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Drug combination using an injectable nanomedicine hydrogel for glioblastoma treatment

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ABSTRACT

Lauroyl-gemcitabine lipid nanocapsules ($GemC_{12}$-LNC) hydrogel, administered intratumorally or perisurgically in the tumor resection cavity, increases animal survival in several orthotopic GBM models. We hypothesized that $GemC_{12}$-LNC can be used as nanodelivery platform for other drugs, to obtain a combined local therapeutic approach for GBM. Paclitaxel (PTX) was selected as a model molecule and PTX-$GemC_{12}$-LNC formulation was evaluated in terms of physicochemical and mechanical properties. The PTX-$GemC_{12}$-LNC hydrogel stability and drug release were evaluated over time showing no significant differences compared to $GemC_{12}$-LNC. The drug combination was evaluated on several GBM cell lines showing increased cytotoxic activity compared to the original formulation and synergy between PTX and $GemC_{12}$. Our results suggest that $GemC_{12}$-LNC hydrogel can be used as nanodelivery platform for dual drug delivery to encapsulate active agents with different mechanisms of action to achieve a better antitumor efficacy against GBM or other solid tumors.

1. Introduction

Glioblastoma (GBM) is a highly malignant brain tumor. Its standard of care therapy includes surgical debulking of the tumor followed, several weeks later, by radiotherapy and concomitant and adjuvant chemotherapy with Temozolomide (TMZ) (Stupp et al., 2009). However, the effective treatment of GBM is limited by its high heterogeneity, rapid proliferation and infiltrating capacity as well as chemoresistance to alkylating agents, which inevitably lead to tumor recurrences. Currently, the median overall survival of GBM patients after treatment is less than two years (16 months with surgery plus chemoradiation alone; 20.9 months in combination with tumor-treating fields) and the long-term survivors are virtually inexistent (Stupp et al., 2017). For these reasons, GBM is an unmet medical challenge and finding effective therapeutic approaches is an urgent global health need. As the blood-brain barrier (BBB) represents a major challenge for brain drug delivery, and 90% of GBM recurrences arise along the resection cavity borders, the local delivery of chemotherapeutic drugs into the resection cavity and the delivery of active molecules through nanomedicines represent two major hopes for the future of these incurable brain tumors (Bianco et al., 2017).

Local drug delivery in the brain would allow to bypass the BBB, increasing the drug dose able to reach the remaining infiltrative cells in the brain and avoiding systemic side effects. Moreover, injecting a drug delivery system in the tumor resection cavity could reduce the incidence of recurrences in the time gap between surgery and standard of care chemoradiation (Bastiancich et al., 2016a; Nam et al., 2018). Gliadel® – a wafer composed of copolymer prolifeprospan 20 loaded with carmustine (BCNU) – is the only local treatment currently available for GBM treatment following surgical resection. This biodegradable scaffold allows for the local delivery of BCNU over a one-week period. However, its use in the clinics is limited by drawbacks such as implant dislodgement, difficulties in adjusting the implants in the resection cavity and local side effects (Bota et al., 2007; Westphal et al., 2003). Moreover, BCNU is an alkylating agent and shares some chemoresistance pathways with TMZ (Sarkaria et al., 2008). Since Gliadel® approval, several physical implants and hydrogels have been developed but none has reached the market yet. An ideal brain local delivery system should be injectable or be sprayed, stick to the resection cavity borders and be adaptable to its shape. It should also be soft and have

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mechanical properties close to the brain to avoid increased intracranial pressure. Finally, its drug content should be high enough to reach the desired local dose without filling the entire cavity (Bastiancich et al., 2016a).

In the last decade several nanocarriers (e.g. liposomes, micelles, polymeric nanoparticles) have been used as delivery tools for the treatment of GBM, as they can deliver drugs at the tumor site by local delivery, passive targeting or active targeting (Ganipineni et al., 2018; Miranda et al., 2017). Our group has recently shown the potential of combining nanomedicines and local delivery systems for the treatment of GBM using Lauroyl-gemcitabine lipid nanocapsules ($GemC_{12}$-LNC). $GemC_{12}$-LNC is a nanomedicine hydrogel uniquely formed by the prodrug Lauroyl-gemcitabine ($GemC_{12}$) and lipid nanocapsules (LNC) (Moysan et al., 2014). $GemC_{12}$ is an amphiphilic derivative of Gemcitabine (Gem), a nucleoside analogue used in various solid tumors, showing improved stability in plasma and cytotoxicity in different cell lines (Moysan et al., 2013). LNC are biocompatible and biomimetic nanocarriers obtained by a soft-energy process, formed of an oily core surrounded by a highly organized membrane of low molecular weight surfactants. LNC formulations generally appear as liquid nanosuspensions, where the lipophilic drug or the reversed micelles containing hydrophilic drugs are incorporated into the LNC structure. However, when $GemC_{12}$ is encapsulated in LNC, the formulation spontaneously forms a hydrogel without the addition of polymers, gelling agents or external stimuli (Moysan et al., 2014). We have recently demonstrated that $GemC_{12}$-LNC as high potential for the local treatment of GBM by delaying the recurrences onset both in mice and rats (Bastiancich et al., 2017; Bastiancich et al., 2018; Bastiancich et al., 2016b). Indeed, the LNC integrity is maintained in the resection cavity for at least one week *in vivo*, allowing for $GemC_{12}$ to be released gradually over time around the resection cavity borders killing residual infiltrating cells. The great advantage of $GemC_{12}$-LNC over conventional hydrogels consists in its simple formulation and complete biodegradation – time and cost-effective, avoiding the use of solvents, gelling agents and polymers avoiding long-lasting residues in the resection cavity – injectability and compatibility for brain implantation. However, even though mice survival was significantly increased by using a single drug ($GemC_{12}$), all developed recurrences (Bastiancich et al., 2017).

We hypothesized that $GemC_{12}$-LNC can be used as nanodelivery platform for other drugs, to obtain a combined local therapeutic approach for GBM. The rationale behind the choice of a dual chemotherapeutic treatment for GBM is to increase response and tolerability and to decrease resistance. To do so (*i*) the single drugs must have a strong cytotoxic activity against GBM cells when used alone; (*ii*) the drugs must act through different mechanisms of action, possess minimal cross-resistance and non-overlapping toxicities; (*iii*) the drug characteristics must be compatible with the formulation (*iv*) the drugs must have a synergic effect (Catarina Pinto et al., 2011).

In the case of $GemC_{12}$-LNC, a lipophilic drug could be incorporated in the oily core of the LNC while an hydrophilic drug could be added in the aqueous phase of the formulation. Based on this, several molecules which have shown promising results against GBM could be tested in combination with $GemC_{12}$. For this work, we used Paclitaxel (PTX) as a model molecule because it has been previously encapsulated in LNC obtaining high encapsulation efficiency and drug loading (Hureaux et al., 2009; Peltier et al., 2006) and its efficacy after local administration has shown promising results for GBM (e.g.(Garcion et al., 2006; Lollo et al., 2015; Vinchon-Petit et al., 2010)). The combination between PTX and Gem is clinically used for breast cancer and several studies show its rationale in other cancers as well (e.g. bladder and pancreatic cancers (de Sousa Cavalcante and Monteiro, 2014; Meluch et al., 2001)). The two drugs act through different mechanisms of action, avoiding cross-resistance or overlapping side effects and their combination might potentiate their activity. Indeed, it has been reported a synergistic effect between these two drugs, probably due to a production of reactive oxygen species by PTX that would inhibit the action of cytidine deaminase, reducing the degradation of Gem into an inactive metabolite and therefore potentiating its activity (Frese et al., 2012).

In this work we aim at exploiting the full hydrogel/nanomedicine potential of $GemC_{12}$-LNC by adding the lipophilic PTX molecule into this formulation. Therefore, we evaluated if the encapsulation of PTX in the LNC oily core alters the physicochemical properties of the LNC and mechanical properties of the hydrogel, and if the combination delivery system is able to increase the efficacy of $GemC_{12}$-LNC *in vitro*.

2. Materials and methods

2.1. Formulation of $GemC_{12}$ lipid nanocapsules hydrogel ($GemC_{12}$-LNC)

The gel formulation PTX-$GemC_{12}$-LNC was prepared using a phase-inversion method previously reported in the literature (Heurtault et al., 2002). Briefly, 0.093 g of $GemC_{12}$ (synthesized as previously described (Moysan et al., 2014)), 0.020 g of PTX (Chemieliva, China), 1.24 g of Labrafac® (Gattefossé, France) and 0.25 g of Span 80 (Sigma-Aldrich, USA) were weighed and stirred in a water bath at 50 °C with 200 µL of acetone (VWR Chemicals, Belgium) until complete dissolution of the drugs. The acetone was then allowed to evaporate and 0.967 g of Kolliphor HS15® (Macrogol (15)-hydroxystearate; Sigma-Aldrich, Germany), 0.045 g of Sodium Chloride (VWR Chemicals, Belgium) and 1.02 g of water for injection (Braun, Germany) were added to the formulation. Three cycles of heating and cooling were performed under magnetic stirring (500 rpm) between 40 and 75 °C. During the last cooling cycle, at the phase-inversion temperature, 2.12 g of injectable water was added, and the formulation stirred for one more minute. The formulation (total volume of 5.7 mL) was then inserted into insulin syringes (BD Micro-Fine™ needle 0.30 mL, Ø 30G; Becton Dickinson, France) before the gelation process occurred, and stored at 4 °C until further use. The PTX-LNC and $GemC_{12}$-LNC were obtained using the same method without adding the active compounds ($GemC_{12}$ and PTX, respectively) and without filling the syringes for the PTX-LNC formulation as no gelation occurred. All the formulations were obtained under aseptic conditions (Bastiancich et al., 2016b).

2.2. Physicochemical characterization of the formulations

The LNC average particle sizes and polydispersity indexes in the different formulations were measured using a dynamic laser light scattering apparatus Zetasizer NanoZS (Malvern Instruments, UK). Zeta potential measurements were performed by laser Doppler velocimetry using the same instrument. For the measurement, each sample was suitably diluted in a ratio of 1:60 with MilliQ water (Merck-Millipore, Germany). The characterization was performed in triplicate on three different batches of $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC.

The quantitative determinations of the drugs were measured by High Performance Liquid Chromatography (HPLC) analysis using a Shimadzu Prominence system (Shimadzu, Japan), under isocratic conditions. The separation was carried out using a C-18 column (for $GemC_{12}$: Thermo Scientific BDS Hypersil C18, 100 × 4.6 mm particle size 3 µm; for PTX: Macherey-Nagel Nucleodur® 100-5 C18 125 × 4.0 mm, particle size 5 µm). For the calibration curves, $GemC_{12}$ and PTX were dissolved in methanol and acetonitrile, respectively, and then diluted in their respective mobile phases to obtain concentrations between 1 and 150 µg/mL.

For $GemC_{12}$, the mobile phase consisted in methanol (VWR Chemicals, France) and MilliQ water in a ratio of 90:10 (v/v), the detection wavelength was set to 248 nm and the flow rate was maintained at 0.8 mL/min (Bastiancich et al., 2016b). For PTX, the mobile phase was acetonitrile (VWR Chemicals, France) and MilliQ water in a ratio of 60:40 (v/v), the detection wavelength was set to 227 nm and the flow rate was maintained at 1 mL/min (Schleich et al., 2013). The total drug content loaded in the hydrogels was evaluated by dissolution of an

amount of $GemC_{12}$-LNC or PTX-$GemC_{12}$-LNC in methanol (dilution ratio 1:240) and quantification by HPLC. The encapsulation efficiency (EE) of $GemC_{12}$ and PTX in the LNC was calculated as the ratio between the total drug content and the initial amount of drug weighed for the formulation. The drug loading was evaluated as the ratio between the total $GemC_{12}$ content and the content of the oil component (Labrafac®) in the formulation (w/w) (Bastiancich et al., 2016b).

To evaluate the stability of the formulations, $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC syringes were stored at 37 °C or 4 °C for six months. At fixed time points, a sample from each batch was used to evaluate its physicochemical properties (size, zeta potential) and its total drug content. The results are presented as the percentages of active ingredient remaining in relation to the initial quantity present in the gel, as a function of time.

2.3. Rheological properties of PTX-$GemC_{12}$-LNC hydrogel extruded from syringes

The viscoelastic properties of $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC hydrogels extruded from 30G needles were measured at 25 °C using a Modular Compact Rheometer MCR 102 (Anton Paar, Austria), with a cone plate geometry (diameter 50 mm, angle: 0.5). At 0.1% constant strain, storage modulus G′ and loss modulus G″ were measured as a function of the angular frequency (0.1–10 Hz). The rheological analysis was performed on three different batches.

2.4. In vitro release of $GemC_{12}$ from the drug-loaded lipid nanocapsules hydrogel

The *in vitro* release of $GemC_{12}$ from the PTX-$GemC_{12}$-LNC hydrogel was obtained during a period of one week in artificial cerebrospinal fluid (aCSF; (Bastiancich et al., 2016b)). To prepare 250 mL of aCSF, Sodium Chloride (1.5 g), Potassium Chloride (0.05 g; VWR Chemicals, Belgium), Magnesium Chloride (0.11 g; Sigma-Aldrich, USA), Calcium Chloride (0.07 g; Sigma-Aldrich, China), Sodium Carbonate (1.5 g; Merck, Germany), Disodium hydrogen phosphate dehydrate (0.01 g; Merck, Germany), D-glucose (0.15 g; Sigma-Aldrich, USA), L-Ascorbic acid (0.05 g; Sigma-Aldrich, China) and Bovine Serum Albumin (0.07 g; Sigma-Aldrich, USA) were weighed. MilliQ water was added and pH was adjusted to 7.35 ± 0.05 with concentrated Hydrochloric acid (VWR Chemicals, France). For the release study of $GemC_{12}$, 200 µL of gel were placed at the bottom of a 5 mL glass tubes (one per incubation time) and 800 µL of aCSF were added. The tubes were incubated at 37 °C and, at 2 h, 8 h, 24 h, 48 h and 7 days, the supernatant and the gel were separated, collected, weighted and appropriately diluted in methanol. The supernatant samples were also centrifuged at 15,000 rpm for 15 min to precipitate the protein residues and avoid interferences before quantification. The samples were then injected in HPLC using the previously described method to quantify $GemC_{12}$.

The *in vitro* release of PTX from the PTX-$GemC_{12}$-LNC hydrogel was obtained during a period of two days in PBS (Gibco Life Technologies, USA). 200 µL of gel were placed at the bottom of 5 mL glass tubes and 500 µL of PBS were added. The tubes were incubated at 37 °C and at 30 min, 2 h, 4 h, 6 h, 8 h, 24 h and 48 h, 200 µL of supernatant were replaced by 200 µL of fresh PBS. The samples were diluted in methanol (1:1 *v/v*) and frozen at −80 °C until further use. At the end of the incubation period (55 h) the supernatant and the residual gel were separated, collected, weighed, and appropriately diluted in methanol. The samples were then injected in HPLC using the previously described method to quantify PTX. The release studies were performed in triplicate.

2.5. Cytotoxicity studies: crystal violet assay on GBM cell lines

Rat glioma cells, 9L (ECACC, UK), were cultured in Eagle's Minimum Essential Medium (EMEM; ATTC, USA) supplemented with 10% Bovine Fetal Serum (Gibco, Life Technologies USA), with 1% of a 10,000 U/mL Penicillin G and 10,000 µg/mL Streptomycin solution (Gibco, Life Technologies, USA) and with 1% Non-Essential Amino Acids (NEAA; Biowhittaker Lonza, Belgium). Murine glioma cells, GL261 (DSMZ, Germany) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies USA) supplemented with 10% Bovine Fetal Serum (Gibco, Life Technologies USA), and with 1% of a 10,000 U/mL Penicillin G and 10,000 µg/mL Streptomycin solution (Gibco, Life Technologies, USA). Cells were subcultured in 75 cm^2 culture flasks (Corning® T-75, Sigma-Aldrich, USA) and incubated at 37 °C and 5% CO_2.

Cytotoxicity assays were performed using crystal violet staining after 48 h of incubation with different concentrations of the formulations. Cells were seeded at a density of 5×10^3 cells/well in 96-wells plates and incubated at 37 °C and 5% CO_2. They were then either incubated with Triton X-100 (Sigma-Aldrich, USA), different concentrations of PTX-LNC, $GemC_{12}$-LNC, PTX-$GemC_{12}$-LNC or left untreated. The treatments were dissolved in PBS and then suitably diluted in complete culture medium. The concentration of active drug ranged between 0.1 and 10,000 nM for $GemC_{12}$ and between 0.011 and 1100 nM for PTX. After 48 h of incubation with the treatments, cells were fixed with 10% formalin solution (Merck, Germany) for 30 min and then stained with Crystal violet solution (0.5% in 20% Methanol) for 30 min. The plates were then rinsed with distilled water multiple times, air-dried and observed at the microscope. Methanol was added to the wells and spectrophotometric readings were performed after 30 min at 545 nm with a MultiSkan EX plate reader (Thermo Fisher Scientific, USA). The *in vitro* cellular studies were carried out in three independent experiments using at least 5 wells per condition at each time and results are expressed as relative percentage of living cells compared to the untreated cells and were not normalized to the Triton X100 values.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, USA) and determined based on $p < 0.05$. Unpaired Wilcoxon test was used to compare LNC size, zeta potential and rheological behaviour. Paired Wilcoxon test was performed to evaluate the stability of the formulations. Finally, two-way ANOVA test with Tukey post-test were used for the *in vitro* cytotoxicity studies, IC_{50} values were calculated through a non-linear regression (curve-fit) mode and Combination Index (CI) values were calculated through Chou, Ting-Chao equation (Chou, 2010) using the obtained IC_{50} values. In the experiments, N corresponds to the number of independent experiments performed while *n* is the total number of replicates for each experiment. Results are expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Physicochemical characterization of the PTX-$GemC_{12}$-LNC formulation

The $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC formulations were prepared using a phase inversion technique process, which is a well-described, cost-effective and easy to scale-up procedure (Heurtault et al., 2002). The amount of PTX to be incorporated in the formulation was decided based on the literature (Lacoeuille et al., 2007; Vinchon-Petit et al., 2010), and it corresponded a quarter of the $GemC_{12}$ amount. A shift in the phase inversion temperature (PIT) – temperature in which the hydrophilic and lipophilic properties of a nonionic surfactant balance – was observed between PTX-$GemC_{12}$-LNC and $GemC_{12}$-LNC (63 °C *vs.* 53 °C, respectively) formulations. As the amount of NaCl is comparable in both formulations, this difference is probably due to a slight change in the physico-chemical behavior of the lipids caused by the dissolution of PTX. No difference was observed in the gelation time of the two formulations (5–7 min after the addition of cold water),

Table 1
Physicochemical characterization and loading efficacy of the formulations.

	Size	PDI	Zeta-potential	EE (%)		DL (%)	
	(nm)		(mV)	$GemC_{12}$	PTX	$GemC_{12}$	PTX
$GemC_{12}$-LNC	58 ± 4	0.12 ± 0.03	−3 ± 1	99 ± 3	–	7.4 ± 0.2	–
PTX-$GemC_{12}$-LNC	60 ± 3	0.17 ± 0.04	−3 ± 1	102 ± 5	101 ± 5	7.6 ± 0.4	1.6 ± 0.1

PDI: polydispersity index; EE: encapsulation efficiency; DL: drug loading. N = 3, *n* = 9; mean ± SD.

allowing the syringes to be filled while the nanosuspensions were still liquid.

No significant difference was observed between $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC formulations in terms of size, polydispersity index (PDI) or zeta potential (Table 1). After dilution, both formulations were monodispersed with an average size of around 60 nm, low PDI (< 0.2) and slightly negative zeta potential. These LNC characteristics are adapted to reduce non-specific binding and diffuse in the brain (Allard et al., 2009). The encapsulation efficiency of $GemC_{12}$ and PTX were around 100% for both drugs, consistent with data previously reported in the literature for $GemC_{12}$-LNC and PTX-LNC (Bastiancich et al., 2016b; Hureaux et al., 2009; Peltier et al., 2006). The drug loading of PTX-$GemC_{12}$-LNC corresponded to 1.6% for PTX and 7.6% for $GemC_{12}$ (similar to the $GemC_{12}$-LNC formulation). The PTX-$GemC_{12}$-LNC formulation presents a PTX concentration of 3.5 mg/mL, which seem appropriate for a local application in the brain. Indeed, some PTX-loaded gels with concentrations ranging from 1.5 to 6.3 mg/mL have already been described in the literature for local use against GBM, showing promising *in vitro* and *in vivo* results (Chen et al., 2017; Tyler et al., 2010; Zhao et al., 2018).

Our results show that adding a lipophilic molecule (PTX) into the $GemC_{12}$-LNC formulation does not alter the physicochemical properties of the LNC, which fit within the standards required for optimal brain drug delivery (e.g. size < 100 nm, biodegradable and biocompatible FDA approved components, polyethylene glycol surface, drug protection and sustained release) (Karim et al., 2016; Miranda et al., 2017).

3.2. Stability of the PTX-$GemC_{12}$-LNC formulation

The stability of the $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC formulations stored in syringes was evaluated at 4 °C and 37 °C for 6 months (Fig. 1). At 4 °C, no significant differences were observed in term of size, zeta potential or drug quantification over time in both formulations. This is in accordance with data in the literature indicating the stability of empty LNCs beyond 18 months in aqueous solution (Heurtault et al., 2002) and the stability of $GemC_{12}$-LNC gel up to 1 year at 4 °C (Moysan et al., 2014).

At 37 °C, significant variations in size and drug quantifications were observed over time. However, zeta potential remained stable throughout the study and was not affected by the storage conditions (Fig. 1B). The $GemC_{12}$-LNC size increased during the first few weeks, and significantly decreased after one month which was also observed in the PTX-$GemC_{12}$-LNC ($^{*}p < 0.05$ compared to day 0; Fig. 1A). The phenomenon of increased size can be explained by a coalescence of LNCs following the change in the crystalline structure of lipids or a change in the arrangement of surfactants on the surface of LNCs (Heurtault et al., 2003). This could later be associated with a release of the oily core from the LNCs, explaining the decrease in size which was associated to macroscopically visible phase separation on gels stored at 37 °C longer than one month. After one month at 37 °C, a decrease in the quantity of $GemC_{12}$ (HPLC quantification) was observed in both formulations compared to the initial total amount. This decrease is associated with the appearance of a new peak on the chromatogram probably representing Gem because of the $GemC_{12}$ amide linkage degradation (Retention time: 1.9 min *vs* 2.8 min of $GemC_{12}$). For the PTX-$GemC_{12}$-LNC formulation, a degradation peak overlapping the one of PTX was observed in the chromatogram after one month at 37 °C (data not shown).

3.3. Mechanical properties of the PTX-$GemC_{12}$-LNC formulation

It has been previously demonstrated that $GemC_{12}$, which is located at the oil-water interface of the LNC, creates inter-nanoparticle association which leads to the spontaneous formation of an hydrogel (Moysan et al., 2014). Therefore, the influence of PTX on the rheology had to be assessed to ensure that the gelification process was not affected by PTX.

LNC are formed of an oily core of tryglicerides (Labrafac®) surrounded by a shell formed of hydrophilic and nonionic surfactants (Kolliphor HS15® and Span80®, respectively). When the drug $GemC_{12}$ is incorporated in the formulation, the alkyl chain of the drug is inserted in the LNC structure while the active part of the molecule is oriented toward the water phase forming H-bond cross linkings that can immobilize the water phase forming a gel (Moysan et al., 2014). When PTX is added to the formulation, it is encapsulated in the oily core of the LNC, without altering the hydrogel properties. Both $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC hydrogels are injectable through insulin syringes. Indeed, as shown in Fig. 2, the addition of PTX in the formulation did not alter the viscoelastic properties of PTX-$GemC_{12}$-LNC which are very close to the ones of $GemC_{12}$-LNC. After PTX-$GemC_{12}$-LNC extrusion from insulin syringes the elastic modulus is always higher than the viscous modulus (G′ 1.12 ± 0.16 kPa and G″ 0.25 ± 0.04 kPa, respectively) and the viscoelastic moduli are relatively independent of the frequency between 0.2 and 1 Hz, showing the rheological behavior of a gel (Mayol et al., 2008; Zuidema et al., 2014). The G′/G″ ratio of 4.5 ± 0.7 indicates a moderate elasticity of the PTX-$GemC_{12}$-LNC gel, which is consistent with the literature (Bastiancich et al., 2016b; Moysan et al., 2014).

The lack of difference between $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC formulations in terms of gelation time, physico-chemical and mechanical properties indicates that PTX is most likely located inside the oily core of the LNC and does not take part in the hydrogel formation, unlike $GemC_{12}$ (Fig. 3).

3.4. In vitro release of the drugs from the PTX-$GemC_{12}$-loaded LNC hydrogel

We have previously reported that the *in vitro* release profile of $GemC_{12}$ from $GemC_{12}$-LNC hydrogel in aCSF is characterized by a burst release of the drug followed by an almost-steady sustained release over several weeks (Bastiancich et al., 2016b). The main advantage of a hydrogel uniquely formed by a drug and a nanocarrier is that the release of the drug corresponds to the dissolution of the hydrogel. Indeed, *in vivo*, we were able to demonstrate by fluorescence resonance energy transfer that, one week after administration of the hydrogel in the brain, there were still intact LNC in the hydrogel core while the others broke-down (Bastiancich et al., 2018). In this study, we aimed at evaluating if the presence of PTX in the formulation had an impact on the hydrogel dissolution behavior. Therefore, we performed a release study of the PTX-$GemC_{12}$-LNC hydrogel in aCSF at 37 °C for one week.

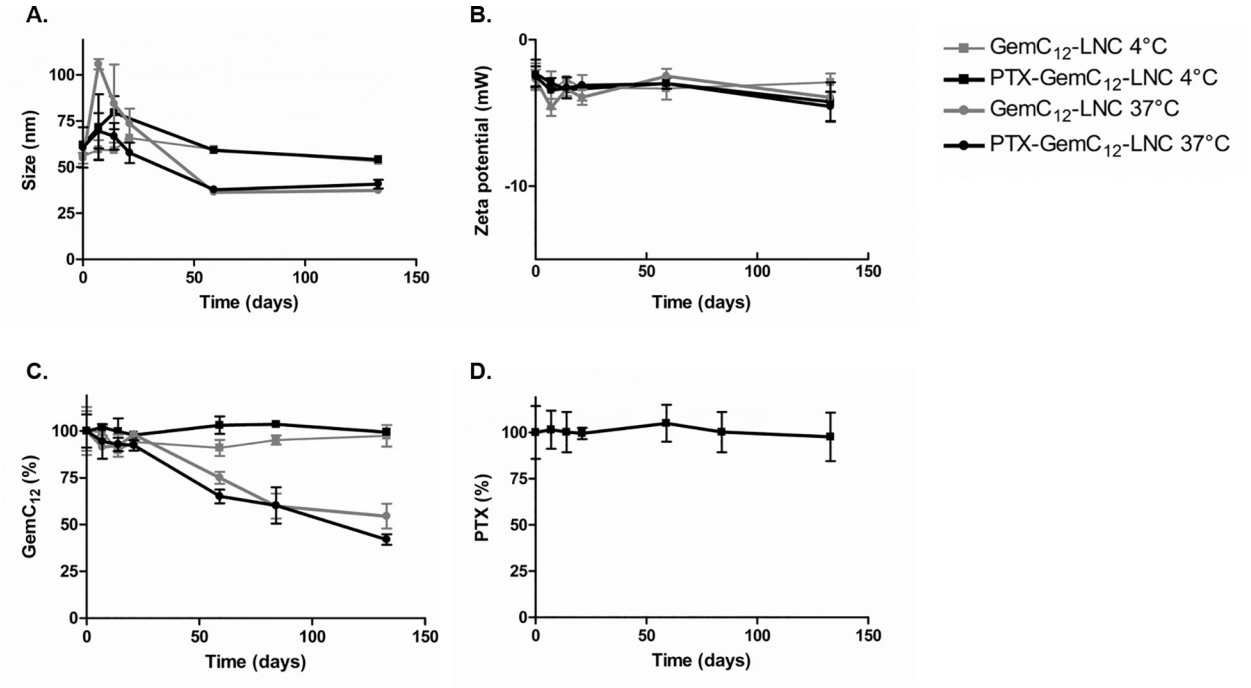

Fig. 1. Stability of the $GemC_{12}$-LNC (grey) and PTX-$GemC_{12}$-LNC (black) formulations over time at 4 °C (square) and 37 °C (circle): LNC size (A) and zeta potential (B) variations over time, $GemC_{12}$ and PTX variations over time normalized to day 0 (C and D, respectively). Unpaired Wilcoxon test, compared with day 0 (mean ± SD; N = 2, *n* = 2).

Our results, presented in Fig. 4A, suggest a burst release of $GemC_{12}$ during the first 24 h (45 ± 6%) followed by a slower release during the next six days (reaching 51 ± 4%). At the end of the incubation period, 37 ± 2% was recovered inside the gel. These data are in accordance with our previous experiments on $GemC_{12}$-LNC, suggesting that the presence of PTX into the formulation does not influence the release of $GemC_{12}$ and therefore the dissolution behavior of the hydrogel. Unfortunately, we were unable to properly quantify the release of PTX in aCSF using this experimental setup (data not shown). We assume that this failure was due either to the binding of PTX with proteins included in the aCSF (Paal et al., 2001) or to its hydrolysis into degradation products (Richheimer et al., 1992; Tian and Stella, 2008), which might explain the appearance of new peaks in our chromatograms. Therefore, we performed the PTX release study from PTX-$GemC_{12}$-LNC in PBS solution over 2 days (Fig. 4B). No degradation peaks were observed in the chromatograms using this protocol and this time frame, and 53 ± 5% of the PTX was released from the gel after 55 h while 61 ± 3% was recovered in the gel showing a similar release behavior compared to $GemC_{12}$.

3.5. *In vitro cytotoxicity of PTX-$GemC_{12}$-LNC in GBM cell lines*

The cytotoxic activity of PTX towards GBM has been previously demonstrated *in vitro* and *in vivo*. Vinchon Petit et al. have studied the cytotoxicity and radiosensitivity of PTX on 9L cells, showing that PTX-LNC possess a hundred times greater biological activity than PTX alone on this cell line (Vinchon-Petit et al., 2010). On the other hand, the cytotoxic activity of $GemC_{12}$ has been widely studied on various GBM cell lines (Bastiancich et al., 2017) and its ability to delay the formation of recurrences in 9L tumor-bearing resected rats has also been demonstrated (Bastiancich et al., 2018). In this work, we aim at evaluating whether the combination of these two drugs in the same hydrogel can give a synergic effect when compared to the single-drug loaded formulations. For this reason, we performed a cell viability assay on GL261 and 9L cell lines in a wide drug concentration range (0.1–10,000 nM $GemC_{12}$; 0.011–1100 nM PTX).

Fig. 5A shows an enhanced cytotoxic response in both cell lines when treated with PTX-$GemC_{12}$-LNC compared to PTX-LNC (in the range 1.1–1100 nM for GL261 and 9L) and $GemC_{12}$-LNC (at 10 nM for GL261 and in the range 10–100 nM for 9L). The IC_{50} values of cells treated with the dual-delivery system PTX-$GemC_{12}$-LNC are lower that

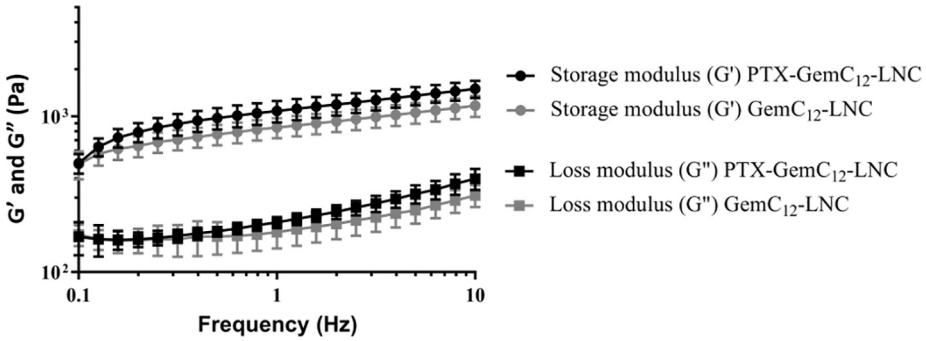

Fig. 2. Viscoelastic property profiles of the $GemC_{12}$-LNC (grey) and PTX-$GemC_{12}$-LNC (black) formulations: storage modulus G′ (circle) and loss modulus G″ (square) *vs* frequency at 25 °C, 0.1% constant strain. (mean ± SD; N = 3, *n* = 3).

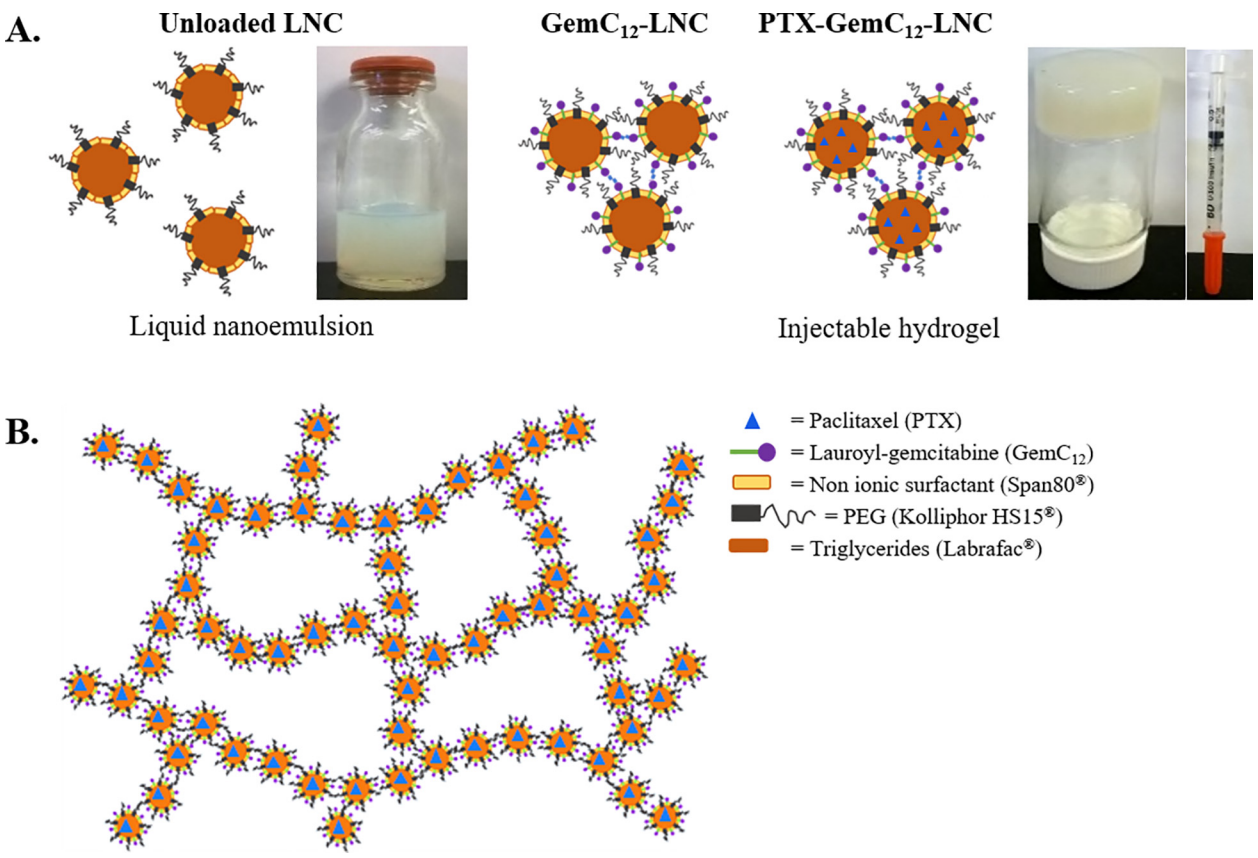

Fig. 3. Pictures taken during the experiments and schematic representation of unloaded LNC (A, left image), $GemC_{12}$-LNC (A, center image) and PTX-$GemC_{12}$-LNC hydrogel (A, right image; B). Part of the content of this figure was adapted with permission from Bastiancich et al. (2016b).

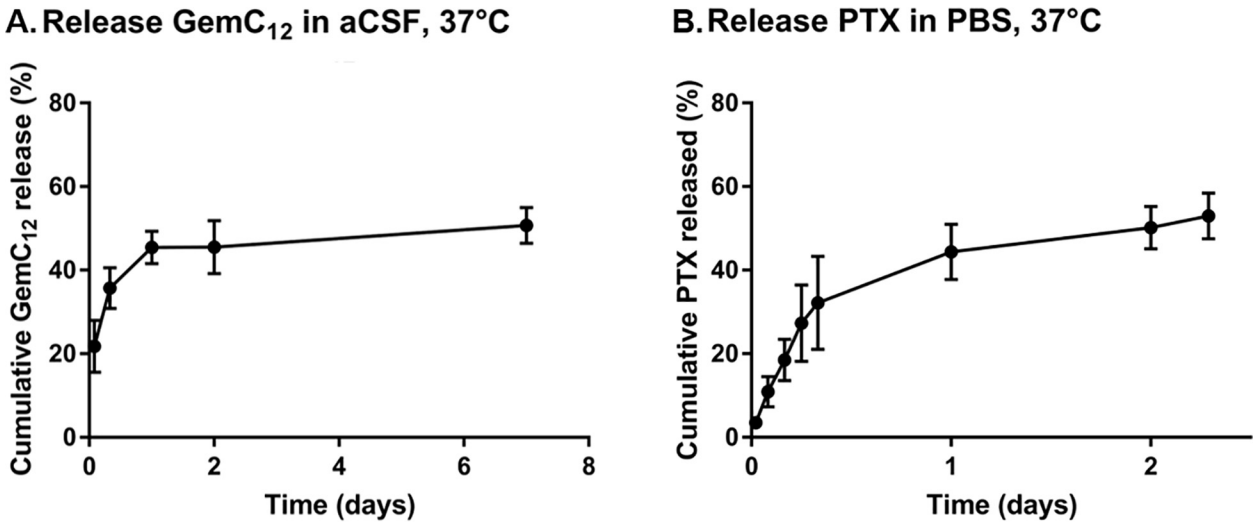

Fig. 4. *In vitro* cumulative release of $GemC_{12}$ and PTX from PTX-$GemC_{12}$-LNC hydrogel: (A) the release study of $GemC_{12}$ was performed in aCSF (pH 7.4) at 37 °C over one week; (B) the release study of PTX was performed in PBS (pH 7.4) at 37 °C over two days. The drugs were quantified by HPLC (N = 1, n = 3; mean ± SD).

the single loaded LNCs for both drugs and both cell lines. However, while the PTX IC_{50} difference is significant between PTX-LNC and PTX-$GemC_{12}$-LNC formulations both in GL261 and 9L, $GemC_{12}$ IC_{50} difference is only significant for the 9L cells (Fig. 5B). Despite this, the CI calculated for both cell lines is below 1, suggesting a synergy between the two encapsulated drugs. This synergy seems stronger for the rat glioma cell line 9L (Fig. 5C).

4. Conclusions

$GemC_{12}$-LNC hydrogel can combine the properties of local delivery systems and nanomedicine, in a unique simple-to-prepare and easy-to-scale-up formulation. It is a nanomedicine hydrogel highly biocompatible, injectable and adapted for an application in the brain. However, to fight a tumor as aggressive and heterogeneous as GBM, combining different active agents seem a more promising strategy than single chemotherapy approach (Ghosh et al., 2018).

Therefore, the objective of this work was to evaluate if $GemC_{12}$-LNC

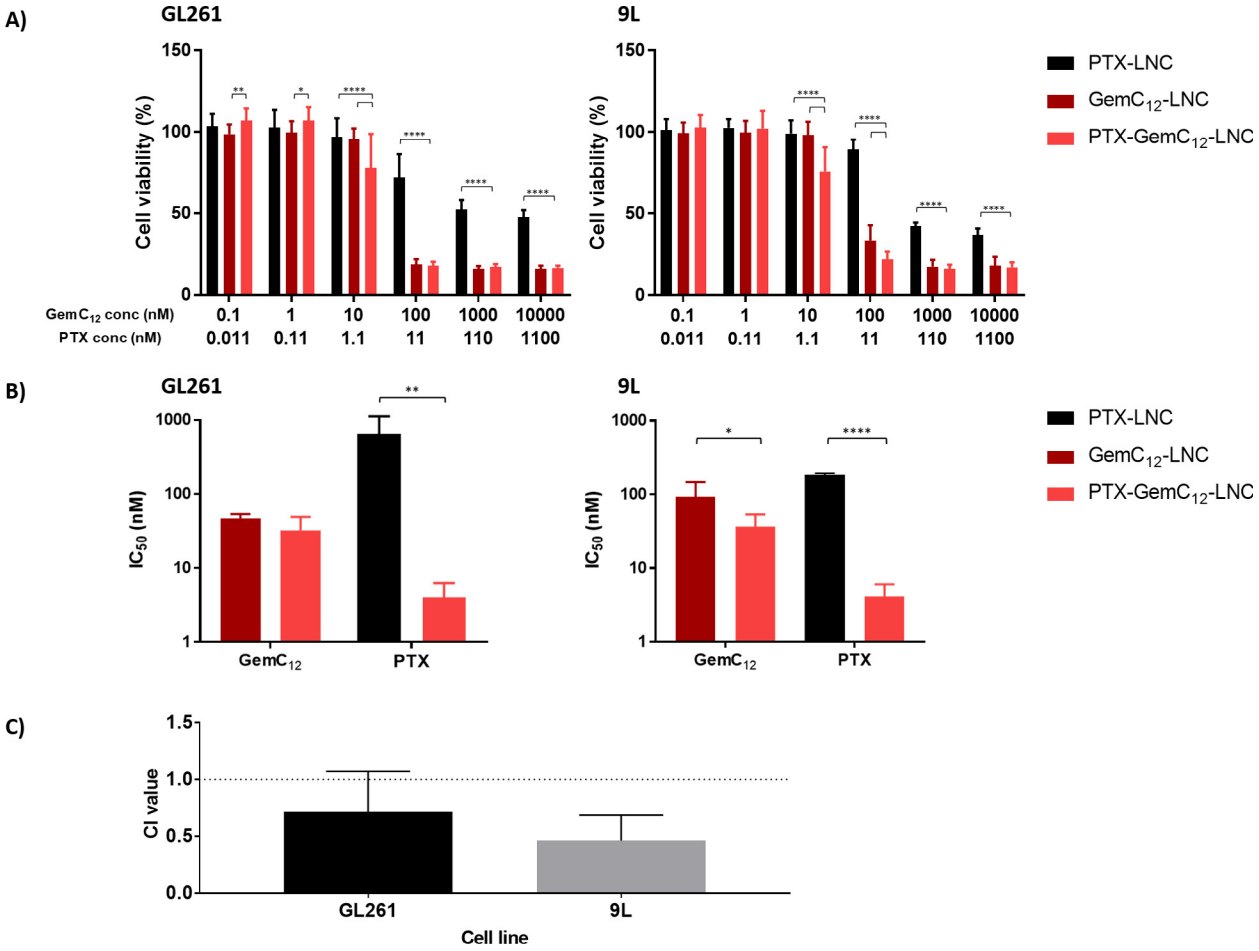

Fig. 5. *In vitro* cytotoxic assay on GL261 (left) and 9L (right): (A) cell viability after 48 h of incubation with the different treatments (PTX-LNC, $GemC_{12}$-LNC and PTX-$GemC_{12}$-LNC); (B) IC_{50} values of $GemC_{12}$ and PTX either alone ($GemC_{12}$-LNC and PTX-LNC formulations) or in combination (PTX-$GemC_{12}$-LNC formulation); (C) calculated CI values (N = 3, n = 16; mean ± SD; two-way ANOVA test with Tukey post-test $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$).

can be used as nanodelivery platform for other drugs, to obtain a combined local therapeutic approach for GBM. PTX was used as a model drug for this scope. We have demonstrated that adding a second lipophilic molecule into the LNC oily core does not alter the stability, degradability, physicochemical and mechanical properties of the hydrogel. *In vitro*, PTX and $GemC_{12}$ show combined efficacy and synergy on GBM cells. Therefore, this combination could be promising for the local treatment of GBM. Further studies will be conducted to select other molecules to be combined with $GemC_{12}$ for the treatment of GBM or for other applications.

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