Receptor-mediated PLGA nanoparticles for Glioblastoma Multiforme treatment

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Abstract

Glioblastoma multiforme is the most lethal type of brain tumor and the established therapy only extends patients survival to approximately one year. Its first-line treatment is based on of chemotherapy with the alkylating agent temozolomide (TMZ). As many other chemotherapeutic drugs, TMZ presents several limitations as high toxicity and low bioavailability. The delivery of TMZ using poly(lactic-co-glycolic acid) nanoparticles is proposed in this work. Stable

nanoparticles functionalized with a OX26 type monoclonal antibody for transferrin receptor were developed, targeting the glioblastoma tumor cells, since these cells are known for overexpressing this receptor. The release profile of TMZ from the nanoparticles was studied mimicking physiological conditions, and targeted cellular internalization was also investigated. Two glioblastoma cell lines - U215 and U87 – were used to evaluate the i*n vitro* cytotoxicity of the drug, showing that the prepared nanocarriers enhance the anticancer activity of TMZ. The functionalization with the monoclonal antibody for transferrin receptor proved to be advantageous in enhancing the cellular internalization in glioblastoma cells.

Keywords

Glioblastoma multiforme, Temozolomide, Poly(lactic-co-glycolic acid), Nanoparticles, Monoclonal antibody, Transferrin receptor

1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of brain tumor. Due to its heterogeneity, anatomic location and high proliferation rate, GBM exhibits high resistance to therapy, resulting in high mortality rates (Bastiancich et al., 2016; Vehlow and Cordes, 2013; Grossman and Batara, 2004). The classic therapy, consisting of a combination of radiation, surgical resection and chemotherapy, fails to successfully treat the disease (Morais et al., 2015), and patients succumb within 12–15 months of initial diagnosis (Weller et al., 2013).

The most commonly used chemotherapeutic drug for the treatment of GBM is the alkylating agent, temozolomide (TMZ). This drug causes cell death by inducing DNA methylation and consequent degradation (Wesolowski et al., 2010). At physiologic pH, TMZ undergoes hydrolysis originating firstly the 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) intermediary, and then 5-amino-imidiazole-4-carboxoamide (AIC), the active form of the drug (Andrasi et al., 2010). Nonetheless, this drug presents some limitations as high toxicity to healthy tissues, exhibiting side-effects as lymphopenia, thrombocytopenia and

myelodysplasia (Stupp et al., 2005). Also, its pharmacological efficiency is reduced due to the existence of DNA repairing mechanisms, namely those mediated by by O-6-methylguanine-DNA methyltransferase enzyme (MGMT) (Jacinto and Esteller, 2007; McLendon et al., 2008) and by multidrug resistance (MDR) mechanisms, such as the p-glycoprotein pump present in GBM tumor cells, which transports chemotherapeutic drugs out of cells (Binkhathlan and Lavasanifar, 2013).

Another limitation in the use of TMZ for GBM treatment, is its low bioavailability in the target tissues, due to the low permeability through the blood-brain barrier (BBB). BBB is still a major challenge in the treatment of brain diseases, preventing the delivery of several drugs. Its function is maintained by tight junctions between the endothelial cells, restricting the passage of 98% of small-molecule drugs and 100% of large-molecules (> 400 Da) into the brain (Abbott et al., 2010). Thus, TMZ is only capable of extending the patients' survival, not being successfully curative (Stupp et al., 2001). Therefore, TMZ delivery by nanocarriers may be a suitable approach to enhance passage across the BBB, in this way increasing its chemotherapeutic efficacy (Coelho et al., 2015).

Several types of nanosystems have been extensively studied for drug delivery. In this work, the use of poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) is proposed. Being biodegradable, biocompatible and FDA-approved (Ramalho et al., 2015), the encapsulation of TMZ in PLGA NPs is a suitable strategy to increase TMZ therapeutic efficacy and to overcome its limitations, as toxicity in healthy tissues by targeting the tumour cells. Also, TMZ encapsulation in PLGA NPs should enhance drug transport across the BBB and avoid drug recognition by p-glycoprotein pump in target cells circumventing drug efflux. Moreover, these NPs exhibit low synthesis complexity (Ramalho and Pereira, 2016) and are able to maintain a controlled drug release for several days, decreasing the need for frequent drug administration and doses, minimizing the side effects in healthy tissues (Makadia and Siegel, 2011). The PLGA polymer can also be easily functionalized for the design of NPs with different targeting moieties (Danhier et al., 2012).

Some PLGA-based nanosystems for the delivery of TMZ were already proposed (Ramalho et al., 2018). Jain and colleagues (2014) developed PLGA NPs able to enhance chemotherapeutic activity of TMZ, although the prepared NPs were not modified to increase the specificity of NPs. (Jain et al., 2014). Two years later, Ananta and colleagues (2016) developed PLGA NPs using three different experimental procedures. However, the prepared NPs were not able to maintain a controlled and sustained release of TMZ (Ananta et al., 2016). Xu and co-workers (2016) intended the simultaneous delivery of TMZ with another chemotherapeutic agent. The group prepared PLGA NPs for the entrapment of both TMZ and paclitaxel. The combination of both drugs showed a synergic effect, increasing their cytotoxicity (Xu et al., 2016). Some authors used active targeting strategies to increase the NPs specificity to tumor cells. In fact, Lee and collaborators (2016) modified PLGA NPs with folate molecules aiming to increase the NPs internalization in target cells. Although the authors tested different methods for the preparation of NPs, the prepared nanosystems did not exhibit good encapsulation efficiency values (Lee and Ooi, 2016). Jain and co-workers (2011) developed PLGA NPs functionalized with transferrin molecules to enhance the transport across the BBB. The obtained NPs proved to enhance the antiproliferative effect of TMZ and increase the accumulation of TMZ in the brain tissue (Jain et al., 2011).

Active targeting strategies are increasingly used to enhance NPs uptake in the target cell or the transport across biological barriers as BBB. For that, the NPs' surface can be modified using receptors usually overexpressed in target cells or biological barriers (Pillai, 2014; Lockman et al., 2002). Instead of using the receptor ligands, immunocarriers can be also developed using monoclonal antibodies (mAb) for the target of chosen receptors. Since mAbs exhibit high specificity for the target receptors, it is possible to achieve high levels of targeting (Loureiro et al., 2014). In this work, transferrin receptor (TfR) is the targeted receptor, using the mAb type OX26 that has proven to efficiently bind cells that overexpress TfRs (Loureiro et al., 2016; Loureiro et al., 2017), as GBM cells (Calzolari et al., 2010) and several other tumor cells types (Frasco et al., 2015).

Here, the main goal was to develop PLGA NPs functionalized with an OX26 mAb, to encapsulate TMZ and to assess its efficiency in the delivery of the drug into GBM tumor cells. The internalization and antiproliferative effect of TMZ entrapped in OX26 mAb-modified and non-modified PLGA NPs was evaluated in GBM cell lines.

2. Materials & Methods

2.1 Materials

PLGA Resomer® RG503H (50:50; MW 24,000 – 38,000), poly(vinyl alcohol) (PVA), Coumarin-6 (C6), phosphate buffered saline (PBS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dichloromethane, ethyl ether, methanol, ethylenediaminetetraacetic acid (EDTA), 2-iminothiolane hydrochloride (Traut's reagent), bovine serum albumin (BSA), citric acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium hydroxide, acetic acid, sulforhodamine B (SRB), trypan blue, holo-Transferrin human (purity ≥ 98%), rhodamine B and Triton X^{TM}-100 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Uranyl acetate was bought from Electron Microscopy Sciences (Hatfield, UK). Polyethylene glycol functionalized with maleimide (mPEG-Mal, Mw 2000) was acquired from Nanocs Inc. (Boston, MA, USA). The OX26 mAb was purchased from Novus Biologicals (Oxfordshire, UK). Transferrin receptor was acquired from Abcam (Cambridge, UK). The secondary antibody Goat anti-Mouse IgG (H+L) Cross Adsorbed Secondary Antibody, HRP conjugate was bought at Thermo Scientific-Pierce Antibodies (Waltham, MA, USA). High-glucose Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), SlowFade Gold Antifade Mountant with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) and Lysotracker DeepRed were acquired from Invitrogen Co. (Scotland, UK). Tricloroacetic acid (TCA) and Tris buffer were purchased from Merck (Darmstadt, Germany). Ringer HEPES (RH) buffer was prepared using 150 mM NaCl, 6 mM $NaHCO_3$, 5.2 mM KCl, 5 mM HEPES, 2.8 mM glucose, 2.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$-$6H_2O$ at pH 7.4). All reagents used for RH buffer composition were acquired from Sigma–Aldrich (St. Louis, MO, USA).

2.2 Cell lines

Two human glioblastoma cell lines, U251 and U87, and an immortalized human astrocyte cell line (NHA) were used in this work. All the used lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. At 80% of confluence, the cells were trypsinized and subcultured.. Both U251 and U87 cell lines were chosen since they show significant similarities with the genetic, immunohistochemical and histological and characteristics of human GBM tumor (Jacobs et al., 2011).

2.3 Preparation of TMZ-loaded PLGA NPs

TMZ-loaded PLGA NPs were prepared using a modification of the single emulsion-solvent evaporation technique previously developed (Ramalho et al., 2015). An organic solution of dichloromethane containing 1 mg of TMZ and 10 mg of PLGA was prepared. A known amount of 2% (w/v) PVA solution was added drop-by-drop to the organic mixture previously prepared. Then, the solution was agitated and emulsified in an ultrasonic bath (45 kHz). The emulsion was then transferred into a known volume of 0.2% (w/v) PVA solution and maintained in continuous agitation, allowing the organic solvent to complete evaporate. The suspension was filtered (200 nm, polyethersulfone membrane syringe filter, VWR, USA) and stored at 4 ºC for 12 h. After, the samples were centrifuged (30 min, 14100 g). to separate NPs from non-encapsulated drug.

2.4 Synthesis of PEGylated PLGA

2 mg of PLGA and 0.04 mg of EDC were dissolved in a known volume of dichloromethane. A mixture of ethyl ether:methanol (70:30) was added to the previously prepared solution to remove the excess of EDC. Then, 0.2 mg of maleimide-PEG-NH_2 were

added to the activated PLGA and the solution was maintained overnight at room temperature (RT) in continuous agitation. The mixture was centrifuged (40 min, 14100 g) and the pellet resuspended in dichloromethane.

2.5 Preparation of TMZ-loaded mAb-PLGA NPs

TMZ-loaded PEG-PLGA NPs were prepared using also the single emulsion-solvent evaporation technique. 8 mg of non-modified PLGA was added to the previously prepared PEG-PLGA. Also 1 mg TMZ was added to the PLGA mixture in dichloromethane, and the remaining protocol was performed as described previously in section 2.3.

The prepared NPs were next modified with OX26 mAb by a covalent coupling reaction at a molecular ratio of OX26 mAb:PEG-PLGA of 1:2. The maleimide group on PEG extremity reacts with thiol groups present on mAbs, after mAb activation by Traut's reagent. A drop of EDTA was added to prevent oxidation of the thiol groups (Loureiro, et al., 2016). The activated mAbs were separated from the excess reagents by size exclusion chromatography (PD Minitrap G-25 columns containing Sphadex Medium, GE Healthcare, Sweden). The activated mAbs were then added to the previously prepared PEG-PLGA NPs and incubated 1 h in the dark at RT, followed by overnight incubation at 4 °C. Non-attached antibody was removed by centrifugation (30 min, 14100 g).

2.6 PLGA NPs physicochemical characterization

The physicochemical features of the prepared NPs, as mean diameter, size distribution and zeta potential values, were assessed by Dynamic Light Scattering (DLS) and by laser doppler velocimetry methods. The measurements were performed in a ZetaSizer Nano ZS, Malvern Instruments, UK. The average NP size was obtained by measuring the fluctuations of scattered light intensity as a function of time. These fluctuations can be related to the NPs diffusion coefficient and size, using the Stokes-Einstein Equation. The attained data is given in intensity distribution. The zeta potential data analysis was performed using the dielectric

constant of water and the zeta potential values were estimated by Smoluchowski approximation from the electrophoretic mobility.

The morphological analysis of the NPs was obtained by Transmission Electron Microscopy (TEM) (TEM JEOL JEM 1400, Japan). The samples were visualized on copper grids (Formvar/Carbon-400 mesh Copper, Agar Scientific, UK) and negatively stained with a 2% (v/v) uranyl acetate solution.

2.7 ELISA studies

The affinity of TfR to OX26 mAb-modified PLGA NPs was analyzed by the enzyme-linked immunosorbent assay (ELISA). The surface of a 96-well plate (Nunc MaxiSorp®) was treated with TfR and incubated overnight at 4 ºC. The plate was then blocked with BSA (bovine serum albumin) and incubated for 2 h at RT, followed by the addition of NPs. After incubation and subsequent washing, the peroxidase-conjugated secondary antibody (goat anti-mouse) was added and incubated for 45 min at RT. The samples were revealed using a solution of citric acid, ABTS and H_2O_2. The absorbance at the wavelength of 405 nm was measured using a fluorescence microplate reader (HT Microplate Spectrophotometer, BioTek). Non-modified NPs were used as a negative control.

2.8 TMZ encapsulation efficiency and release from PLGA NPs

The TMZ encapsulation efficiency (EE) and its release from PLGA NPs were determined by UV–Vis spectrophotometry at λmax 265 nm. Free TMZ was obtained from centrifugation of NPs suspension (30 min, 14100 g),and quantified using a calibration curve of known TMZ concentrations (UV-1700 PharmaSpec, Shimadzu, Japan). The TMZ loading capacity of the prepared PLGA NPs was also determined by the following equation: (amount of encapsulated TMZ/polymer weight*100).

In vitro temozolomide release studies were performed, over 20 days at 37 ºC, using a cellulose dialysis membrane diffusion technique. A sufficient amount of TMZ-loaded PLGA

NPs was diluted in 2 mL of release buffer (PBS, pH 7.4, 0.01 M) and placed into a dialysis device (Float-A-Lyzer G2, CE, 10KDa, SpectrumLabs (Laguna Hills, CA)). The outside space of the dialysis device was filled with 4 mL of release buffer. At predetermined times, samples were collected from the outside medium and, after measurement by UV-Vis spectrophotometry, returned to the release medium. A solution of TMZ in PBS was used as control.

2.9 Cellular imaging studies

The NPs *in vitro* uptake in the human cells lines (U251, U87 and NHA) was assessed using laser scanning confocal microscopy (LSCM) (Leica TCS SP5 II, Leica Microsystems, Germany). For that purpose, C6-loaded NPs were used. This fluorescent marker is distributed in the NPs matrix, allowing the visualization of the NPs inside the cellular compartments (Holzer et al., 2009). 1000 cells/well were seeded in 12-well plates (Ibidi, Germany) and allowed to attach for 24 h (37 °C, in a humidified 5% CO_2 incubator). Cells were then treated with mAb-modified and unmodified C6-loaded NPs for 2 and 72 h. After the incubation period and subsequent wash with PBS, the cells were fixed with 4% (w/v) paraformaldehyde. The acidic cell compartments (as endosomes/lysosomes) were stained with LysoTracker® Red and cell nuclei were marked with DAPI. At least six images from different areas of each cell lines were acquired in emission mode. Untreated cells were also used control.

2.10 Quantification of *in vitro* cellular uptake of mAb-PLGA NPs

Cellular uptake of mAb-modified and unmodified PLGA NPs was quantified by a fluorescence assay using C6 loaded PLGA NPs. U251, U87 and NHA cells were seeded in 96-well plates at a density of 8000 cells per well and allowed to attach for 24 h. Six NPs samples were diluted in cell culture medium at final polymer concentration of 50 μg/mL, and the cells were incubated with these samples for 0.5 h and 2 h. At the end of the incubation period, the cells were washed with cold PBS to remove the non-internalized NPs. The cells were then lysed with 0.1% Triton X-100 in 0.1 N NaOH solution. The fluorescence intensity from C6-loaded

NPs was measured using a fluorescence microplate reader (HT Microplate Spectrophotometer, BioTek) with excitation and emission wavelengths set at 430 and 485 nm, respectively.

2.11 Transferrin competitive binding assay

Transferrin blocking assay was used to confirm whether the mAb-PLGA NPs are internalized through transferrin receptor-mediated endocytosis. Competitive binding to TfRs was achieved using an excess of transferrin, and cellular uptake of PLGA NPs was quantified by fluorescence using C6 as mentioned above. U251, U87 and NHA cells were seeded in 96-well plates at a density of 8000 cells per well and allowed to attach for 24 h. Transferrin was diluted in cell culture medium and added to the cells at six final different concentrations ranging from 1 to 10 mg/mL. The same range of concentrations of folate was used as control. After 1 h, cells were incubated for 2 h with C6-NPs samples at final polymer concentration of 50 µg/mL. At the end of the incubation period, the cells were washed and lysed as described in the previous section. The fluorescence intensity from C6-loaded NPs was measured with excitation and emission wavelengths set at 430 and 485 nm, respectively.

2.12 Effects of NPs on human brain-like endothelial cells (HBLECs) monolayer integrity

In accordance with French legislation, the donors' parents gave their informed consent for the collection of human umbilical cord blood. The protocol was approved by the French Ministry of Higher Education and Research (CODECOH DC2011-1321). All experiments were carried out in accordance with the approved protocol. The *in vitro* HBLEC model consists of a co-culture of endothelial cells (derived from $CD34^+$-cells) and brain pericytes. Briefly, $CD34^+$-cells were isolated from human umbilical cord blood and then prompted to differentiate into endothelial cells via exposure to endothelial cell medium supplemented with 50 ng/mL vascular endothelial growth factor (PeproTech Inc., Rocky Hill, NJ, USA) (Pedroso et al., 2011). The $CD34^+$-derived endothelial cells thus obtained were seeded onto Matrigel (BD Biosciences, San Jose,

CA, USA)-coated filters (Costar Transwell inserts, Corning Inc., Corning, NY, USA, pore size 3 µm, $8x10^4$cells/cm^2) (Cecchelli et al., 2014). These inserts with cells were maintained with a dry bottom for one week (500 µL of medium in the upper compartment, changed every other day) to avoid the cells crossing the membrane and forming a non-physiological second layer on the lower face of the insert. Next, the inserts were transferred onto pericytes (50,000 cells per well, seeded in 12-well plates two days before the transfer). The resulting co-culture was cultured with endothelial cell medium supplemented with 5% heat inactivated fetal calf serum (GIBCO, Life Technology SAS, Saint Aubin, France) and 50 µg/mL gentamicin. The medium was changed every two days. These culture conditions were maintained for 5 days and enabled the $CD34^+$-derived endothelial cells to acquire a true BBB phenotype (i.e. HBLECs). Under these conditions, the model was stable for 30 days and was then ready for experiments (Cecchelli, et al., 2014; Kuntz et al., 2015).

The effect of non-modified and OX26 mAb-modified PLGA NPs on HBLECs cells was evaluated in Ringer HEPES buffer at 40 µM and 80 µM as previously described (Loureiro, et al., 2017). Culture medium was removed and replaced with NPs and with 1,5KBq/mL of ^{14}C-sucrose, in the luminal compartment. ^{14}C-sucrose was used as an indicator for the integrity of the BBB model.. After 120 min, aliquots were taken in abluminal compartments of the cell culture and samples were analyzed using Liquid Scintillation Analyser (Tri-carb 2100TR) for ^{14}C-sucrose. The endothelial permeability coefficient (Pe) of ^{14}C-sucrose was determined in cm/min.

2.13 In vitro cytotoxicity studies

The efficiency of non-modified and OX26 mAb-modified PLGA NPs in enhancing the antiproliferative effect of TMZ on different human cell lines was evaluated by the Sulforhodamine B (SRB) colorimetric method (Skehan et al., 1990).

U251, U87 and NHA cells were seeded at a density of 1000 cells/well, in 96-well assay plates and incubated for 24 h (37 °C, in a humidified 5% CO_2 incubator) to allow complete cell adhesion. Then, TMZ, TMZ-PLGA NPs and mAb-TMZ-PLGA NPs, diluted in DMEM medium

at ten final concentrations of TMZ ranging from 0.1 to 800 µM, were added to the cells. After an incubation period of 72 h, the cells were fixed with 10% (w/v) TCA for 1 h at 4 ºC, and stained with 0.4% (w/v) SRB dye for 30 min. After repeatedly washing the cells with 1% (v/v) acetic acid to remove unbound dye, the cells air-dried and the protein-bound stain was solubilized with 10 mM Tris solution for UV-VIS absorbance quantification at 560 nm using the (BioTek Synergy HT Microplate Reader, BioTek, UK). The GI_{50} value - the concentration inhibiting the net cell growth by 50% - for TMZ was calculated from the dose–response curve.

Unloaded PLGA and mAb-PLGA NPs were added as control to assess if they affect cell growth in control cells. Not exposed cells were also included in all assays as no-treatment controls (null controls).

2.14 Statistical analysis

All results are presented as mean and standard deviation, for at least three independent experiments. Statistical analysis was performed using Non-parametric test Kruskal-Wallis and Mann-Whitney, with a 95% confidence interval.

3. Results and discussion

3.1 PLGA NPs physicochemical properties

Different TMZ-loaded PLGA NPs were prepared in this work. In all the prepared formulations, PVA was used as an emulsion stabilizer since it has a high affinity to PLGA and forms an uniform layer on the NPs' surface, conferring stability against aggregation (Gelperina et al., 2010). Maleimide-functionalized PEG was also used to allow covalent coupling of OX26 mAb at the PEG terminus with the chemically reactive end-group. The physicochemical properties of the prepared NPs were evaluated and are presented in Table 1.

[insert table 1 here]

The prepared unloaded NPs exhibited a mean diameter of 176 ± 3 nm, and a zeta potential value of -29 ± 3 mV, negative as expected due to the negative charge of the carboxylic groups of PLGA polymer (Table 1). The TMZ molecules accommodation in the polymeric matrix caused a significant ($p<0.05$) increase in size for the TMZ-PLGA NPs (181 ± 2 nm). Moreover, NPs mean size was also significantly increased with OX26 mAb modification (194 ± 1 nm) ($p<0.05$), indicating an efficient conjugation since the diameter of the globular antibody is approximately 15 nm (Dammer et al., 1996).The prepared nanocarriers exhibited a small size distribution ($PdI \leq 0.1$). Loading of the NPs with TMZ did not significantly affect their zeta potential, however a small decrease was observed due to the adsorption of TMZ molecules on PLGA NPs surface, exerting a masking effect on the superficial net charge (Musumeci et al., 2006). The modification of the NPs' surface with mAb also does not significantly alter their zeta potential, due to the negative nature of this immunoglobulin ($p>0.05$). The colloidal stability of the prepared NPs is a result of electric charge of the carboxylic groups at the NP surfaces, and the PVA layer on the NP's surface causing steric repulsions. In fact, Figure 1 shows a stabilizer layer surrounding PLGA NPs. TEM images (Figure 1) also show uniform PLGA NPs with a spherical form. The mean size of the NPs revealed by TEM are in agreement with those determined by DLS analysis.

[insert figure 1 here]

Figure 1 -TEM image of the prepared PLGA NPs. Scale bar is 200 nm.

NP dimensions and zeta potential are key parameters that affect the efficiency of NP systemic circulation, and uptake by target cells (Wohlfart et al., 2012). Also transport across the BBB will depend on these parameters. Although mAb modification of the NPs' surface is expected to enhance the transport of NPs across the BBB through receptor-mediated transport, these two physiochemical characteristics also are determining factors. Thus, these parameters were considered during the design of this nanosystem. The prepared mAb-modified PLGA NPs exhibit suitable physicochemical characteristics for cancer cell uptake and transport across the

BBB. Since neutral and anionic NPs are more easily transported across the BBB and are not associated to BBB toxicity (Wu et al., 2015), most of the NP formulations described in the literature for brain delivery have moderate to high (between −1 to −45 mV) negative zeta potentials, as the one prepared in this work (Saraiva et al., 2016). It has also been proven that NPs with dimensions up to 200 nm are more easily accumulated in brain tissue (Veszelka et al., 2015) since tumor microenvironment exhibits vascular fenestrations in the range of 40–200 nm (Wu, et al., 2015). Also NPs with dimensions up to 200 nm are more efficiently taken up by receptor-mediated endocytosis, as it was intended in this work with the use of TfR (Masserini, 2013).

The stability of the prepared NPs was assessed in terms of size and zeta potential, and no changes were observed (data not shown), proving that the developed system is stable at storage conditions (4 ºC), for at least one month.

The binding ability of the mAb-modified NPs to TfR was evaluated by ELISA assays. OX26 mAb-modified NPs showed significantly higher absorbance at 405 nm (0.81 ± 0.05) than non-modified NPs (0.29 ± 0.08) ($p<0.05$). Therefore, OX26 mAb demonstrated that the bioactivity for the TfR is preserved after the mAb conjugation with the NPs.

3.2 TMZ encapsulation efficiency and release from PLGA NPs

PLGA NPs loaded with TMZ showed encapsulation efficiencies ranging from 48 ± 10% for mAb-PLGA NPs to 44 ± 3% for non-modified NPs, respectively. Drug loading varied from 10 ± 2% for mAb-PLGA NPs to 9 ± 1% of the polymer weight for non-modified NPs. These NPs were subsequently evaluated for their ability to sustain the release of TMZ for cancer cell therapy. The *in vitro* release profile of TMZ from PLGA NPs was evaluated at 37 °C in PBS (pH 7.4, 0.01 M) to mimic the physiological pH and salt concentrations. The attained release curve is presented in Figure 2.

[insert figure 2 here]

Figure 2 - *In vitro* release of TMZ from mAb-modified and non-modified PLGA NPs in PBS (pH 7.4, 0.01 M) at 37 °C. Free TMZ was used as control. Results are represented as mean ± SD (n=3).

The TMZ release can be justified by two possible mechanisms. In aqueous medium, the esters bonds of PLGA are hydrolyzed causing the erosion of the polymeric matrix, allowing the release of the entrapped TMZ molecules. TMZ is also released by diffusion through the polymeric matrix. Thus, the release rate of a drug will depend upon different factors such as drug physicochemical properties and geometry of drug-loaded PLGA NPs (size and shape) (Cohen et al., 1991).

The dialysis method was used to compare the release of encapsulated TMZ with free TMZ. TMZ molecules were released in a biphasic release pattern, composed of an initial rapid released followed by a slower and controlled release, characteristic of PLGA NPs. As Figure 2 shows, 36± 6% of the total TMZ was released at the first 24 h for mAb-modified PLGA NPs and 43 ± 1% for non-modified NPs, respectively. When in aqueous medium, the surface-adsorbed TMZ molecules are rapidly released from the NPs, explaining the verified burst release. The TMZ entrapped in NPs polymeric matrix exhibited a slower and controlled release for several days. The conjugation of mAbs on the surface of the PLGA NPs affected the TMZ release from the PLGA NPs, since it was observed a higher release from non-modified PLGA in comparison to mAb-PLGA NPs. Non-modified PLGA NPs exhibited a total release of TMZ (98 ± 2%) after 9 days, while mAb-modified NPs only released about 78 ± 2% of entrapped TMZ at day 20 (Figure 2). These results may be explained by the mAb molecules linked to the surface of the NPs that may obstruct water permeation, hindering the diffusion of the drug molecules as previously reported (Loureiro, et al., 2016).

3.3 In vitro cellular uptake of mAb-PLGA NPs

Coumarin-6 was used as fluorescence marker to visualize the cellular uptake of the prepared PLGA NPs by confocal microscopy, in malignant glioma cell lines – U87 and U251 - and in immortalized human astrocytes - NHA. This fluorescent dye is distributed in the NPs matrix, allowing their visualization inside the cellular compartments since during the time of the experiment only about 13% of the dye is released from the NPs (data not shown). The cell nuclei were stained with DAPI and the acidic compartments, as endosomes and lysosomes, with LysoTracker Deep Red. The confocal images of non-treated and mAb-C6-PLGA NPs treated cells are presented in Figure 3.

[insert figure 3 here]

Figure 3 - LSCM images of cells treated with mAb-C6-PLGA NPs. U251 cells: (A) control cells (without NPs treatment); cells after (B) 2 h and (C) 72 h of incubation with mAb-C6–PLGA NPs. U87 cells: (D) control cells (without NPs treatment); cells after (E) 2 h and (F) 72 h of incubation with mAb-C6–PLGA NPs. NHA cells: (G) control cells (without NPs treatment); cells after (H) 2 h and (I) 72 h of incubation with mAb-C6–PLGA NPs. The nuclei are marked in blue, the acidic compartments (late endosomes/lysosomes) in red and the C6-NPs in green. The colocalization of PLGA NPs within the late endosomes/lysosomes is represented by the yellow/orange color. Scale bar: 25 μm.

After 2 h incubation, the NPs were uptaken by all the treated cells. The colocalization of C6-NPs and lysotracker-stained lysosomes is represented by the yellow/orange dots, due to the combined fluorescence of green and red emission, respectively (Dunn et al., 2011), suggesting that the NPs are internalized by an endocytic mechanism (Figures 3B, 3E and 3H). Also, as shown in Figures 3C, 3F, 3I, after 72 h incubation it is possible to visualize the NPs in the cytoplasm, suggesting that the NPs can escape the endo-lysosomal compartments. The images

after 72 h incubation also show a decrease in the number of cells, due to the antiproliferative activity of the TMZ entrapped in the NPs. All the attained results suggest that these mAb-PLGA NPs, being internalized by endocytosis mechanism, are able to efficiently perform as cytoplasmic drug delivery vehicles, avoiding multidrug resistance mechanisms such as TMZ efflux from target cells.

3.4 Selective cellular uptake of mAb-PLGA NPs

C-6 was also used to quantify the cellular internalization of the PLGA NPs in U251, U87 and NHA cell lines. The cellular internalization of mAb-modified and non-modified PLGA NPs was measured after 0.5 and 2 h of incubation, respectively. The results are expressed as the fluorescent intensity of C6 in Figure 4.

[insert figure 4 here]

Figure 4 - Quantification of cellular uptake of mAb-modified and non-modified PLGA NPs after 0.5 and 2 h incubation, in three human cell lines: (A) U251 cell line, (B) U87 cell line and (C) NHA cell line. (D) Quantification of cellular uptake of mAb-modified and non-modified PLGA NPs with TfR blocking with increase concentrations of transferrin. The cellular internalization of PLGA NPs is represented as the fluorescent intensity of coumarin-6. Data represented as mean ± SD (n=3).

As the attained results show, the NPs were efficiently internalized by the cells, showing that the longer the incubation time, the higher the cellular uptake ($p<0.05$). Also, modification of the PLGA NPs surface with mAb for TfR significantly increased the cellular internalization in all the studied cell lines for 2 h incubation period ($p<0.05$). Cellular uptake of mAb-NPs by

U251 cells after 0.5 h and 2 h of incubation was found to be 1.89 and 1.37-fold higher than unmodified NPs, respectively. For U87 cells, the cellular uptake for mAb-NPs was 1.70 and 1.41-fold higher in 0.5 and 2 h incubation, respectively. Also, in NHA cells the cellular internalization of NPs increased with mAb modification, exhibiting uptake rates 1.67 and 1.21-fold higher in 0.5 h and 2 h incubation, respectively.

Tf competitive binding assay was used to investigate how TfR impacts the mAb-PLGA NPs cell internalization. Cells were pretreated with increasing doses of Tf to block the TfR before the incubation with NPs. As Figure 4D shows, cellular internalization of mAb-NPs significantly decreased with blockage of TfR in a dose-depend manner, in all the studied cell lines ($p<0.05$). It was observed a decrease between 43 and 48% in the internalization of mAb-NPs when using 10 mg/mL of Tf. Blockage of the TfR did not affect the internalization of the non-modified NPs. The same blocking experiment, using folate instead of TfR, was used as control, showing that pretreatment with folate did not exhibit any effects on the mAb-NPs uptake (data not shown). Confocal studies displaying NPs in late endosomes/lysosomes suggest that these NPs are selectively uptaken by TfR-mediated endocytosis.

3.5 Effects of NPs on brain-like endothelial cells (HBLECs) monolayer integrity

An *in vitro* model for the human BBB was used to assess whether the prepared NPs exhibited harmful effects on on the monolayer integrity of the BBB-like cells. The *in vitro model* is composed of endothelial cells derived from hematopoietic umbilical cord blood and brain pericytes and is named Brain-like endothelial cells (HBLECs). Thus, the HBLECs model retains most of the features of the human BBB such as expression of membrane receptors, high transepithelial/transendothelial electrical resistance and low permeability to generally used permeability-markers, like sucrose molecules (Cecchelli, et al., 2014). The obtained results are shown in Figure 5.

[insert figure 5 here]

Figure 5 – *In vitro* BBB model monolayer integrity assays. The effects of the studied NPs on the cellular monolayer integrity was assessed in terms of permeability of sucrose (a marker for BBB paracellular integrity). NPs were tested at two different concentrations, 40 and 80 µM, respectively. The results are expressed as mean and standard deviation (n=3).

Using the permeability of sucrose as a marker for integrity of the BBB model (Deli et al., 2005; Cecchelli et al., 1999), the results showed that non-modified and OX26 mAb-modified NPs, at a concentration of 40 µM, exhibited no harmful effects on the integrity of the BBB cellular model. Thus, at 80 µM, HBLEC monolayer integrity to sucrose has been increased, suggesting that in future work is necessary to use concentrations not higher than 40 µM to avoid any dramatic changes in BBB integrity (Figure 5). These results suggest that its possible to use these NPs for TMZ delivery to GBM cells without causing harmful effects on the BBB integrity.

3.6 Cell growth inhibition by TMZ-loaded NPs

The *in vitro* antiproliferative activity of the TMZ-loaded NPs in comparison with free TMZ, was evaluated in two human glioma cell lines, U251 and U87. NHA cell line was also used as control. Bare PLGA NPS, at a concentration of 50 $\mu g.mL^{-1}$, showed no significant antiproliferative effect on the studied cell lines (data not shown), proving that PLGA NPs are biocompatible.

The effect of TMZ at different concentrations, ranging from 0.1 to 800 µM, was tested and the results are presented in Figure 6 and Table 2. It was verified that both free and encapsulated TMZ induce a concentration-related decrease in cell growth in all the studied cell lines (Figure 6). TMZ entrapped in NPs inhibited the cellular growth more efficiently than free TMZ in all the cell lines, resulting in significantly lower GI_{50} values for both mAb-modified and unmodified NPs as shown in Table 3 ($p<0.05$). Also, these results demonstrated that the TMZ

deleterious effect is significantly lower in the non-tumor cell line ($p<0.05$). Although the NPs also potentiate the TMZ effect on NHA cells, these cells show more resistance to TMZ toxicity than U251 and U87 glioma cells, whether encapsulated or not.

[insert figure 6 here]

Figure 6 - Cytotoxic effects of TMZ, free or entrapped in mAb-modified and unmodified PLGA NPs, after 72 h treatment on three human cell lines by SRB assay. (A) U251 cell line, (B) U87 cell line and (C) NHA cell line. Growth inhibition is presented as percent [(%)=((T-T0)/(C-T0)) x 100]. Data represented as mean ± SD (n=3).

U251 cell line exhibited the highest sensitivity to TMZ. TMZ-mAb-PLGA NPs are significantly more effective than free TMZ, exhibiting a GI_{50} value (15±2 μM) about 4-fold lower than for free TMZ (61±1 μM) in U251 cell line ($p<0.05$).

[insert table 2 here]

The entrapment of a TMZ in PLGA NPs improves its intracellular delivery, since free TMZ is expected to be transported into cells by a passive diffusion mechanism, suffering a multi-drug resistance mechanism mediated by the membrane p-glycoprotein pump (Panyam and Labhasetwar, 2003; Vasir and Labhasetwar, 2007). Instead, NPs may be more efficiently transported by receptor-mediated endocytosis. Although internalization of mAb-modified NPs was enhanced, as shown by the *in vitro uptake* studies suggesting that these NPs are transported into the cells through transferrin receptor-mediated endocytosis, the cytotoxic effect of TMZ was decreased with the mAb-modification of the NPs' surface (Figure 6 and Table 2) ($p<0.05$). In fact, TMZ-PLGA NPs was the formulation that exhibited higher toxicity, with for example a GI_{50} value of 4±1 for the U251 cell line. The decrease in the cytotoxic effect when using mAb-NPs, comparatively with non-modified NPs, is probably due to the lower release rate of the drug, already mentioned in section 3.2. Still, both formulations – mAb-modified and

unmodified NPs – are able to improve the *in vitro* therapeutic efficacy of TMZ, indicating that the use of the mAbs should present advantages in *in vivo* conditions, allowing an increased transport of the drug across the BBB.

4. Conclusion

The use of PLGA NPs were proposed to improve the brain delivery of TMZ. For that, NPs functionalized with a OX26 mAb for TfR were developed for GBM tumor cells targeting, since these cells are known for overexpressing this receptor. Stable NPs were prepared with suitable physicochemical features for brain delivery, such as mean dimensions smaller than 200 nm and net negative charge. The developed nanocarriers exhibited a good TMZ encapsulation efficiency and were able to maintain a controlled and sustained release of the drug for up to 20 days. Cytotoxicity studies showed that the entrapment of the drug in PLGA NPs significantly improves the antiproliferative activity of TMZ. The use of the monoclonal antibody for the transferrin receptor proved to be advantageous in enhancing the cellular internalization of the NPs in the target cells, suggesting that these are selectively uptaken by a transferrin receptor-mediated endocytosis mechanism. Although the modification of the NPs with OX26 mAb apparently decreased the cytotoxic potential in GBM cells, the use of this antibody could enhance the permeability across the BBB during *in vivo* conditions, since BBB cells are also known for overexpressing this receptor. As such, we propose that NPs functionalized with a OX26 mAb for TfR could be efficiently used for dual-targeting of both BBB and GBM cells. Future *in vivo* studies will allow evaluating the potential of the developed nanocarriers for the treatment of GBM.

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6. Declaration of interests

None

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