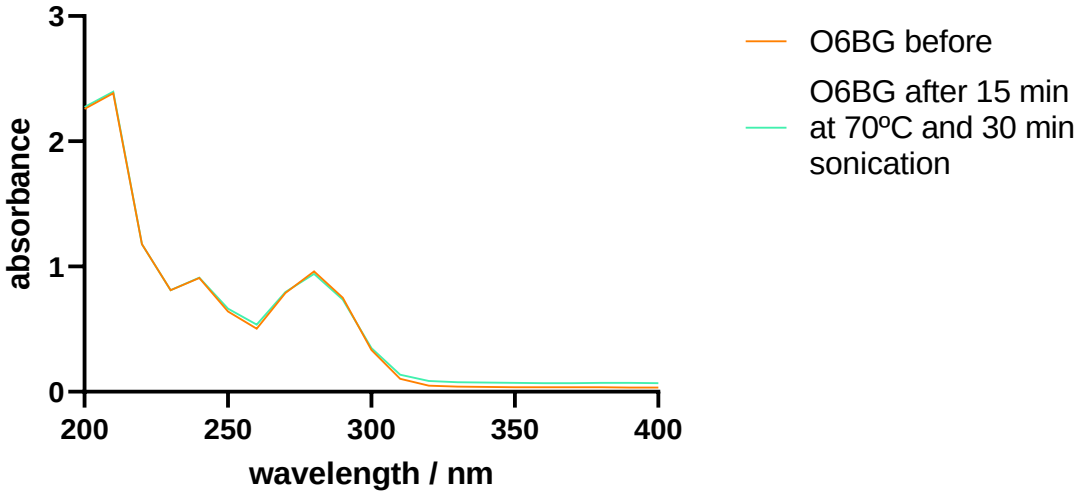

Figure 1 – Stability tests for O6BG after 15 minutes at 70ºC and 30 minutes of sonication

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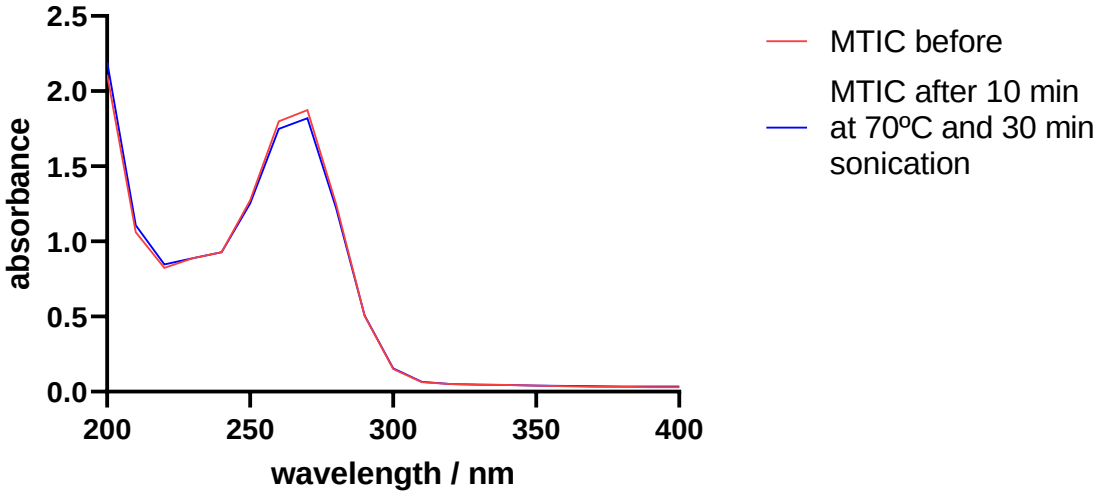

Figure 2 - Stability tests for MTIC

Figure 1 shows that no major changes occurred in the absorbance spectrum of O6BG, suggesting that O6BG will be stable during the production process.

2. Calibration curves

To obtain the encapsulation efficiency, the absorbance was read and the drug concentration was obtained using the calibration curves found in equations 1 and 2.

$$MTIC: absorbance = 0{,}0546 * concentration - 0{,}1375 \quad (1)$$

$$O6BG: absorbance = 0{,}0606 * concentration - 0{,}1949 \quad (2)$$

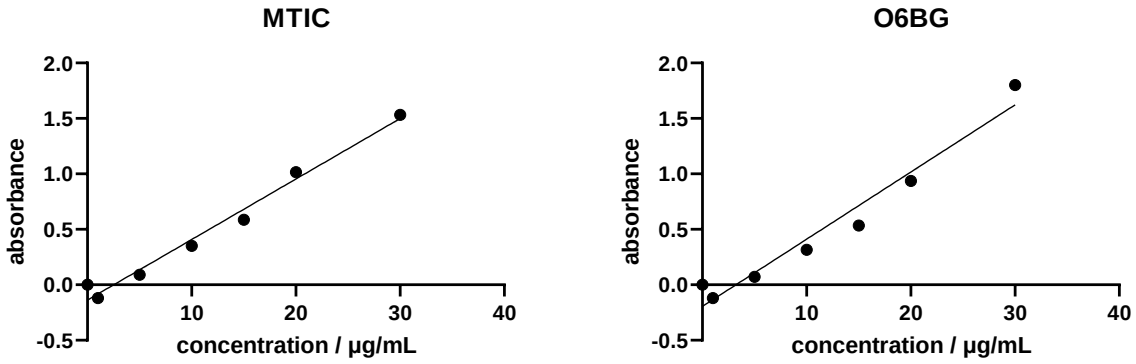

Figure 3 – Calibration curves for MTIC and O6BG in Pluronic

In order to obtain the amount of drug released, the drug concentration was calculated with the following equations.

$$MTIC: absorbance = 0{,}0508 * concentration + 0{,}0245 \quad (3)$$

$$O6BG: absorbance = 0{,}039 * concentratio + 0{,}0263 \quad (4)$$

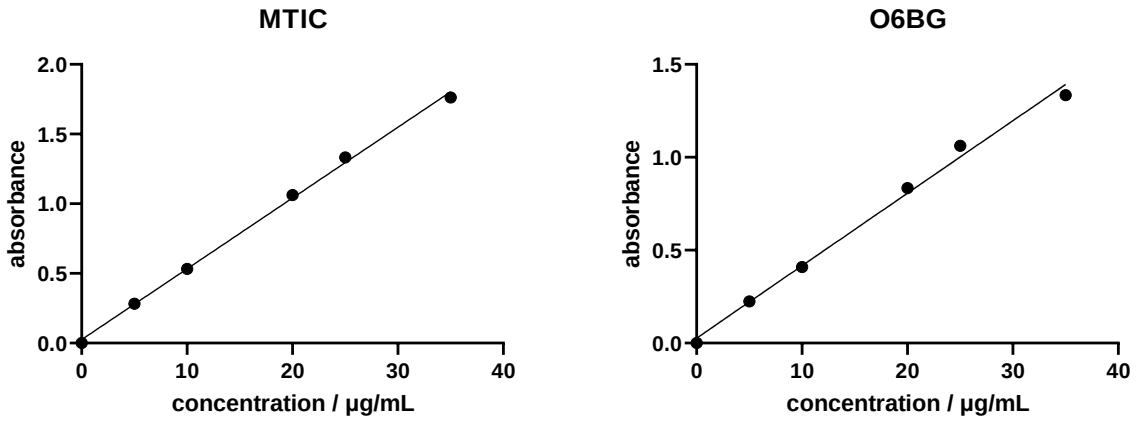

Figure 4 – Calibration curves for MTIC and O6BG in PBS

The drug released in FaSSGF was calculated using the following equations:

$$MTIC: absorbance = 0{,}0996 * concentration + 0{,}0385 \quad (5)$$

$$O6BG: absorbance = 0{,}1735 * concentration - 0{,}0024 \quad (6)$$

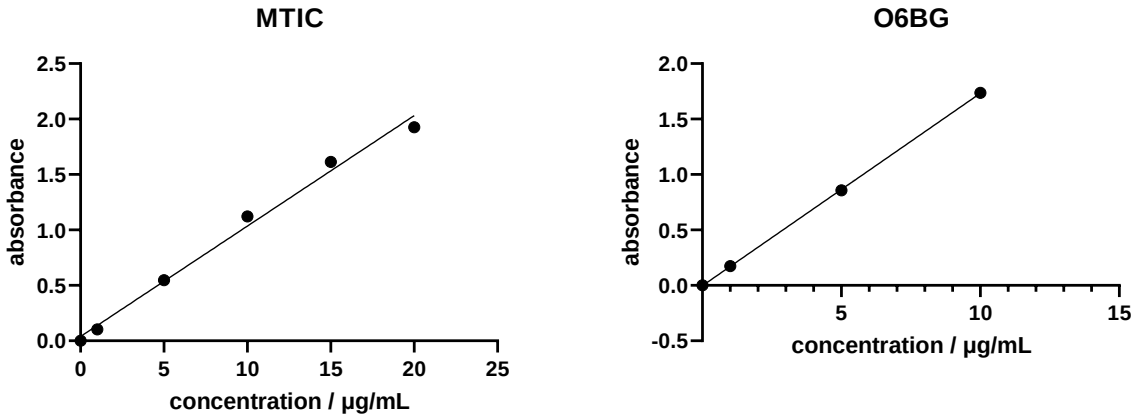

Figure 5 – Calibration curves in FaSSGF

The drug released in FaSSIF was calculated using the following equations:

$$MTIC: absorbance = 0{,}0811 * concentratio - 0{,}0168 \quad (7)$$

$$O6BG: absorbance = 0{,}065 * concentration + 0{,}00227 \quad (8)$$

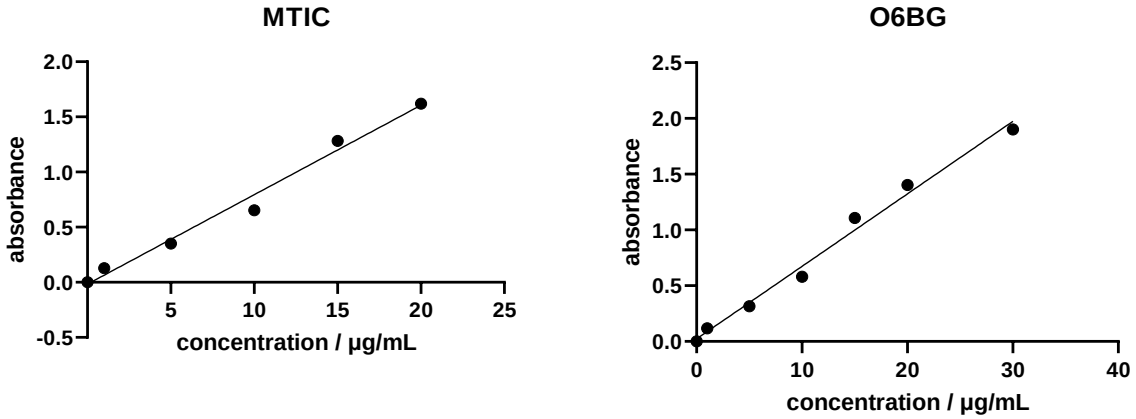

Figure 6 – Calibration curves in FaSSIF