Temozolomide-loaded photopolymerizable PEG-DMA-based hydrogel for the treatment of glioblastoma

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A B S T R A C T

Glioblastoma is the most frequent primary malignant brain tumor in adults. Despite treatments including surgery, radiotherapy and chemotherapy by oral Temozolomide (TMZ), the prognosis of patients with glioblastoma remains very poor. We hypothesized that a polyethylene glycol dimethacrylate (PEG-DMA) injectable hydrogel would provide a sustained and local delivery of TMZ. The hydrogel photopolymerized rapidly (~2 min) and presented a viscous modulus (~10 kPa). TMZ release kinetic presented two phases: a linear burst release of 45% of TMZ during the first 24 h, followed by a logarithmic release of 20% over the first week. The in vivo tolerability study showed that the unloaded hydrogel did not induce apoptosis in mice brains nor increased microglial activity. In vivo, the anti-tumor efficacy of TMZ-hydrogel was evaluated on xenograft U87MG tumor-bearing nude mice. The tumor weight of mice treated with the photopolymerized TMZ hydrogel drastically decreased compared with all other groups. Higher apoptosis (located at the center of the tumor) was also observed. The present study demonstrates the potential of a photopolymerizable TMZ-loaded hydrogel to treat glioblastoma.

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1. Introduction

Glioblastomas are highly malignant brain tumors that arise from astrocytes or supportive brain tissue. Glioblastoma multiforme is the most common malignant tumor of the central nervous system in adults. These tumors show a high proliferation rate, variability in tumor histopathology and diffusely infiltrate adjacent brain tissue [1], making glioblastoma a very challenging cancer to treat. No curative treatment has yet been found [2].

Current conventional therapeutic procedures, aiming to increase the patient life expectancy, focus on surgical resection combined with adjuvant radiotherapy and/or orally delivered Temozolomide (TMZ) chemotherapy [3]. However, tumor recurrences are inevitable, leading to a median survival of about 14 months with a 5 year-life expectancy of 10 to 27% of patients [2]. Additionally, hematologic toxicity often requires discontinuation of TMZ therapy or prevents dose escalation as dictated by the treatment therapy [7]. Current anti-glioma therapies have less-than-optimal efficiency, mainly due to the reduced accumulation of TMZ into the tumor, systemic toxicity and limited diffusion through the blood–brain barrier (BBB) [8].

Due to obvious consequences on the patient quality of life, the extent of brain tissue that can be resected is often limited and in most cases, cancerous cells infiltrate brain parenchyma, leading to relapses and development of a new tumor. Although curing glioblastoma will likely depend on the discovery of anti-cancer drugs that will efficiently kill remaining cancer cells or make them sensitive to destruction (i.e., by radiotherapy), innovative methods of drug delivery will have to be developed to deliver the drug at the tumor site and to reduce its impact on the healthy tissues.

Direct administration into the brain parenchyma of locally implanted drug delivery systems is a powerful strategy that will ensure direct contact of the drug with remaining tumor cells, controlled and sustained drug release and a limited exposition of healthy tissues to the cytotoxic molecule [9]. Local delivery will also preserve the drug activity and thus potency by avoiding degradation by the liver [10]. So far, only one local drug delivery system, Gliadel®, has been approved for the treatment of glioblastoma. Gliadel® wafers are implanted into the tumor bed following surgical removal of the tumor. Gliadel wafers have been shown to release carmustine over a period of approximately 5 days. The median post-operative survival of the patients implanted with Gliadel® was 34 weeks compared to 23 weeks in the placebo group [11]. Nevertheless, side effects frequently appear, including seizures, brain edema, incomplete wound healing and intracranial infections, all mainly due to the wafer migration post-implantation [9].

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In this context, researchers developed novel strategies for local delivery to improve the efficacy of anti-cancer drugs. Camptothecin and vincristine were encapsulated in poly-(lactic-co-glycolic) acid (PLGA) nanoparticles and embedded in a thermoreversible hydrogel. This system was evaluated in the C6 rat Glioma model and produced long-term survivors [12]. In another study, paclitaxel was loaded in PLGA foams and their in vivo anti-tumor efficacy on a subcutaneous tumor model was correlated with the in vitro release profiles [13]. Tunable diblock copolymer hydrogels were evaluated in vivo for the local delivery of tamoxifen [14]. Slow-releasing TMZ microspheres showed significant tumor reduction both in subcutaneous and orthotopic xenograft glioma models [15]. To our knowledge, despite the growing number of publications in the field of photopolymerizable hydrogels, up to now, no clinical trial is ongoing or has been performed for cancer or tissue engineering applications.

The main challenge to treat glioblastoma lies in killing the cancer cells that infiltrate the brain tissue and remain in the parenchyma after the resection of the main primary tumor. A system based on a photopolymerizable polyethylene glycol dimethacrylate (PEG-DMA) hydrogel delivering TMZ locally would present several advantages. Indeed, PEG-DMA can be injected and easily and quickly photopolymerized in brain tumor resection bed using a commercial UV light [16], and should be well tolerated in the host tissues. PEG-DMA-based hydrogel properties (chemical composition and small pore size) should prevent cell infiltration, avoiding to provide a favorable environment for cancerous cell proliferation [17]. In addition, this polymer is commercially available (GMP) which should facilitate a future potential clinical translation. It was the first time PEG-DMA hydrogel was used for local delivery of TMZ to treat glioblastoma tumors. Indeed, photopolymerizable dimethacrylate is initially used as a component of dental resins (commercialized or in clinical trials). To the extent of our knowledge there is only one study describing in situ photopolymerization of polyethylene glycol diacrylate in the arterial intima to deliver proteins to the arterial media [18].

Hence, we hypothesized that an injectable hydrogel, loaded with TMZ, photopolymerized in situ and thus closely fitting the borders of the resected zone, would provide a sustained and local delivery. A therapeutic concentration would be maintained at the resection borders with a sustained diffusion in the surrounding tissue, killing cancer cells infiltrating the tissue and thus reducing the occurrence of local recurrences. We aimed to develop a new injectable delivery system that will (i) fit the resection cavity, (ii) be easily and quickly polymerized in situ via a commercial device, (iii) localize and sustainably release any anti-cancer drug directly to the invaded parenchyma and (iv) significantly reduce the occurrence of glioblastoma recurrence (Fig. 1). Our main objective was to demonstrate the feasibility, safety and efficiency of TMZ local delivery via a PEG-DMA-based photopolymerizable hydrogel on tumor growth. Hydrogel setting properties and TMZ release were characterized in vitro. The tolerability and anti-tumor efficacy of the hydrogel were evaluated in vivo, in the brain and, on a subcutaneous human glioblastoma tumor model, respectively.

2. Material and methods

2.1. TMZ formulation

2.1.1. Preparation of TMZ-loaded polymeric micelles (M-TMZ)

To counter its low water solubility, TMZ (10 mg, Sigma-Aldrich, USA) was dispersed in 500 mg of PEG750 poly((ε-caprolactone-co-trimethylene carbonate)) PEG750-p(CL-co-TMC) (Johnson & Johnson Center for Biomaterials and Advanced technologies, USA). Three different polymer ratios were tested for the solubilization of TMZ: 30:70, 50:50 and 70:30. After 30 min of magnetic stirring, PBS (5 mL) was...
2.1.2. Preparation of M-TMZ-loaded hydrogels (M-TMZ/PEG-DMA)

M-TMZ was added to a poly(ethylene glycol) dimethacrylate (PEG-DMA) solution (average Mn = 550 g/mol) (Sigma-Aldrich, USA) at a 75:25 vol ratio in water. 0.5% of Lucirin-TPO® (BASF, USA) was used as photoinitiator. 200 μL/well of the M-TMZ/PEG-DMA solution was placed in a black 96-well plate. Each well was irradiated at 750 mW/cm² during 15 s with an AURA light (Lumenor, USA). The irradiation time and intensity were optimized to obtain a fast setting with the lowest light intensity (750 mW/cm²) (kinetics measured with rheology, preliminary data not shown). The narrow light was centered at 400 nm (40 nm bandwidth). Unloaded PEG-DMA hydrogel was prepared as aforementioned, where M-TMZ was replaced by PBS.

2.1.3. Physico-chemical characterization of M-TMZ and M-TMZ/PEG-DMA formulations

Average particle size and size distribution of polymeric micelles were measured by dynamic laser light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). Zeta (ζ) potential was measured by Laser Doppler Electrophoresis (Zetasizer Nano ZS, Malvern Instruments, UK). Samples were diluted in 10 nM NaCl (Sigma-Aldrich, USA). The column was a CC 250/4.6 Nucleosil 300-5 C18 (Macherey-Nagel).

TMZ released from the gels was quantified by reverse-phase high performance liquid chromatography (HPLC) (Agilent 1100 series, Agilent Technologies). 200 μL of TMZ released from the hydrogel (prepared as previously described) were eluted with a gradient of water containing 0.1% Trifluoroacetic acid (TFA) and acetonitrile (ACN) (95:5 gradient to 90:10 from 0 to 3 min; 90:10 gradient to 0:100 from 3 to 6 min; 0:100 to 6 to 9 min; gradient to 95:5 from 9 to 11 min). The column was a CC 250/4.6 Nucleosil 300-5 C18 (Merck). The flow rate was set at 1.0 mL/min and the detection wavelength was 227 nm. The injection volume was 20 μL. Calibration was performed with standard solutions of 1 to 100 μg/mL of TMZ dissolved in water/ACN 1:1 at pH = 5 (correlation coefficient of R² = 0.9992). The limit of quantification was 1 μg/mL.

2.2. Mechanical properties of the PEG-DMA hydrogel

Mechanical properties were evaluated by rheological measurements carried out with a kinexus pro rheometer (Malvern Instruments). The following experiments were performed using the unloaded PEG-DMA solution. A parallel plate geometry was selected (8 mm diameter) and a constant 100 μm gap was maintained between the two plates. 120 μL of PEG-DMA solution was gently introduced and held in place between the two plates due to capillary forces. The light was placed under the sample, beneath a transparent glass plate. The distance between the light guide tip (5 mm diameter) and bottom surface of the sample was 1 cm. Directly after placing the PEG-DMA solution, the plate cartridge was closed and the storage and loss modulus (G’ and G″) along with the complex viscosity were monitored under a shear strain of 5% for 120 s. The first 10 s was used to determine the initial modulus (G’₀), after which the lamp was turned on for 15 s. The complete monitoring duration was 130 s. G’final and G″final were determined as the average G’ values over the last 10 s of the run. Measurements were performed in quintuplicate at 25 °C.

2.3. Monitoring of temperature elevation during PEG-DMA photopolymerization

In order to evaluate the temperature increase due to irradiation and polymerization of PEG-DMA, 60 μL of the unloaded PEG-DMA solution was placed in a white Teflon mold (6 mm diameter, 2 mm thickness), which accommodated a temperature sensor (RTD sensor, HSRTD-3-100, Omega). The data was acquired using a 4-channel RTD module (Omega). Similarly to the rheological measurement, the lamp was placed at a 1 cm from the mold. Irradiation parameters were kept as aforementioned. After 10 s of monitoring to determine the initial temperature (ambient temperature = 21 °C), the lamp was turned on and temperature variations were monitored for 120 s. The maximum variation (ΔT) was reported. Measurements were performed in triplicates.

2.4. In vitro release

2.4.1. Non cross-linked PEG-DMA release from photopolymerized hydrogels

After photopolymerization (as described above) of 100 μL/well of PEG-DMA hydrogel in a black 96-well plate, 200 μL of PBS containing 10 mg/mL of bovine serum albumin (BSA) (Sigma-Aldrich, USA) was added on the hydrogels as a release medium. Samples were incubated at 37 °C. 22 h, 100 μL of the release medium was sampled and mixed with 100 μL of acetonitrile (ACN) to precipitate BSA. Samples were centrifuged at 15,000 g for 15 min and the supernatants were collected. Non-reacted PEG-DMA was quantified by HPLC (n = 3). Samples were eluted with a gradient of water and ACN (60:40 gradient to 25:75 from 0 to 8 min; 25:75 gradient to 10:90 from 8 to 9 min; 10:90 from 9 to 10 min; gradient to 60:40 from 10 to 12 min; 60:40 to 15 min). The reverse phase column was a BDS Hypersil C18 100 × 4.6 (Thermo Scientific). The flow rate was set at 1.0 mL/min and the detection wavelengths were 215 and 285 nm. Samples were diluted by a factor 80 before injection (injection volume = 50 μL). The standard curve was performed between 2 and 500 μg/mL of PEG-DMA dissolved in water/ACN 1:1 (correlation coefficient of R² = 0.99). The limit of quantification was 2 μg/mL.

2.4.2. TMZ release from M-TMZ/PEG-DMA hydrogel

200 μL/well of M-TMZ/PEG-DMA hydrogels was photopolymerized in a black 96-well plate, as aforementioned. Unloaded PEG-DMA hydrogels were also prepared to obtain background values. 200 μL of the release medium (citrate buffer at pH = 5.3 containing 10 mg/mL BSA) was added in each well and the samples were incubated at 37 °C. At predetermined time intervals, 100 μL of release medium was sampled and replaced by fresh medium and 100 μL of ACN was added to precipitate the BSA. Samples were centrifuged at 15,000 g for 15 min and the supernatants were collected. TMZ concentration was assessed by reverse-phase high performance liquid chromatography (HPLC) (Agilent 1100 series, Agilent Technologies). 200 μL of the release medium (citrate buffer at pH = 5.3 containing 10 mg/mL BSA) was added in each well and the samples were incubated at 37 °C. A standard curve was performed between 2 and 500 μg/mL of TMZ dissolved in water/ACN 1:1 (correlation coefficient of R² = 0.99). The limit of quantification was 2 μg/mL.

2.5. In vivo tolerability and anti-cancer efficacy

All experiments were performed in compliance with guidelines set by National Regulations and were approved by the ethical committee for animal care of the faculty of medicine of the Université catholique de Louvain.

2.5.1. Tolerability

The in vivo tolerability of unloaded PEG-DMA hydrogel was assessed by evaluation of microglia activation and apoptosis in the surrounding brain tissue, using a lb-1 staining and a TUNEL assay, respectively. Female NMRI mice, aged 8 weeks, were obtained from Janvier (France). Animals were anesthetized by an intraperitoneal injection of ketamine/xylazine (62.56.25 mg/kg, respectively). After 5 day, mice skulls were drilled and a cavity was created in the cortex of the left frontal lobe by aspiration (Fig. S1). Mice received an intracranial treatment of 3 μL into the cavity, using a 5 μL Hamilton® syringe with a 26-G needle. Mice were then anesthetized. Mice were killed after 3 days (n = 3) and Group 1: cavity without treatment; Group 2: in-situ irradiation at 380 nm with an intensity of 720 mW/cm² for 15 s and Group 3: Unloaded hydrogel + in-situ irradiation. After 1 week, mice were sacrificed. Brains were removed and embedded in cryo-embedding compound OCT (Tissue Tek). Sections were collected on charged super-frost-plus glass slides and stored at −80 °C until staining.
Microglia activation was evaluated by Iba-1 immunostaining. Slides were rehydrated in PBS and fixed in a paraformaldehyde 4% solution. Then they were permeabilized by a 0.1% Triton X-100 solution. Sections were incubated for 30 min with 10% goat serum to block non-specific binding sites before incubation with a goat anti-human Iba-1 antibody (1:100; Novus Biologicals, USA) for 60 min at room temperature. Then they were incubated for 60 min at room temperature with rabbit anti-goat IgG biotinylated antibody (1:100; Vector Laboratories, USA). Sections were then counterstained with hematoxylin and mounted with DPX neutral mounting medium (Prosan). Image acquisition was performed using a SCN400 Leica slide scanner, allowing the acquisition of entire sections and image analysis was performed with Digital Image Hub (Leica) \((n = 3, N = 3)\).

A fluorescent TUNEL assay (In Situ Cell death detection kit (Roche, Switzerland)) was conducted following supplier instructions. Nuclei were stained using DAPI (Sigma-Aldrich, USA). Slides were mounted with Vectashield (Vector Laboratories, USA) and examined under an inverted fluorescent microscope (Apointe, Zeiss) with 350 nm (blue, DAPI) and 748–789 nm (green, TUNEL) excitation filters \((n = 3, N = 3)\).

2.5.2. Anti-tumor efficacy on a subcutaneous human glioblastoma tumor model

Human U87MG glioblastoma cells were injected subcutaneously in the right flank of 6 week old female athymic nude mice (Janvier, France) \((3 \times 10^6 \text{ cells per mouse})\). Before injection, animals were anesthetized with ketamine/xylazine \((62.5 \text{ and } 6.25 \text{ mg/kg, respectively})\). Tumors were allowed to grow until they reached a volume of \(2.5 \pm 0.5 \text{ mm}^3\). Initial tumor volume was measured using an electronic caliper using the formula for a prolate ellipsoid: volume \(= \pi/6 \times \text{length} \times \text{width}^2\). Treatments were then applied. Animals were randomized into six groups \((n = 5)\): Group 1: Control group (no treatment) \((n = 6)\); Group 2: unloaded PEG-DMA hydrogel photopolymerized \(\text{ex situ}\) (photopolymerized before subcutaneous implantation) \((n = 7)\); Group 3: M-TMZ/PEG-DMA hydrogel photopolymerized \(\text{ex situ}\) \((n = 6)\); and Group 4: M-TMZ solution, injected in the tail vain of mice \((n = 5)\). It is important to note that the ideal situation would be to deliver and polymerize the liquid directly after incision. Nevertheless, it was practically impossible to perform. After the incision through the skin, the liquid dispersed everywhere and it was not possible to polymerize it homogeneously. Then, the alternative was to polymerize the hydrogel “\text{ex situ}” and to implant it on the tumor, as proof of concept. The volume of each hydrogel was 70 μL and the TMZ dose was 4.75 mg/kg \([22]\). For groups 2 and 3, hydrogels were implanted by aseptic surgery as follows: after anesthesia with ketamine/xylazine, a small incision was made into the skin and the hydrogel disk (already photopolymerized) was implanted on the tumor. The skin was closed by subcutaneous suturing. For group 4, animals were injected intravenously with a dose equivalent to the total amount of TMZ administered using the hydrogel \((52.5 \mu \text{L of M-TMZ solution})\). As the hydrogel covered the tumor, it was not possible to measure the tumor growth with an electronic caliper. Hence, after 7 days, mice were sacrificed and tumors were extracted and weighed. The volume of tumors before treatment was converted into tumor weight, considering that tumor density was equal to 1 \((\text{tumor density was previously determined on extracted tumors (data not shown)})\). Then, tumor weight post-treatment was normalized to tumor weight before treatment.

2.5.3. Immunohistochemistry

After extraction and weighting, tumors were fixed in paraformaldehyde 4% in PBS. After fixation, tumors were dehydrated, embedded in paraffin and serially sectioned \((5 \mu \text{m-thick sections})\).

Apoptosis within the tumors was evaluated using a TUNEL assay to detect DNA fragmentation (DeadEnd™ Fluorometric TUNEL System, Promega). Sections were dewaxed with Histosafe, rehydrated with isopropanol, and washed in running deionized water. The fluorescent TUNEL assay was then conducted following supplier instructions.

Image acquisition was performed using a fluorescent microscope (Apointe, Zeiss, Zaventem, BE).

Endothelial cells and vessel formation were assessed by CD34 immunostaining, while human glioblastoma proliferating cells were identified by Ki67 staining. Sections were processed for immunohistochemical analysis. Primary antibodies against endothelial cells (rat anti-mouse CD34 antibody, 1:100 (Hycheck Biotech)) and proliferating cells (mouse anti-human Ki67, 1:100 (Dako, Denmark) \([23]\)) were used in combination with a secondary immunoperoxidase stain (rabbit anti-rat IgG biotinylated antibody, 1:100 (Vector Laboratories, USA) and a horse anti-mouse IgG biotinylated antibody, 1:100 (Vector Laboratories)). Endogenous biotin was blocked using a blocking kit (SP2001, Vector Laboratories). The staining was performed with an ABC Elite kit (Vector Laboratories) and DAB (Sigma). Hematoxylin was used as a counterstain. Negative controls were generated by omitting the primary antibodies. Image acquisition was performed using a SCN400 Leica slide scanner, allowing the acquisition of entire sections.

2.6. Statistics

Results were expressed as mean ± standard deviation for Fig. 2 and as mean ± standard error of the mean \([19]\) for Fig. 5. Unpaired t test was performed to demonstrate statistical differences between groups \((p < 0.05)\), using the software GraphPad Prism.

3. Results and discussion

3.1. Physicochemical characterization of the hydrogel formulation

PEG750–p(CL-co-TMC) polymeric micelles were used to solubilize the poorly-water soluble TMZ. The influence of the copolymer composition \((30:70; 50:50 \text{ and } 70:30 \text{ PEG750–p(CL-co-TMC)})\) on micelle physico-chemical characteristics is summarized in Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>Influence of PEG750–p(CL-co-TMC) polymer ratio on physico-chemical characterization of polymeric micelles.</td>
</tr>
<tr>
<td>CL/TMC ratio</td>
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<tr>
<td>TMZ concentration (mg/mL)</td>
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<tr>
<td>Size (nm)</td>
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<tr>
<td>PDI</td>
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<td>ζ potential (mV)</td>
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<sup>a</sup> \( n = 3 \)
<sup>b</sup> \( n = 3 \)
<sup>c</sup> \( n = 3 \)
The 50:50 ratio allowed the solubilization of 2 mg/mL of TMZ, while the 30:70 and 70:30 ratios allowed the solubilization of 0.9 and 1.3 mg/mL TMZ, respectively. The size of M-TMZ varied from 60 (30:70) to 30 nm (50:50 and 70:30), which are consistent with sizes described in the literature [24]. The lowest polydispersity index (PDI) was obtained with the 50:50 ratio. ζ potentials were equivalent for the three studied ratios and can be considered as neutral. Size and ζ potential of M-PTX (50:50) are consistent with those previously described for the solubilization of PTX [19,25]. Additionally, the 30:70 and 70:30 ratio-based formulations presented some aggregates one week after preparation, while the 50:50 ratio-based formulations remained stable. The presence of TMZ in the formulation compared to unloaded micelles did not influence the size of ζ potential of polymeric micelles (data not shown). The 50:50 copolymer ratio was thus selected to solubilize TMZ for the study.

The major advantage of our PEG_{750-}p(CL-co-TMC) micelles is that they are self-assembling upon gentle mixing with water and that neither organic solvent nor dialysis or evaporation step is required for drug encapsulation. PEG_{750-}p(CL-co-TMC) copolymers are shown to be biocompatible (slug test — data not shown), non-cytotoxic and non-hemolytic (up to 10% w/v) [19]. Additionally, PEG_{750-}p(CL-co-TMC) micelles display a high kinetic and thermodynamic stability (CMC = 20 μg/mL). Given these properties, PEG_{750-}p(CL-co-TMC) micelles may constitute an attractive vehicle to solubilize and deliver a poorly soluble drug like TMZ.

3.2. PEG-DMA hydrogel setting properties

The complex viscosity of a 75:25 (v/v) PEG-DMA:water with 0.5% of Lucirin-TPO® solution increased 3-fold during photopolymerization (15 s of irradiation), from an initial value of 6.1 ± 2.210³ Pa to 2.1 ± 0.410⁴ Pa after setting [Fig. 2]. The maximum rate of change in complex viscosity, corresponding to a maximum in reaction speed, was observed after 18 s, while 95% of G^f0 was only reached after 76 s. No crossover was observed and G' remained slightly higher than G" excluding the determination of the gel point through this method. However final moduli values were similar: G^f0 was determined at 2.0 ± 0.410⁴ Pa. During the first 10 s of irradiation, no change in G' was observed, while ΔT increased close to a maximum value. ΔT^max was 5.6 °C and was reached after 16 s, followed by a continuous decrease to a ΔT of 1 °C after 120 s.

The monomer and photoinitiator used to produce the hydrogel were be considered. The photoinitiator Lucirin TPO® used in this work is used by manufacturers to stabilize their monomers), quickly followed by the start of propagation and linear polymer growth. In addition, as there was a direct correlation between temperature increase/decrease and the lamp being turned on (immediate increase of temperature) and off, it is likely that the temperature increase observed during the photopolymerization was mainly due to the irradiation, with little contribution of the exothermic polymerization reaction. The actual start of crosslinking (the formation of bonds between different polymer chains) corresponded to the time when G' started increasing, where the rigidity of the forming gel increased due to increasing molecular weight and decreased mobility. The activation step was rather slow compared to a system where monomer density would be much higher. However due to a rather low initial viscosity, the activated radicals would probably easily diffuse to the dispersed and available monomers, hence supporting fast kinetics. The combination of irradiation and polymerization resulted in a ΔT of 5.6 °C. In cats and dogs, thermal damage to brain tissues was observed for temperature increases of 3 °C (38 °C baseline) [28]. With an increase of 6 °C, 100 min of exposure would be required to observe necrosis in human muscle tissue [28]. Since the temperature increase we observed was due to light irradiation and was below 2 °C after 60 s, the setting conditions to form a PEG-DMA hydrogel in situ should be safe. Additional investigation could focus on further reducing the light dose. Moreover, the ΔT was determined inside the hydrogel and temperature can be expected to be lower at the interface with the tissue. Under shear deformation, the order of magnitude of brain tissue moduli was determined to be around 1 kPa, with values varying depending on studies [29]. The PEG-DMA hydrogel G’ remained in this range, suggesting that the material would accommodate well in the resected brain tissue cavity, unlikely inducing mechanically injury.

3.3. Non cross-linked PEG-DMA and TMZ in vitro release

Sixteen ± 3% of the total weight of PEG-DMA used to form the hydrogels was released within 24 h of incubation. This is in accordance with results reported in the literature for other soft materials [21]. Since monomer elution is inversely correlated with the degree of conversion, further reduction of this release could be obtained by increased Lucirin-TPO® proportion [30].

TMZ release kinetic presented two phases: a linear burst release of 45% was first observed during the first 24 h, followed by a release of 20 more % over the first week [Fig. 3]. Then the release plateaued to reach 70%. The burst effect was most likely due to TMZ diffusion through the hydrogel while the 30% still remaining after 2 weeks were trapped in the hydrogel. However, the unaccounted TMZ fraction could also either have been integrated to the PEG-DMA polymer chains during photopolymerization or degraded and not detected at the selected wavelength with HPLC.

TMZ release profile was similar to the release profile of carmustine (Gliadel®) [31]. Ideally, TMZ should be released over a period of 1 month to cover the gap between surgery and the beginning of conventional treatment (oral administration of TMZ combined with radiotherapy). Modification of the initiator and/or the crosslinker (molecule or concentration) could be used to slow down TMZ release. The PEG-DMA hydrogel could also be chemically modified but to facilitate future clinical translation, chemical modification of the polymer will not be considered. The photoinitiator Lucirin TPO® used in this work is photocleaved and reacts with a monomer molecule to form a growth center, and therefore should not be available for diffusion. According to The Committee for Risk Assessment of the European Chemical Agency, the “non observed adverse effect level” (NOAEL) established by a 90-day study was considered to be 100 mg/kg/day. A dose of

![Fig. 3. Cumulative TMZ release from M-PTX/PEG-DMA hydrogels. Release was performed at 37 °C in citrate buffer (pH = 5.3) containing 10 mg/mL BSA (n = 3). Released TMZ was quantified by HPLC after BSA precipitation in BSA (n = 3).](image-url)
50 mg/kg was introduced in the hydrogel. So, even if the total amount of Lucirin TPO® was released, which is unlikely, the global dose was below the NOAEL. Hence, the Lucirin TPO® photoinitiator release and potential toxicity were not studied.

3.4. In vivo tolerability of PEG-DMA hydrogel

Microglial activation was observed for all the groups (Fig. 4). As expected, brain tissue resection itself induced microglia activation. Previous studies dealing with traumatic brain injury have shown elevated levels of Iba-1 staining years after occurrence of the initial brain injury [32].

Microglial activation was similar to what was reported following biomaterial implantation [33]. The presence of activated microglia is not necessarily a portent of irreversible damage [33]. Some cell infiltration can be observed at the interface between the hydrogel and the cavity border but none inside the hydrogel. Prevention of cell invasion was one of the main reasons a PEG-based hydrogel was selected for our application [17] so very limited cell infiltration in the hydrogel was expected.

PEG-DMA tolerability was studied by evaluating its impact on brain cell apoptosis. Neither the resection itself nor the irradiation did induce significant apoptosis around the cavity (Fig. 5). When PEG-DMA hydrogel was implanted and photopolymerized in the surgical cavity, some apoptotic cells could be observed around the lesion but not deeper in the brain parenchyma.

The PEG-DMA used to deliver TMZ was similar to co-polymers used in dentistry (i.e., triethylene glycol dimethacrylate (TEG-DMA)). PEG-DMZ was preferred to PEG-TMA for its longer PEG chain (Mn = 550) making the resulting hydrogel softer and more biocompatible. Indeed, unlike PEG-DMA [34], monomers of low molecular weight such as TEG-DMA tend to be more cytotoxic [17]. In addition, even if the hydrogel modulus was one order of magnitude higher than the brain modulus (+/− 1 kPa), no adverse effect was observed so we assume that a modulus of 10 kPa remains acceptable.

3.5. In vivo anti-tumor efficacy of M-TMZ loaded PEG-DMA hydrogel

3.5.1. Tumor weight

Tumor weight of mice treated with M-TMZ/PEG-DMA hydrogel (group 3) drastically decreased compared with all other groups (p < 0.0001) (Fig. 6A). For 2 animals, the tumor completely disappeared (Fig. 6B). No other treatment was significantly different than the control (p > 0.05). Unloaded PEG-DMA hydrogel (group 2) and the M-TMZ solution (group 4) tended to decrease tumor weight but the difference with the control group (group 1) was not significant (p > 0.05). No inflammation was observed in tissues surrounding the unloaded hydrogel.

![Fig. 4. Effect of unloaded-PEG-DMA hydrogel photopolymerization in situ on microglia activation. Mouse brains were retrieved 7 days post-surgery and treated for immunohistochemistry. The effect of the (A) lesion, (B) the in-situ irradiation (380 nm, 720 mW/cm², 15 s) and (C) the unloaded-PEG-DMA hydrogel photopolymerization in situ on microglial activation was assessed by Iba-1 staining (brown) (n = 3, N = 3). Sections were counterstained with hematoxylin. c = cavity of surgery; f = cortex of the left frontal lobe of brain; h = hydrogel; arrows show the activated microglia zones. Scale bar = 100 μm.](image)

![Fig. 5. Effect of unloaded-PEG-DMA hydrogel photopolymerization in situ on brain cell apoptosis. Mouse brains were retrieved 7 days post-surgery and treated for TUNEL. The effect of the (A) lesion, (B) the in-situ irradiation (380 nm, 720 mW/cm², 15 s) and (C) the unloaded-PEG-DMA hydrogel photopolymerization in situ on cell apoptosis was assessed by TUNEL (green) (n = 3, N = 3). Cell nuclei were stained with DAPI (blue). Scale bar = 100 μm.](image)
hydrogels while TMZ-hydrogels sporadically induced a local inflammation (data not shown).

Ideally, an in vivo glioma model should closely resemble human glioblastoma in its morphology, invasiveness and angiogenic behavior, to allow clinically relevant studies of tumor behavior and therapeutic drug efficiency. The subcutaneous human U87-MG tumor model in immunosuppressed mice is known to exhibit moderate to high invasiveness, tumor-induced necrosis and vascular alterations, which make it a relevant human glioblastoma model, particularly used for therapeutic agent studies [35]. Mice treated with intravenous injection of M-TMZ did not present significant reduction of tumor weights when compared to untreated mice. This was likely due to (i) the low

![Graph](image)

**Fig. 6.** Anti-tumor efficacy of M-TMZ/PEG-DMA hydrogels. (A) Ratio between tumor weights 7 days after treatment and initial tumor weights of xenografted human U87MG tumor-bearing nude mice untreated (control), treated with unloaded hydrogel, M-TMZ-loaded hydrogel, or intravenous injection of M-TMZ. TMZ dose was 4.75 mg/kg. Results are expressed as the tumor weight at day 7/initial tumor weight ratio ± SEM, ***p < 0.0001. (B) Pictures of tumors after 7 days post-implantation (n = 5 to 7). Stars: Complete regression of tumors.

![Images](image)

**Fig. 7.** Effect of M-TMZ/PEG-DMA hydrogel on U87MG cell apoptosis. Tumors were retrieved 7 days post-treatment and treated for immunohistochemistry. TUNEL (green) was performed on tumor section. DAPI staining was used to label nuclei (blue). Scale bar = 1 mm (n = 3, N = 3).
dose of TMZ (4.75 mg/kg), adjusted to be equivalent to the dose administered locally by the hydrogels, compared to the dose classically used for intravenous injection (40 mg/kg) combined with (ii) low TMZ accumulation into the tumor following intravenous administration. The lack of inflammation at the hydrogel implantation site supports the previously reported PEG-DMA biocompatibility [9]. The subcutaneous U87-MG model was used to establish the proof-of-concept of our system and to evaluate its impact on tumor growth. Since we have demonstrated a significant tumor reduction when treated with M-TMZ-loaded PEG-DMA hydrogel, the system will be tested in an orthotopic model over a longer period.

3.5.2. In vivo glioblastoma cell apoptosis and proliferation

Apoptotic cells were observed in the center of tumors only for tumors treated with TMZ-loaded hydrogels (Fig. 7). This observation corroborated the tumor weight reduction reported in Section 3.5.1. Apoptotic cells were located in the center of the tumor, while no apoptotic cells were located in the periphery. CD34 positive staining was observed in and at the periphery of tumors whatever the condition (Fig. 8). Proliferating cells were detected in and at the periphery of tumors untreated or treated with the unloaded PEG-DMA hydrogel, while no proliferating cells were observed in the tumors for the TMZ-loaded PEG-DMA hydrogel (Fig. 9).

As U87MG tumors are very well vascularized tumors [35], we could hypothesize that TMZ released from the M-TMZ/PEG-DMA hydrogel reached the center of the tumor. However, it does not explain why dead cells were detected in the tumor center but not at the periphery. This illustrates the problem that glioblastoma presents. Most solid tumors present heterogeneous distribution of the tumor vasculature, associated with a hypoxia region in the center of the tumor. In that case, reaching the center of the tumor with chemotherapy is the main challenge [36]. Anti-angiogenic therapies have been studied to normalize the tumor vasculature, making the whole tumor more accessible to chemotherapy [20]. On the contrary, glioblastoma is very well vascularized [35]. Hence, chemotherapies could diffuse and accumulate in the whole tumor. So, despite the fact that the TMZ/PEG-DMA hydrogel induced apoptosis only at the tumor center and that proliferative cells can be detected at its periphery, our system can still cause tumor reduction/disappearance, and within a short period of time (1 week). It is thus a promising system to locally treat glioblastoma.

4. Conclusion

In conclusion, we designed and characterized an injectable photopolymerizable PEG-DMA-based hydrogel for the local release of TMZ that fit many requirements of a local drug delivery for the treatment of glioblastoma: (i) a fast and easy in situ photopolymerization, easily translated to a clinical practice, (ii) mechanical properties compatible with brain implantation, (iii) a sustained release of TMZ over 1 week, (iv) a good tolerability with brain tissues and (v) most of all, a potent in vivo anti-tumor efficacy. We established the proof-of-concept for a system based on a commercially available photopolymerizable PEG-DMA hydrogel that could be easily applied in the tumor resection cavity and maintain a therapeutic concentration of TMZ at the resection borders as well as ensuring a sustained diffusion in the surrounding tissue.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.05.272.
Fig. 9. Effect of M-TMZ/PEG-DMA hydrogel on cancer cell proliferation. Tumors were retrieved 7 days post-treatment and treated for immunohistochemistry. Human cancer proliferating cells were detected by Ki67 staining (brown). Sections were counterstained with hematoxylin. (A) Control (no treatment); (B) unloaded hydrogel and (C) M-TMZ-loaded hydrogel. White scale bar = 100 μm, black scale bar = 10 μm (n = 3, N = 3).

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