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1 **Receptor-mediated PLGA nanoparticles for Glioblastoma Multiforme treatment**

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21

22 **Abstract**

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

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

36

37 **Keywords**

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

40

41 **1. Introduction**

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

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

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

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

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

83 Some PLGA-based nanosystems for the delivery of TMZ were already proposed
84 (Ramalho et al., 2018). Jain and colleagues (2014) developed PLGA NPs able to enhance
85 chemotherapeutic activity of TMZ, although the prepared NPs were not modified to increase the
86 specificity of NPs. (Jain et al., 2014). Two years later, Ananta and colleagues (2016) developed
87 PLGA NPs using three different experimental procedures. However, the prepared NPs were not
88 able to maintain a controlled and sustained release of TMZ (Ananta et al., 2016). Xu and co-
89 workers (2016) intended the simultaneous delivery of TMZ with another chemotherapeutic
90 agent. The group prepared PLGA NPs for the entrapment of both TMZ and paclitaxel. The
91 combination of both drugs showed a synergic effect, increasing their cytotoxicity (Xu et al.,
92 2016). Some authors used active targeting strategies to increase the NPs specificity to tumor
93 cells. In fact, Lee and collaborators (2016) modified PLGA NPs with folate molecules aiming to
94 increase the NPs internalization in target cells. Although the authors tested different methods for
95 the preparation of NPs, the prepared nanosystems did not exhibit good encapsulation efficiency
96 values (Lee and Ooi, 2016). Jain and co-workers (2011) developed PLGA NPs functionalized
97 with transferrin molecules to enhance the transport across the BBB. The obtained NPs proved to
98 enhance the antiproliferative effect of TMZ and increase the accumulation of TMZ in the brain
99 tissue (Jain et al., 2011).

100 Active targeting strategies are increasingly used to enhance NPs uptake in the target cell
101 or the transport across biological barriers as BBB. For that, the NPs' surface can be modified
102 using receptors usually overexpressed in target cells or biological barriers (Pillai, 2014;
103 Lockman et al., 2002). Instead of using the receptor ligands, immunocarriers can be also
104 developed using monoclonal antibodies (mAb) for the target of chosen receptors. Since mAbs
105 exhibit high specificity for the target receptors, it is possible to achieve high levels of targeting
106 (Loureiro et al., 2014). In this work, transferrin receptor (TfR) is the targeted receptor, using the
107 mAb type OX26 that has proven to efficiently bind cells that overexpress TfRs (Loureiro et al.,
108 2016; Loureiro et al., 2017), as GBM cells (Calzolari et al., 2010) and several other tumor cells
109 types (Frasco et al., 2015).

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

114

115 **2. Materials & Methods**

116 **2.1 Materials**

117 PLGA Resomer® RG503H (50:50; MW 24,000 – 38,000), poly(vinyl alcohol) (PVA),
118 Coumarin-6 (C6), phosphate buffered saline (PBS), 1-Ethyl-3-(3-dimethylaminopropyl)
119 carbodiimide (EDC), dichloromethane, ethyl ether, methanol, ethylenediaminetetraacetic acid
120 (EDTA), 2-iminothiolane hydrochloride (Traut's reagent), bovine serum albumin (BSA), citric
121 acid, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium
122 hydroxide, acetic acid, sulforhodamine B (SRB), trypan blue, holo-Transferrin human (purity \geq
123 98%), rhodamine B and Triton XTM-100 were obtained from Sigma–Aldrich (St. Louis, MO,
124 USA). Uranyl acetate was bought from Electron Microscopy Sciences (Hatfield, UK).
125 Polyethylene glycol functionalized with maleimide (mPEG-Mal, Mw 2000) was acquired from
126 Nanocs Inc. (Boston, MA, USA). The OX26 mAb was purchased from Novus Biologicals
127 (Oxfordshire, UK). Transferrin receptor was acquired from Abcam (Cambridge, UK). The
128 secondary antibody Goat anti-Mouse IgG (H+L) Cross Adsorbed Secondary Antibody, HRP
129 conjugate was bought at Thermo Scientific-Pierce Antibodies (Waltham, MA, USA). High-
130 glucose Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), SlowFade
131 Gold Antifade Mountant with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) and
132 Lysotracker DeepRed were acquired from Invitrogen Co. (Scotland, UK). Trichloroacetic acid
133 (TCA) and Tris buffer were purchased from Merck (Darmstadt, Germany). Ringer HEPES (RH)
134 buffer was prepared using 150 mM NaCl, 6 mM NaHCO₃, 5.2 mM KCl, 5 mM HEPES, 2.8
135 mM glucose, 2.2 mM CaCl₂ and 0.2 mM MgCl₂·6H₂O at pH 7.4). All reagents used for RH
136 buffer composition were acquired from Sigma–Aldrich (St. Louis, MO, USA).

137

138 **2.2 Cell lines**

139 Two human glioblastoma cell lines, U251 and U87, and an immortalized human
140 astrocyte cell line (NHA) were used in this work. All the used lines were cultured in DMEM
141 supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37 °C in
142 a humidified 5% CO₂ incubator. At 80% of confluence, the cells were trypsinized and
143 subcultured.. Both U251 and U87 cell lines were chosen since they show significant
144 similarities with the genetic, immunohistochemical and histological and characteristics
145 of human GBM tumor (Jacobs et al., 2011).

146

147

148 **2.3 Preparation of TMZ-loaded PLGA NPs**

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

159

160 **2.4 Synthesis of PEGylated PLGA**

161 2 mg of PLGA and 0.04 mg of EDC were dissolved in a known volume of
162 dichloromethane. A mixture of ethyl ether:methanol (70:30) was added to the previously
163 prepared solution to remove the excess of EDC. Then, 0.2 mg of maleimide-PEG-NH₂ were

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

167

168 **2.5 Preparation of TMZ-loaded mAb-PLGA NPs**

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

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

182

183 **2.6 PLGA NPs physicochemical characterization**

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

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

193 The morphological analysis of the NPs was obtained by Transmission Electron
194 Microscopy (TEM) (TEM JEOL JEM 1400, Japan). The samples were visualized on copper
195 grids (Formvar/Carbon-400 mesh Copper, Agar Scientific, UK) and negatively stained with a
196 2% (v/v) uranyl acetate solution.

197

198 **2.7 ELISA studies**

199 The affinity of TfR to OX26 mAb-modified PLGA NPs was analyzed by the enzyme-
200 linked immunosorbent assay (ELISA). The surface of a 96-well plate (Nunc MaxiSorp®) was
201 treated with TfR and incubated overnight at 4 °C. The plate was then blocked with BSA (bovine
202 serum albumin) and incubated for 2 h at RT, followed by the addition of NPs. After incubation
203 and subsequent washing, the peroxidase-conjugated secondary antibody (goat anti-mouse) was
204 added and incubated for 45 min at RT. The samples were revealed using a solution of citric
205 acid, ABTS and H₂O₂. The absorbance at the wavelength of 405 nm was measured using a
206 fluorescence microplate reader (HT Microplate Spectrophotometer, BioTek). Non-modified NPs
207 were used as a negative control.

208

209 **2.8 TMZ encapsulation efficiency and release from PLGA NPs**

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

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

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

223

224

225 **2.9 Cellular imaging studies**

226 The NPs *in vitro* uptake in the human cells lines (U251, U87 and NHA) was assessed
227 using laser scanning confocal microscopy (LSCM) (Leica TCS SP5 II, Leica Microsystems,
228 Germany). For that purpose, C6-loaded NPs were used. This fluorescent marker is distributed in
229 the NPs matrix, allowing the visualization of the NPs inside the cellular compartments (Holzer
230 et al., 2009). 1000 cells/well were seeded in 12-well plates (Ibidi, Germany) and allowed to
231 attach for 24 h (37 °C, in a humidified 5% CO₂ incubator). Cells were then treated with mAb-
232 modified and unmodified C6-loaded NPs for 2 and 72 h. After the incubation period and
233 subsequent wash with PBS, the cells were fixed with 4% (w/v) paraformaldehyde. The acidic
234 cell compartments (as endosomes/lysosomes) were stained with LysoTracker® Red and cell
235 nuclei were marked with DAPI. At least six images from different areas of each cell lines were
236 acquired in emission mode. Untreated cells were also used control.

237

238 **2.10 Quantification of *in vitro* cellular uptake of mAb-PLGA NPs**

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

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

248

249 **2.11 Transferrin competitive binding assay**

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

261

262 **2.12 Effects of NPs on human brain-like endothelial cells (HBLECs) monolayer**

263 **integrity**

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

273 CA, USA)-coated filters (Costar Transwell inserts, Corning Inc., Corning, NY, USA, pore size
274 3 μm , 8×10^4 cells/cm²) (Cecchelli et al., 2014). These inserts with cells were maintained with a
275 dry bottom for one week (500 μL of medium in the upper compartment, changed every other
276 day) to avoid the cells crossing the membrane and forming a non-physiological second layer on
277 the lower face of the insert. Next, the inserts were transferred onto pericytes (50,000 cells per
278 well, seeded in 12-well plates two days before the transfer). The resulting co-culture was
279 cultured with endothelial cell medium supplemented with 5% heat inactivated fetal calf serum
280 (GIBCO, Life Technology SAS, Saint Aubin, France) and 50 $\mu\text{g}/\text{mL}$ gentamicin. The medium
281 was changed every two days. These culture conditions were maintained for 5 days and enabled
282 the CD34⁺-derived endothelial cells to acquire a true BBB phenotype (i.e. HBLECs). Under
283 these conditions, the model was stable for 30 days and was then ready for experiments
284 (Cecchelli, et al., 2014; Kuntz et al., 2015).

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

293

294 **2.13 *In vitro* cytotoxicity studies**

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

298 U251, U87 and NHA cells were seeded at a density of 1000 cells/well, in 96-well assay
299 plates and incubated for 24 h (37 °C, in a humidified 5% CO₂ incubator) to allow complete cell
300 adhesion. Then, TMZ, TMZ-PLGA NPs and mAb-TMZ-PLGA NPs, diluted in DMEM medium

301 at ten final concentrations of TMZ ranging from 0.1 to 800 μ M, were added to the cells. After
302 an incubation period of 72 h, the cells were fixed with 10% (w/v) TCA for 1 h at 4 °C, and
303 stained with 0.4% (w/v) SRB dye for 30 min. After repeatedly washing the cells with 1% (v/v)
304 acetic acid to remove unbound dye, the cells air-dried and the protein-bound stain was
305 solubilized with 10 mM Tris solution for UV-VIS absorbance quantification at 560 nm using
306 the (BioTek Synergy HT Microplate Reader, BioTek, UK). The GI₅₀ value - the concentration
307 inhibiting the net cell growth by 50% - for TMZ was calculated from the dose–response curve.

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

311

312 **2.14 Statistical analysis**

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

316

317

318 **3. Results and discussion**

319 **3.1 PLGA NPs physicochemical properties**

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

326 **[insert table 1 here]**

327

328 The prepared unloaded NPs exhibited a mean diameter of 176 ± 3 nm, and a zeta
329 potential value of -29 ± 3 mV, negative as expected due to the negative charge of the carboxylic
330 groups of PLGA polymer (Table 1). The TMZ molecules accommodation in the polymeric
331 matrix caused a significant ($p < 0.05$) increase in size for the TMZ-PLGA NPs (181 ± 2 nm).
332 Moreover, NPs mean size was also significantly increased with OX26 mAb modification ($194 \pm$
333 1 nm) ($p < 0.05$), indicating an efficient conjugation since the diameter of the globular antibody
334 is approximately 15 nm (Dammer et al., 1996). The prepared nanocarriers exhibited a small size
335 distribution ($PDI \leq 0.1$). Loading of the NPs with TMZ did not significantly affect their zeta
336 potential, however a small decrease was observed due to the adsorption of TMZ molecules on
337 PLGA NPs surface, exerting a masking effect on the superficial net charge (Musumeci et al.,
338 2006). The modification of the NPs' surface with mAb also does not significantly alter their
339 zeta potential, due to the negative nature of this immunoglobulin ($p > 0.05$). The colloidal
340 stability of the prepared NPs is a result of electric charge of the carboxylic groups at the NP
341 surfaces, and the PVA layer on the NP's surface causing steric repulsions. In fact, Figure 1
342 shows a stabilizer layer surrounding PLGA NPs. TEM images (Figure 1) also show uniform
343 PLGA NPs with a spherical form. The mean size of the NPs revealed by TEM are in agreement
344 with those determined by DLS analysis.

345 **[insert figure 1 here]**

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

347

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

355 BBB. Since neutral and anionic NPs are more easily transported across the BBB and are not
356 associated to BBB toxicity (Wu et al., 2015), most of the NP formulations described in the
357 literature for brain delivery have moderate to high (between -1 to -45 mV) negative zeta
358 potentials, as the one prepared in this work (Saraiva et al., 2016). It has also been proven that
359 NPs with dimensions up to 200 nm are more easily accumulated in brain tissue (Veszeka et al.,
360 2015) since tumor microenvironment exhibits vascular fenestrations in the range of 40–200 nm
361 (Wu, et al., 2015). Also NPs with dimensions up to 200 nm are more efficiently taken up by
362 receptor-mediated endocytosis, as it was intended in this work with the use of TfR (Masserini,
363 2013).

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

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

371

372

373 **3.2 TMZ encapsulation efficiency and release from PLGA NPs**

374 PLGA NPs loaded with TMZ showed encapsulation efficiencies ranging from $48 \pm 10\%$
375 for mAb-PLGA NPs to $44 \pm 3\%$ for non-modified NPs, respectively. Drug loading varied from
376 $10 \pm 2\%$ for mAb-PLGA NPs to $9 \pm 1\%$ of the polymer weight for non-modified NPs. These
377 NPs were subsequently evaluated for their ability to sustain the release of TMZ for cancer cell
378 therapy. The *in vitro* release profile of TMZ from PLGA NPs was evaluated at 37 °C in PBS
379 (pH 7.4, 0.01 M) to mimic the physiological pH and salt concentrations. The attained release
380 curve is presented in Figure 2.

381

382 [insert figure 2 here]

383

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

387

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

394 The dialysis method was used to compare the release of encapsulated TMZ with free
395 TMZ. TMZ molecules were released in a biphasic release pattern, composed of an initial rapid
396 released followed by a slower and controlled release, characteristic of PLGA NPs. As Figure 2
397 shows, $36 \pm 6\%$ of the total TMZ was released at the first 24 h for mAb-modified PLGA NPs
398 and $43 \pm 1\%$ for non-modified NPs, respectively. When in aqueous medium, the surface-
399 adsorbed TMZ molecules are rapidly released from the NPs, explaining the verified burst
400 release. The TMZ entrapped in NPs polymeric matrix exhibited a slower and controlled release
401 for several days. The conjugation of mAbs on the surface of the PLGA NPs affected the TMZ
402 release from the PLGA NPs, since it was observed a higher release from non-modified PLGA in
403 comparison to mAb-PLGA NPs. Non-modified PLGA NPs exhibited a total release of TMZ (98
404 $\pm 2\%$) after 9 days, while mAb-modified NPs only released about $78 \pm 2\%$ of entrapped TMZ at
405 day 20 (Figure 2). These results may be explained by the mAb molecules linked to the surface
406 of the NPs that may obstruct water permeation, hindering the diffusion of the drug molecules as
407 previously reported (Loureiro, et al., 2016).

408

409

410 **3.3 *In vitro* cellular uptake of mAb-PLGA NPs**

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

419 **[insert figure 3 here]**

420

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

429

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

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

441

442

443 **3.4 Selective cellular uptake of mAb-PLGA NPs**

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

448

449 **[insert figure 4 here]**

450

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

457

458

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

463 U251 cells after 0.5 h and 2 h of incubation was found to be 1.89 and 1.37-fold higher than
464 unmodified NPs, respectively. For U87 cells, the cellular uptake for mAb-NPs was 1.70 and
465 1.41-fold higher in 0.5 and 2 h incubation, respectively. Also, in NHA cells the cellular
466 internalization of NPs increased with mAb modification, exhibiting uptake rates 1.67 and 1.21-
467 fold higher in 0.5 h and 2 h incubation, respectively.

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

478

479 **3.5 Effects of NPs on brain-like endothelial cells (HBLECs) monolayer integrity**

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

488

489 **[insert figure 5 here]**

490

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

495

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

504

505

506 **3.6 Cell growth inhibition by TMZ-loaded NPs**

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

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

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

521 **[insert figure 6 here]**

522

523 **Figure 6** - Cytotoxic effects of TMZ, free or entrapped in mAb-modified and unmodified
524 PLGA NPs, after 72 h treatment on three human cell lines by SRB assay. (A) U251 cell line, (B)
525 U87 cell line and (C) NHA cell line. Growth inhibition is presented as percent $[(\%) = ((T - T_0) / (C -$
526 $T_0)) \times 100]$. Data represented as mean \pm SD (n=3).

527

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

531 **[insert table 2 here]**

532

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

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

548

549 **4. Conclusion**

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

567

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579

580 **6. Declaration of interests**

581 None

582

583 **7. References**

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