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Post-resection treatment of glioblastoma with an injectable nanomedicine-loaded photopolymerizable hydrogel induces long-term survival

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor. Despite available therapeutic options, the prognosis for patients with GBM remains very poor. We hypothesized that the intra-operative injection of a photopolymerizable hydrogel into the tumor resection cavity could sustain the release of the anti-cancer drug paclitaxel (PTX) encapsulated in poly (lactic-co-glycolic acid) (PLGA) nanoparticles and prevent GBM recurrence. The tumor was resected 13 days after implantation and a pre-gel solution composed of polyethylene glycol dimethacrylate (PEG-DMA) polymer, a photoinitiator and PTX-loaded PLGA nanoparticles (PTX PLGA-NPs) was injected into the tumor resection cavity. A solid gel filling the whole cavity was formed immediately by photopolymerization using a 400 nm light. PTX *in vitro* release study showed a burst release (11%) in the first 8 h and a sustained release of 29% over a week. *In vitro*, U87 MG cells were sensitive to PTX PLGA-NPs with IC_{50} level of approximately 0.010 μg/mL. The hydrogel was well-tolerated when implanted in the brain of healthy mice for 2 and 4 months. Administration of PTX PLGA-NPs-loaded hydrogel into the resection cavity of GBM orthotopic model lead to more than 50% long-term survival mice (150 days) compared to the control groups (mean survival time 52 days). This significant delay of recurrence is very promising for the post-resection treatment of GBM.

1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive and lethal type of brain tumor. GBM exhibits a high proliferation rate, high tumor cell infiltration into adjacent brain tissue, resistance to conventional treatment and the ability to quickly develop recurrences, which are responsible for its poor prognosis (Milano et al., 2010). The current standard therapy includes surgical resection, balanced between the need to remove a maximum of the tumor and to limit any resulting impairments, followed by radiotherapy and oral alkylating chemotherapy with temozolomide (TMZ;Temodar®) (Davis, 2016; Wilson et al., 2014). Massive and thorough surgical resection of GBM is frequently not achievable because GBM frequently infiltrates the brain parenchyma beyond the main mass of the tumor, or the brainstem, the diencephalon and neocortical areas that control speech, motor function and sensation (Davis, 2016). If achievable, the macroscopically complete resection of the primary tumor is not curative since infiltrating tumor cells invariably remain in the surrounding brain parenchyma, leading to later tumor recurrences. As a consequence, approximately 70% of GBM patients experience local recurrence within one year of diagnosis (Stupp et al., 2005); the 2-year survival rate is only 27%, and the long-term survival rate is less than 5% (Ostrom et al., 2014; Stupp et al., 2009).

The blood brain barrier (BBB), which acts very effectively to protect the brain from harmful molecules, limits the entry of most systemically administered drugs to the brain (Bastiancich et al., 2016a). To overcome this issue, one of the promising strategies to limit or delay GBM recurrences is the direct administration into tumor resection cavity of a local drug delivery system, ensuring sustained release of cytotoxic drugs. Indeed, a local drug delivery system will ensure (*i*) a direct

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contact of the drug with tumor cells, (*ii*) a sustained drug release, (*iii*) a limited drug degradation and (*iv*) a reduction of off-target effects in healthy tissues (Bastiancich et al., 2016a). The rationale for this approach is that biocompatible materials can be introduced directly into the brain inside the surgical tumor resection cavity. Indeed, after surgical resection, patients have to wait a period of time before starting the radio/chemotherapy regimen because of the duration of the post-surgical wound healing process; thus, residual infiltrative cells will keep proliferating during this time (Patel et al., 2015). Consequently, in this time gap, a local drug delivery system could be administered before starting the conventional radio/chemotherapy regimen. In this approach, cytotoxic agents diffuse into the brain parenchyma to kill residual tumor cells around the resection cavity borders, which are responsible for recurrences. The only system applying this strategy approved by FDA is Gliadel®, a polymeric wafer loaded with carmustine. However, Gliadel® showed minimal and controversial advantages. Although a significant survival benefit for patients who received Gliadel® treatment was reported, the short sustained intracerebral release of most drug (1 week) and the local sides effects are involved in the major limitations of Gliadel® wafer (Bota et al., 2007; Engelhard, 2000; Perry et al., 2007).

Recently, we developed an innovative hydrogel uniquely comprised of photopolymerizable polyethylene glycol dimethacrylate (PEG-DMA) delivering TMZ for the local treatment of GBM (Fourniols et al., 2015). This injectable hydrogel presented mechanical properties compatible with brain implantation. In vivo, this system was well tolerated over one week in a healthy mouse brain and reduced tumor growth in a subcutaneous human U87 MG GBM model. However, O6-methylguanine-DNA methyltransferase (MGMT) is involved in the drug resistance mechanism of GBM, which limits the efficacy of TMZ (Kitange et al., 2009). To bypass this issue, other anti-cancer drugs were investigated for the treatment of GBM, such as lauroyl-gemcitabine ($GemC_{12}$) and doxorubicin (Bastiancich et al., 2016b; Fourniols et al., 2015; Qi et al., 2014; Vinchon-Petit et al., 2010). Among them, paclitaxel (PTX) could be an alternative as it is an efficient anti-tumor agent that inhibits cell proliferation and induces apoptosis (Gupta et al., 2003). Also, it kills GBM cells via a MGMT-independent mechanism (Shen et al., 2017; Xin et al., 2010; Zhan et al., 2010). However, the anti-glioma effect of PTX is very limited when administered systemically as it cannot cross the BBB and, in consequence, do not accumulate in the CNS at therapeutic doses (Chang et al., 2001). We hypothesized that PTX would be a promising drug for the local delivery before radio- and chemotherapy.

Encapsulation of a bioactive molecule in nanoparticles (NPs) is one of the strategies widely applied to solubilize lipophilic drugs, to protect them from degradation and to obtain a sustained delivery. Poly (lactic-co-glycolic acid) (PLGA) is one the most successful polymers developed to formulate polymeric NPs for the delivery of anti-cancer drugs. PLGA has attracted considerable attention due to the following favorable attributes: (*i*) biodegradability and biocompatibility, (*ii*) FDA/EMA approval for parenteral administration, (*iii*) possibility of sustained drug release and (*iv*) efficient encapsulation of poorly soluble drugs (Danhier et al., 2012). Additionally, PTX-loaded PLGA-NPs have been previously developed with good encapsulation and loading efficiencies and significant regrowth delays of various *in vivo* tumor models (Amoozgar et al., 2014; Cui et al., 2013; Danhier et al., 2009b; Luo et al., 2016).

The aim of the present study was to develop an injectable photopolymerizable hydrogel capable of sustaining the release of PTX over a period to fill the gap between surgical resection and the conventional radio/chemotherapy regimen (Fig. 1). The hydrogel consists of a PEG-DMA polymer, the Lucirin-TPO® photoinitiator and PTX PLGA-NPs. PLGA NPs are incorporated to slow down the PTX release from the hydrogel. The pre-gel solution is injected into the resection cavity right after surgery and then photopolymerized under blue light (400 nm) irradiation. The PTX release as well as the cytotoxic effect of PTX PLGA-NPs on U87 MG cells were studied, the mid- and long-term tolerability in healthy mouse brain and anti-tumor efficacy after local injection in the resection cavity in an orthotopic U87 MG model were investigated *in vivo*.

2. Materials and methods

2.1. Hydrogel formulation

2.1.1. Formulation of PTX PLGA-NPs

PTX PLGA-NPs were formulated using a modified emulsion-evaporation method as previously reported (Danhier et al., 2009a). Briefly, PLGA (Resomer® RG 502, Mw = 7000–17,000 g/mol, 56 mg, Sigma-Aldrich, USA), PLGA-PEG (M_W = 4600–10040 g/mol, 12 mg), PCL-PEG (M_W = 5000–13,100 g/mol, 12 mg) (synthesized as previously described (Schleich et al., 2013)) and PTX (6 mg, Chemieliva, China) were dissolved in dichloromethane. This organic solution was then added to an aqueous solution containing 3% (w/v) PVA (M_W = 30–70 kDa, Sigma-Aldrich, USA), emulsified using a vortex for 2 min, and then sonicated (4 × 30 s, 50 W). The mixture was rotary evaporated for 1 h to remove the organic solvent. To remove the non-encapsulated drug, the suspension was filtered (1.2 µm), washed using centrifugation (15,000 rpm, 45 min) and suspended in water.

2.1.2. Physico-chemical characterization of PTX PLGA-NPs

The average particle size and polydispersity index of the PTX PLGA-NPs were measured by dynamic light scattering and zeta (ς) potential measurements performed by laser Doppler velocimetry using a Zetasizer NanoZS (Malvern Instruments, UK) (n = 3).

2.1.3. Quantitative determinations of the PTX in PTX PLGA-NPs

PTX was quantified by high-performance liquid chromatography (HPLC) using a Shimadzu Prominence system (Shimadzu, Japan). The separation was conducted using a BDS Hypersil C18 (Thermo Scientific, USA) (100 × 4.6 mm; particle size 3 µm) column with a mobile phase comprising acetonitrile (VWR Chemicals, France) and water at a ratio of 45:55 (v/v). The detection wavelength was set to 227 nm, and the flow rate was maintained at 1.2 mL/min. Under these conditions, the retention time of PTX was approximately 6.5 min. A calibration curve was obtained by diluting PTX in acetonitrile at concentrations between 1 and 100 µg/mL (correlation coefficient of R^2 = 0.9993). The limit of quantification was 0.84 µg/mL, and the limit of detection was 0.28 µg/mL. The total quantity of PTX loaded in the PTX PLGA-NPs was evaluated by the dissolution of an amount of nanoparticles in acetonitrile (dilution ratio 1:100) and quantification by HPLC. Encapsulation efficiency was calculated by final drug amount in nanoparticles divided by the total drug added, while the drug loading is calculated by final drug amount in nanoparticles divided by the initial amount of polymers as previously described (Danhier et al., 2009a).

2.1.4. Preparation of PTX PLGA-NPs-loaded hydrogel

PTX PLGA-NPs were diluted in water to reach 3.5 mg/mL of PTX and mixed with PEG-DMA (average Mw = 550 g/mol) (Sigma-Aldrich, USA) at a 75:25 v/v ratio. Next, 0.5% of Lucirin-TPO® (BASF) was added as the photoinitiator. Then, the pre-gel solution was irradiated at 750 mW/cm^2 for 15 s with a blue light (Lumencor, USA). The hydrogel formation was re-optimized with different concentrations of PEG-DMA and photoinitiator (Supplementary data, Fig. S1). Unloaded and blank PLGA-NPs-loaded PEG-DMA hydrogels were also prepared as controls by adding water or blank PLGA-NPs instead of the PTX PLGA-NPs in the pre-gel solution. The stability of PTX under blue light exposure as well as that of PTX in the PTX PLGA-NPs-loaded PEG-DMA hydrogel was assessed by HPLC by measuring the recovery of the total amount of PTX, the PTX peak integrity and retention time (Supplementary data, Fig. S2).

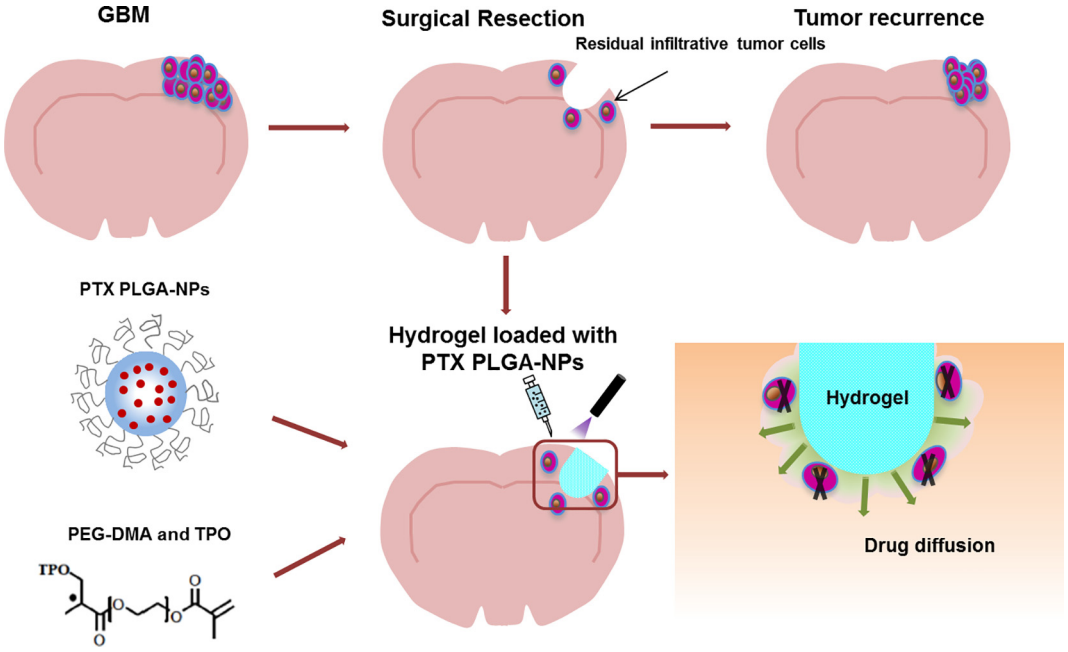

Fig. 1. Schematic representation of the main objective of the study.

Table 1
Physico-chemical characterization of blank PLGA-NPs and PTX PLGA-NPs (n = 3; mean ± SD).

	Size (nm)	Polydispersity index	ζ potential (mV)	Encapsulation efficiency (%)	Drug loading (%)
PLGA-NPs	192 ± 4	0.07 ± 0.02	−16.9 ± 2.7	–	–
PTX PLGA-NPs	190 ± 4	0.13 ± 0.03	−16.1 ± 2.3	53 ± 8	4 ± 0.6

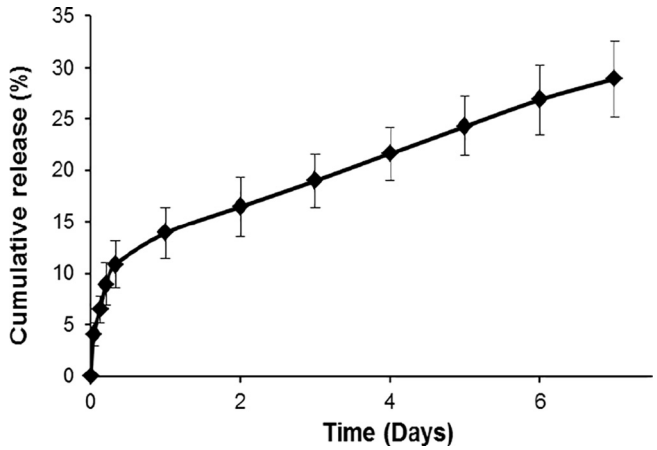

Fig. 2. *In vitro* cumulative release of PTX from PTX PLGA-NPs/PEG-DMA hydrogels. Release was performed at 37 °C in PBS (pH = 7.4). The amount of PTX released was quantified by HPLC (mean ± SD; n = 3).

2.2. PTX release from PTX PLGA-NPs/PEG-DMA hydrogel

The *in vitro* release of PTX from the PTX PLGA-NPs/PEG-DMA hydrogel was performed over one week. Two hundred microliters of gel were placed at the bottom of a 50 mL tube and covered with 5 mL of PBS (pH 7.35). The samples were incubated at 37 °C and, at fixed time intervals, all the release buffer was collected and replaced with 5 mL of fresh medium. Samples were frozen at −80 °C and then lyophilized. The release samples containing free PTX and PTX PLGA-NPs were diluted in acetonitrile to break the PLGA nanoparticle structure and solubilize PTX. The supernatant was analyzed by HPLC (see Section 2.1.3) (n = 3).

2.3. In vitro cytotoxicity studies of PTX PLGA-NPs

2.3.1. Cell cultures

U87 MG GBM cells (ATCC, USA) were cultivated in Eagle's Minimum Essential Medium (EMEM, ATCC, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and a 1% penicillin/streptomycin mixture (Gibco, USA). Cells were cultured in 75 cm^2 culture flasks (Corning® T-75, Sigma-Aldrich, USA) and incubated at 37 °C and 5% CO_2.

2.3.2. Cytotoxicity studies

The PTX PLGA-NPs cytotoxicity on U87 MG cells was assessed by the MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich, USA) assay (Bastiancich et al., 2016b). Briefly, 96-well plates were coated with 0.1 mg/mL poly (D) lysine (MP Biomedicals, USA) and cells were seeded at 5 × 10^3 cells/well. After 24 h, different concentrations of PTX PLGA-NPs (1 ng/ml–10 µg/ml) were added and incubated for 48 h. Cells without treatment and cells treated with Triton X-100 1% (Sigma-Aldrich, USA) served as controls (100% and 0% viability, respectively). After 48 h of incubation, cells were washed and incubated with MTT solution (0.5 mg/mL). Formazan crystals were then solubilized in DMSO (Merck, USA), and spectrophotometric readings were performed at 560 nm using a MultiSkan EX plate reader (Thermo Scientific, USA) (n = 6).

The PTX PLGA-NPs cytotoxicity on U87 MG cells was also evaluated by a clonogenic assay adapted from Franken et al. (2006). Briefly, 1 × 10^5 U87 MG cells/well were seeded in a 12-well plate and incubated for 24 h. The next day, different concentrations of PTX PLGA-NPs (0.0032–1 µg/mL) were added and incubated for 48 h with the cells. Then, U87 MG cells were detached and 1000 cells/well were seeded in six-well plates in EMEM complete media for 12 days. The medium was then discarded and the clones were fixed by adding 50%

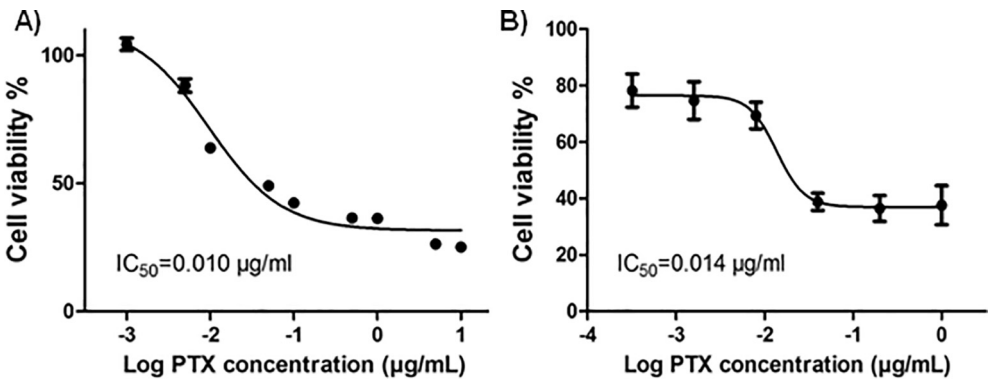

Fig. 3. *In vitro* cytotoxicity study. U87 MG glioma cells were treated with PTX PLGA-NPs at different PTX concentrations. The cytotoxic effect of PTX PLGA-NPs was assessed by the MTT (A) and clonogenic assays (B). Data are presented as the percentage of cell survival (untreated cells were assumed to have 100% survival). Mean ± SEM, $n = 6$ for MTT assay and $n = 3$ for clonogenic assay.

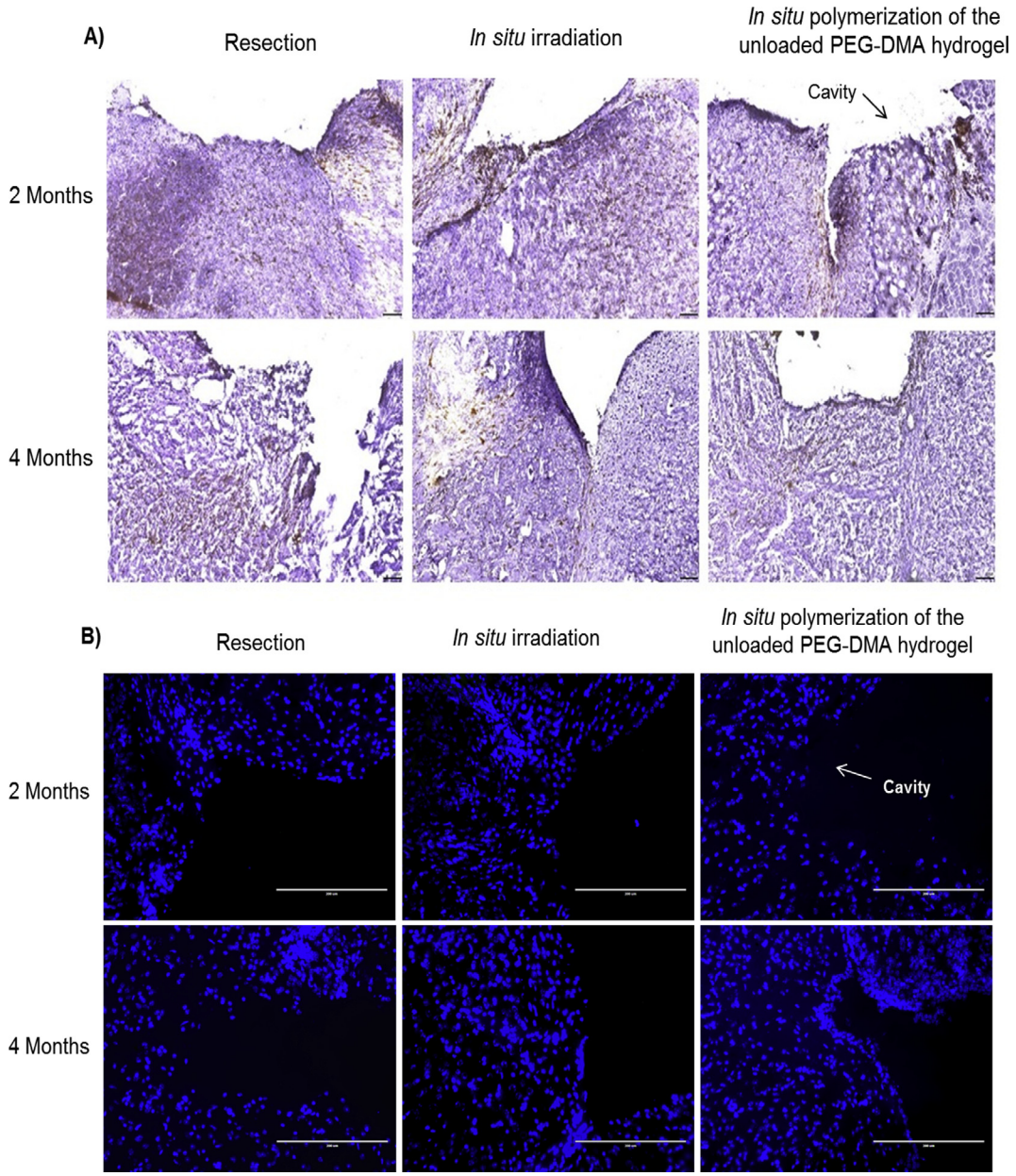

Fig. 4. (A) Mid- and long-term effects of the *in situ* photopolymerization of unloaded PEG-DMA hydrogels on microglial activation. Mouse brain was extracted at 2 and 4 months post-surgery and counterstained with hematoxylin and Iba-1 immunostaining. The groups are the resection alone, *in situ* irradiation (400 nm, 750 mW/cm^2, 15 s) and *in situ* photopolymerization of the unloaded PEG-DMA hydrogel ($n = 3$). Scale bar = 100 μm (B) Mid- and long-term effects of the *in situ* photopolymerization of unloaded PEG-DMA hydrogels on brain cell apoptosis. Mouse brain was extracted at 2 and 4 months post-surgery and evaluated with a TUNEL assay. The groups are resection alone, *in situ* irradiation (400 nm, 750 mW/cm^2, 15 s), and *in situ* photopolymerization of unloaded PEG-DMA hydrogel ($n = 3$). Scale bar = 200 μm.

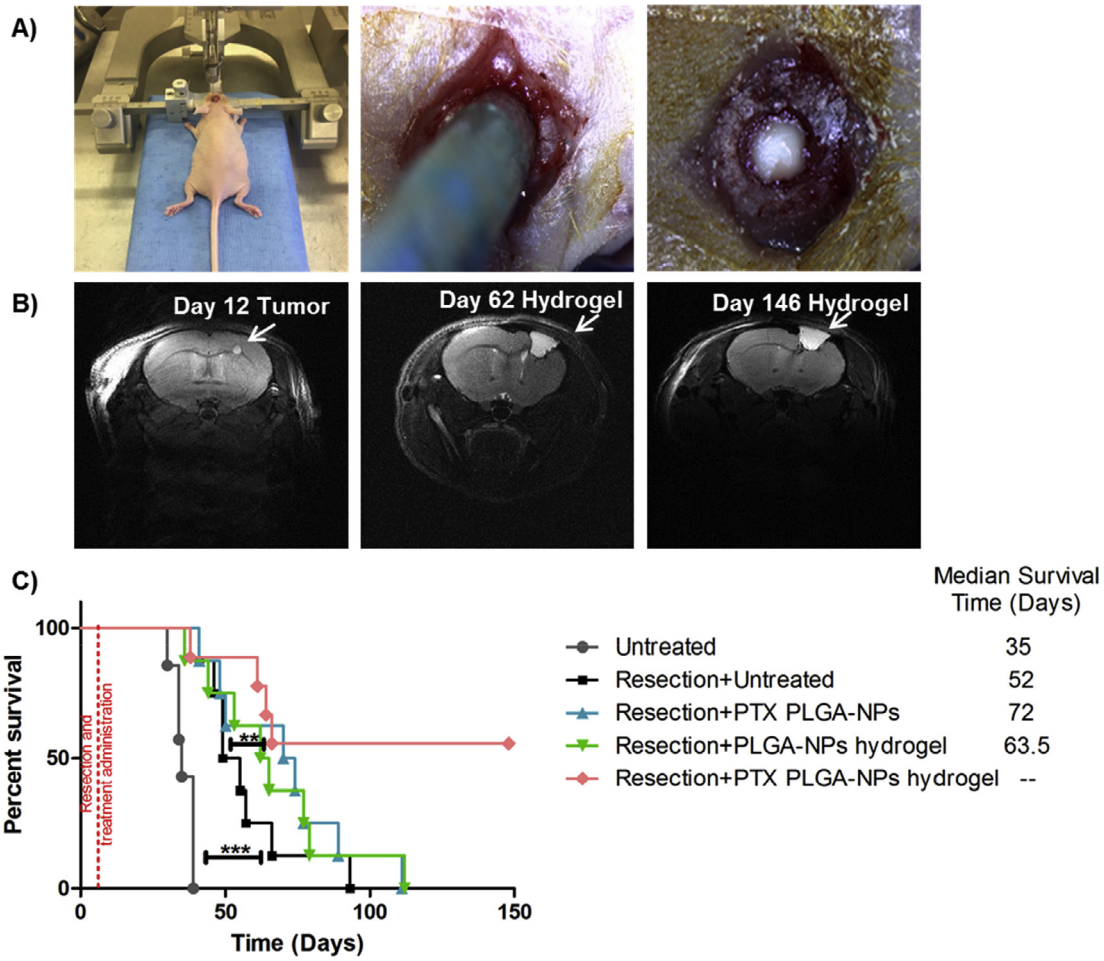

Fig. 5. (A) Pictures of the tumor resection surgeries and treatment administration. Briefly, mice were fixed on a stereotactic frame for the tumor cell injections (left), biopsy punch resections of tumor tissue (middle) and *in situ* photopolymerization of PEG-DMA hydrogels with PTX PLGA-NPs (right). (B) Axial (T2-weighted) images of a mouse brain: brain tumor before resection (day 12, left), treatment with PTX PLGA-NPs/PEG-DMA hydrogel (day 62 post-tumor inoculation, middle; day 146 post-tumor inoculation, right). (C) Kaplan-Meier survival curves for mice after different treatments in the resection cavity. (n = 7–9 for all groups). $^{**}p < 0.01$, $^{***}p < 0.001$.

ethanol, 5% acetic acid and 0.5% crystal violet for 30 min at room temperature. Then, the cells were washed twice with water and air-dried. The clones were counted manually and the number of clones was compared to untreated cells (n = 3).

2.4. In vivo studies

All experiments were performed following the Belgian national regulations guidelines in accordance with EU Directive 2010/63/EU and were approved by the ethical committee for animal care of the Université catholique de Louvain medicine faculty (2014/UCL/MD/004). The animals had free access to water and food. Animal body weight was monitored daily throughout the experiments.

2.4.1. In vivo tolerability assays of hydrogel in healthy mouse brain

Seven-week-old female NMRI mice (Janvier, France) were randomly divided into 3 groups: (*i*) resection alone, (*ii*) resection + irradiation, and (*iii*) resection + photopolymerized PEG-DMA hydrogel. The resection was performed as previously described (Bianco et al., 2017). Five microliters of unloaded PEG-DMA hydrogel were injected into the resection cavity and polymerized as described in part 2.1.4. The cranial window was then covered with Neuro-Patch® (Aesculap, USA) previously soaked with fibrin glue (Baxter Innovations, Austria). Mice were then sutured and monitored the behavior for 2 or 4 months. At the end of the experiment, mice were sacrificed and their brains were extracted, fixed in 10% formalin solution (Merck, USA) overnight, washed with PBS and incubated in 30% sucrose for 2 days at 4 °C. The brains were then embedded in OCT (Tissue-Tek, USA) and sectioned at 18 µm using a Leica CM 1950 cryostat (Leica, Germany).

Microglial activation was evaluated by Iba-1 immunostaining as described (Fourniols et al., 2015). Slides were scanned using a SCN400 Leica slide scanner, and image analysis was performed on selected zone with Digital Image Hub (Leica, Germany). Apoptosis was evaluated with the Fluorometric TUNEL System kit® (Promega, USA) according to the manufacturer's instructions. Slides were examined under an inverted fluorescence microscope (Evos, USA) with 350 nm (blue, DAPI) and 450–500 nm (green, TUNEL) excitation filters. The stainings were performed on 3 animals (n = 3).

2.4.2. Orthotopic U87 MG human GBM tumor resection model

Six-week-old female NMRI nude mice (Janvier, France) were anesthetized and positioned in a stereotactic frame. As previously described (Bianco et al., 2017), a five microliters Hamilton syringe fitted with a 26 G needle was used to inject two to three microliters of complete culture medium containing 3×10^4 U87 MG glioma cells into the right frontal lobe. The injection coordinates were 0.5 mm anterior, 2.1 mm lateral from the bregma and 2.5 mm deep from the outer border of the cranium.

The presence, volume and location of the tumors were determined by magnetic resonance imaging (MRI) using the 11.7T Bruker Biospec

MRI system (Bruker) for all mice on day 11 or 12 post-tumor cell implantation. The tumor volume was assessed using the rapid acquisition with relaxation enhancement (RARE) sequence (TR = 2500 ms; effective echo time (TE_{eff}) = 30 ms; RARE factor = 8; FOV = 2 × 2 cm; matrix 256 × 256; twenty-five contiguous slices of 0.3 mm, NA = 4).

On day 13 post-tumor injection, tumor resection was performed based on a biopsy-punch resection model (Bianco et al., 2017). A 2 mm Ø biopsy punch (Kai Medical, Germany) was then inserted 3 mm deep and twisted for 15 s to cut the tumor site. Once withdrawn, the tumor and brain tissues were aspirated using a diaphragm vacuum pump. Five microliters of hydrogel was photopolymerized into the resection cavity before sealing the cranial window with a 4 mm × 4 mm square piece of Neuro-Patch® (Aesculap, Germany) impregnated with a reconstituted fibrin glue (Baxter Innovations, Austria).

2.4.3. *In vivo anti-tumor efficacy of PTX PLGA-NPs-loaded hydrogel on the U87 MG glioblastoma model after tumor resection*

The *in vivo* anti-tumor efficacy of PTX PLGA-NPs-loaded hydrogel was conducted post-tumor injection by administration of the treatment in the resection cavity as follows: Group 1: untreated group (n = 7), Group 2: resection and untreated group (n = 8), Group 3: PTX PLGA-NPs-treated group (n = 8), Group 4: blank PLGA-NPs-loaded PEG-DMA hydrogel *in situ* photopolymerized treated group (n = 8) and Group 5: PTX PLGA-NPs-loaded PEG-DMA hydrogel *in situ* photopolymerized treated group (n = 9). The dose of PTX administered was 0.525 mg/kg. The delivered dose of drug without the PEG-DMA gel was the same as that within the PEG-DMA hydrogel. The mice were sacrificed when they presented a 20% body weight loss or 10% body weight loss associated with clinical signs of distress (paralysis, arched back, apathy).

2.5. *Statistical analysis*

The results are expressed as the mean ± standard deviation (SD) or mean ± standard error (SEM) of at least three replicates. Statistical analysis was performed using GraphPad Prism and Excel software based on $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ for significant differences. In these experiments, n is the number of replicates for each experiment. For Fig. 5, one-way ANOVA test was performed to demonstrate statistical differences between groups using the software GraphPad Prism.

3. Results and discussion

3.1. *Physico-chemical characterization of PTX PLGA-NPs*

The PTX PLGA-NPs formulation was prepared using a modified emulsion-diffusion technique. The blank PLGA-NPs and PTX PLGA-NPs were characterized in terms of their physico-chemical properties and encapsulation capacities. As shown in Table 1, the size of both the blank PLGA-NPs and PTX PLGA-NPs was approximately 190 nm, and the polydispersity indices showed a narrow size distribution (0.07 and 0.13, respectively). The zeta potential values were negative for the two formulations (approximately −17 mV). The presence of PTX in the formulation did not influence the size or zeta potential of the nanoparticles. The PTX encapsulation efficacy and drug loading, assessed after breaking the PLGA nanoparticle structure in acetonitrile, were approximately 53% and 4%, respectively. The physico-chemical parameters are consistent with those previously described for the encapsulation of PTX (Amoozgar et al., 2014; Jin et al., 2009; Schleich et al., 2013).

3.2. *PTX release from PTX PLGA-NPs-loaded PEG-DMA hydrogel*

The hydrogel system was developed to fill the cavity after the surgical resection of the tumor to act as a PTX drug depot and kill the residual infiltrating GBM cells. Thus, we evaluated the *in vitro* release profile of PTX. The *in vitro* release kinetics of PTX PLGA-NPs from the PEG-DMA hydrogel was evaluated after incubation at 37 °C in PBS pH = 7.35. An initial burst release of 10.9 ± 2.3% was observed in the first 8 h, followed by a sustained release of the drug over 1 week (28.9 ± 3.7%) (Fig. 2). Over 90% of the drug was recovered when the total amount of released PTX was sum up with the quantity of PTX that remained in the hydrogel. Comparing to the release of TMZ free drug (45% burst release and 65% sustained release over 1 week) in PEG-DMA hydrogel, the small burst and continuous PTX release may be attributed to the diffusion of PTX localized in the PLGA core of the nanoparticles (Danhier et al., 2012). After resection, as most tumor cells were removed, the PTX initial burst release may kill the closer tumor cells, while the continued release could kill the remaining infiltrative cells (Bastiancich et al., 2016a). Longer sustained release could not be measured *in vitro* due to the low recovery of PTX but based on the one week release, we expect the system to release PTX for at least 4 weeks.

3.3. *In vitro cytotoxicity of PTX PLGA-NPs in U87 MG GBM cells*

To evaluate the cytotoxicity of PTX PLGA-NPs, an MTT assay was conducted on U87 MG GBM cells after 48 h of incubation with different concentrations of PTX (0.001–10 µg/mL). The results are illustrated in Fig. 3A. PTX PLGA-NPs induced an obvious cytotoxic effect on U87 MG cells, demonstrating concentration-dependent inhibitory effects. The IC_{50} value of PTX PLGA-NPs was 0.010 µg/mL. To confirm this result, a modified clonogenicity study was conducted to evaluate the proliferative and reproductive ability of U87 MG cells after incubation with PTX PLGA-NPs for 48 h (Fig. 3B). The numbers of clones were counted, and the resulting IC_{50} value (0.014 µg/mL) was close to that obtained in the MTT test. Some authors previously evaluated the cytotoxic effect of PTX on GBM cells, showing a similar sensitivity of U87 MG GBM cells to PTX (from 0.003 µg/mL to 4.5 µg/mL) (Maleki et al., 2017; Najlah et al., 2016; Ruan et al., 2017; Xu et al., 2016). No cytotoxic activity was observed for the drug-free nanoparticles at a polymer concentration below 2 mg/mL (data not shown).

3.4. *In vivo tolerability of PEG-DMA hydrogel in heathy mouse brain*

The influence of the PEG-DMA on brain microglial activation and apoptosis was evaluated 2 and 4 months after implantation in healthy mice. After 2 and 4 months, none of the animals with brain resection with or without PEG-DMA hydrogel implantation showed behavioral changes or loss of body weight. Microglial activation was observed whatever the condition both after 2 and 4 months (Fig. 4A), probably due to the surgery. No positive TUNEL signal was observed whatever the timing and the treatment (Fig. 4B). We previously observed that after 1 week exposure to the same hydrogel, the brain resection itself induced microglial activation and some cell apoptosis at the cavity margin (Fourniols et al., 2015). Our results show that there is also inflammation but no significant apoptosis resulting from surgical resection alone after 2 and 4 month. It has been previously shown that microglial activation followed by resection or biomaterial implantations into the brain can last much longer time (Bastiancich et al., 2017; Bjugstad et al., 2010). There is still Iba-1 staining at 4 months indicating that the microglia activation was not resolved. However, it remains controversial whether microglia activation is beneficial or detrimental. On one hand, microglial activation may be considered as a positive factor as it could remove cell debris and recover tissue integrity in the injured brain (Zhang et al., 2017). Other author even proposed the restoration of the microglial anti-tumor function as a treatment for GBM (Chiu et al., 2012). On the other hand, excessive activation may cause neuronal damage through the secretion of cytotoxic molecules (Dheen et al., 2007). Irradiation and hydrogel implantation did not increase the extent of microglia activation, so we assume the PEG-DMA hydrogel is brain-compatible. In conclusion, the *in situ* photopolymerization of PEG-DMA hydrogel is well tolerated in healthy mice brains and is

therefore suitable for local administration into the mouse brain.

3.5. In vivo anti-tumor efficacy of PTX PLGA-NPs-loaded hydrogel on the U87 MG glioblastoma model after tumor resection

To evaluate the anti-tumor efficacy of the hydrogel and its capacity to slow down tumor recurrences in a clinically relevant model, the GBM was surgically resected on day 13 post-tumor inoculation, and the treatments were intra-operatively injected into the resection cavity (Fig. 5A). The tumors were well-demarcated and visible by MRI imaging (Fig. 5B). The hydrogel was visible and stayed in place during the entire course of the experiment (Fig. 5B). The median survival times of mice with or without resection were significantly different (52 and 35 days, respectively; $p < 0.001$), demonstrating the effect of surgical resection (Fig. 5C). Injection of PTX-NPs and blank NPs-loaded hydrogel in the resection cavity failed to significantly prolong the median survival time of mice compared to that of resected mice ($p > 0.05$). However, the survival time of the PTX PLGA-NPs hydrogel-treated mice was significantly enhanced ($p < 0.01$) compared to that of mice in the resection untreated group, and more than 50% of the mice were still alive 150 days after the injection of tumor cells. One mouse out of 5 surviving mice showed the sign of tumor recurrence on days 147 by MRI imaging (data not shown).

The improvement of animal survival was reported with the convection enhanced delivery (CED) or local implantation of hydrogels (Bastiancich et al., 2017; Chen et al., 2017; Sawyer et al., 2011; Stephen et al., 2014; Tyler et al., 2010; Zhang et al., 2016). For example, when camptothecin was encapsulated in PLGA-NPs and delivered via CED in rats, 30% of the group treated with the system experienced long-term survival until 60 days, the median survival of which was significantly improved compared to the control (Sawyer et al., 2011). More recently, in a similar experimental model, lauroyl-gemcitabine lipid nanocapsule-based hydrogel was delivered intra-surgically in the resection cavity in U87 MG-bearing mice, and a significantly prolonged medial survival of 62 days was observed compared to that of the untreated group (35.5 days) (Bastiancich et al., 2017). In comparison, our promising results (150 days long-term survivals) highlight the high potential of PTX-loaded PLGA-NPs/PEG-DMA hydrogel for local implantation after surgical resection to significantly improve the overall survival of mice bearing GBM.

4. Conclusion

In conclusion, we designed and characterized an injectable photopolymerizable PEG-DMA hydrogel containing PTX-loaded PLGA-NPs that fits many requirements of a local drug delivery system for the treatment of GBM. We demonstrated the sustained release of PTX over one week. The *in vitro* cytotoxicity study confirmed U87 MG cells were sensitive to PTX. The *in vivo* tolerability studies showed that the implantation of hydrogel into the brains of healthy mice was well tolerated for 4 months. The *in vivo* anti-tumor efficacy studies performed on a clinically relevant tumor model (orthotopic U87 MG resection model on mice) showed that PTX-loaded PLGA NPs in PEG-DMA hydrogel significantly prolonged mouse survival, and 50% of the mice were alive at 5 months after tumor implantation. Future pre-clinical studies may take advantage of the potential therapeutic benefits of this system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpharm.2018.07.033.

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