Local Chemotherapy

Liposomes Encapsulated in an Injectable Hydrogel for Hyperthermia-Induced Drug Delivery from Thermosensitive Liposomes

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One of the main challenges in cancer treatment is the administration of active doses of chemotherapeutic to a tumor site while minimizing severe side effects. On this basis, researchers have developed different materials for achieving both spatial and temporal effective release of the active molecule at the therapeutic target.[1–5] Among these materials, the locoregional administration of drug-loaded, in situ gelling hydrogels overcomes the pharmacokinetic restrictions of intravenous injection and effectively enhances the therapeutic ratio.[6,7] Following this principle, the present work describes the design of a composite material, which enables a thermally triggered and localized release of a chemotherapeutic (doxorubicin), achievable through incorporation of drug-loaded thermosensitive liposomes in a thermoresponsive chitosan/β-glycerophosphate (C/β-GP) hydrogel for local treatment. Doxorubicin (DOX) is sequentially released from the gel by 1) passive diffusion of entrapped free drug and a small portion of drug-loaded liposomes, and 2) external thermal activation of the drug-loaded liposomes irreversibly trapped in the gel. The effect of this on-demand scheduled dosing is assayed in vitro with human ovarian carcinoma cells, and is proposed as a way to challenge some of the compensatory mechanisms available to tumor cells. By reducing the exposure to sublethal doses of chemotherapeutic, the growth of cells with a short doubling time is inhibited while also potentially avoiding the development of drug resistance.[8–10]

Our proposed approach combines the in situ gelation of thermoresponsive C/β-GP hydrogels and the on-demand release achievable using thermosensitive liposomes, with the aim of providing a localized, optimal delivery of chemotherapeutic. C/β-GP-based gelling systems have been widely studied because of their biocompatible and biodegradable properties.[11,12] However, a feature which makes these hydrogels especially attractive is that they can be formulated as a syringable solution at working temperatures that undergoes a gelation at body temperature, enabling a minimally invasive delivery and localized cohesion and release of encapsulated agents. Studies investigating intratumoral injections of anticancer drug-releasing C/β-GP hydrogels in vivo have shown encouraging results.[13] Lysolipid thermally sensitive liposomes (LTSLs) are bilayered spherical vesicles that rapidly change structure upon mild hyperthermia (41–43 °C), creating openings in the liposome, which release the drug payload.[14] DOX has been efficiently loaded into LTSLs by the pH gradient method,[15] in which the creation of a transmembrane proton gradient induces the accumulation of the drug into the acidic interior of the liposome. This mechanism of drug uptake also allows a pH-sensitive release when the liposome is subjected to an acidic pH, such as in the endosomal compartments after cell internalization.[16]

The combination of C/β-GP hydrogels with DOX-loaded LTSLs gives rise to a homogeneous dispersion (Scheme 1a) that, upon local injection, will become a cross-linked gel entrapping the liposomes (Scheme 1c). As a result of its composition, the release of drug from this formulation, denoted Lipogel, demonstrates a multistep profile. Initially, a rapid increase in DOX release above a therapeutic concentration takes place due to a combination of the diffusion of free drug from the gel bolus and a limited release from encapsulated liposomes. Afterward, to maintain levels of released DOX within an optimal and efficacious concentration window, liposomal release can

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be activated externally through minimally invasive application of hyperthermia treatments (Scheme 1e) using radiofrequency, microwaves or high-intensity-focused ultrasound (HIFU). Precisely controlling the drug release profile from the hydrogel in this way may enhance efficacy against tumor cells with minimal systemic side effects. In addition, the inclusion of hypertermic stimulation in the treatment may enable chemosensitization in the tumor.[20,21]

DOX-loaded thermosensitive liposomes (hydrodynamic diameter: 250 nm) with a transition temperature of 41 °C were prepared. 10% of the DOX within the liposome dispersion was unencapsulated (Supporting Information), thus enabling an initial burst release from liposome-entrapping gels. Next, the liposomes were dispersed within a chitosan/β-GP solution and upon heating to 37 °C, a stable gel was formed (Figure 1a). Rheological measurements of the hydrogels are presented in Figure 1b. As shown, the introduction of the liposomes into the gel reduced the temperature of gelation from 35 to 33 °C. However, this is well within an acceptable range, whereby gels are liquid and syringable at working temperatures of ≈25 °C.

Discrete release at different time points up to 7 d is presented in Figure 1c. An early burst effect is displayed by Lipogel at a constant 37 °C, as a result of unencapsulated DOX in combination with a fraction of DOX released from liposomes under physiological conditions. Thereafter, the release rate steadily decreases until the end of the assay. In contrast, the amount of DOX released from the materials significantly increases after incubation at 42 °C for 1 h at day 2, due to the activation of the thermosensitive liposomes (approximately sevenfold increase in release compared with non-pulsed samples at day 3). This fact indicates that both liposomal temperature sensitivity and structural integrity are maintained in the Lipogel environment. As a control, it was shown that hydrogels loaded with free DOX (without liposomes) did not have an enhanced release at 42 °C, and that free DOX was rapidly released from the gel at 37 °C (Figure S5, Supporting Information), in contrast with the controlled release achievable with Lipogel.

The cumulative release profile from the samples is displayed in Figure 1d. After 2 d, when DOX levels have reached a plateau, triggered release provides further dosing for 3 d. Additionally, as mentioned above, although the majority of the thermosensitive liposomes is irreversibly trapped in the gel, a certain amount of them diffuse through the large pores of the C/β-GP matrix. This phenomenon has been observed before,[22] and can be tuned by changes in the liposome size. For our selected hydrodynamic diameter, approximately 20% of the liposomes are released from Lipogel in a sustained way over 7 d (Supporting Information). These liposomes are intact, as confirmed by DLS measurements (data not shown), and have the capability to release their DOX content upon cellular internalization due to the pH changes in the endocytic apparatus of the cell.[16,23]

Ideally, the initial efficacy produced by the burst release from Lipogel would initiate apoptosis in a significant portion of gel-exposed tumor cells, resembling a tumor priming mechanism.[24–26] Thereafter, a fraction of released liposomes could further penetrate into deep areas of the solid tumor by maximizing the drug delivery distance from the gel implant, the release of these liposomes could overcome a major limitation to the efficacy of intratumoral treatments.[10]

In order to assess the bioactivity of the Lipogel formulation, human A2780 ovarian carcinoma cells were incubated along with gels added to hanging inserts above the cell culture well. In this way, DOX and DOX-loaded liposomes released from the sequestered gels diffuse across the insert membrane into the growth media. The growth and viability of cells were assessed visually, through Live/Dead staining (Figure 2a) and quantitatively via a PicoGreen double-stranded (ds)DNA assay (Figure 2b). After 48 h incubation, a significant decrease in dsDNA was observed, demonstrating the efficacy of the first passive diffusion of free DOX and drug-loaded liposomes from
Afterward, the gel-containing inserts were transferred into another well with nontreated cells to independently evaluate the activity of DOX released after the external thermal activation of the drug-loaded liposomes irreversibly entrapped in the gel. Two days after a 1-h hyperthermic pulse at 42 °C, the efficacy of the gels was drastically enhanced when compared with non-pulsed samples. In fact, pulsed Lipogel reduced dsDNA to levels comparable to a free DOX control, in which cells were incubated with free DOX for 4 d. The heat pulse itself did not result in a significant reduction in cell viability. These results demonstrate that efficacy was effectively extended and enhanced through hyperthermia-triggered release and that DOX released from Lipogel retained its full bioactivity.

In summary, here we have reported the design of a novel thermosensitive liposome/hydrogel composite that can facilitate an on-demand, localized release of chemotherapeutics. This system enables a local control of anticancer drug scheduling and sequencing, which are key parameters in oncological treatments. The possibility to tune the release profiles of different therapeutics independently without the compounded side effects associated with combination therapies, may lead to more powerful oncologic regimes and synergistic treatment options.

**Experimental Section**

Fabrication of DOX-Loaded Hyperthermia-Sensitive Liposomes:

Thermosensitive liposomes were prepared as described by Negussie et al. with slight changes. Briefly, dipalmitoyl phosphatidylcholine (DPPC), monostearoyl phosphatidylcholine (MSPC), and distearoyl phosphatidylethanolamine-poly(ethylene)glycol 2000 (DSPE-PEG2000) in a molar ratio of 85.3:9.7:5.0 were dissolved in chloroform and a lipid film was formed in a rotavapor under vacuum, at 40 °C. The film was kept under N2 gas in order to remove the remaining solvent residue. Liposomes were prepared by hydrating the lipid film with 300 × 10⁻³ M citrate buffer (pH 4.0) at 60 °C, aiming for a final lipid concentration of 50 mg mL⁻¹. The resulting liposomes were extruded through polycarbonate membrane filters at 60 °C to achieve a final liposome size of 250 nm and a PDI of 0.18, as measured by DLS using a Malvern CGS-3 multangle goniometer (Malvern Ltd., Malvern, UK, with a JDS Uniphase 22 mW He-Ne laser operating at 632 nm, an optical fibre-based detector.
Figure 2. a) Fluorescent images of A2780 ovarian carcinoma cells, which were live/dead stained after exposure to Lipogels with and without the hyperthermic pulse. Free DOX was employed as positive control. b) dsDNA levels of the same samples, as assessed by PicoGreen assay. A significant reduction in cell number is apparent in samples treated with free DOX and Lipogel at day 2 or day 4 after a pulse.
and a digital LV/LSE-5003 generator, measurement angle: 90°). In order to create a pH gradient for active DOX loading, the exterior of liposomes in the resulting suspension was neutralized to pH 7.4 by adding 500 × 10^{-3} \text{ M} sodium carbonate buffer. Subsequently, liposomes were loaded with DOX by incubation at 37°C for 1 h (DOX: lipid 5:10 w/w, encapsulation efficiency > 90%). The unencapsulated DOX molecules were to a large extent removed from the resulting suspension by PD10 column purification, while permitting 10% of the DOX within the resulting liposome dispersion to remain unencapsulated.

**Preparation of Chitosan/β-glycerophosphate Gels:** Preparation of thermoresponsive C/β-GP gels has been described elsewhere.[20] Briefly, ultra-pure chitosan (100 mg, UP CL214 from Pronova Biomedical, Norway) was dissolved in dH_{2}O (4.5 mL) at pH 8–9. β-Glycerophosphate (350 mg) was dissolved in dH_{2}O (0.5 mL) also at pH 8–9 and added dropwise to the chitosan solution. To each gel (5 g), the liposome dispersion (208 μL) was added and gently mixed, corresponding to a final DOX concentration of 116 μg per gram of gel and a final lipid concentration of 2.5 mg per gram of gel. Gels containing free DOX were prepared by dissolving DOX in the constituent dH_{2}O, prior to gel preparation, to give a final concentration of 116 μg per gram of gel.

**Rheological Testing:** The rheological properties of chitosan/β-GP gels were assessed using oscillatory measurements on an AR-1000 cone and plate rheometer (TA Instruments). The thermoresponsiveness of the gels was assessed as a function of temperature, with storage modulus (G') being used as an indicator of gel structure. The temperature was increased by 1°C min^{-1} using a temperature sweep mode extended between 20 and 50°C at a frequency of 0.5 Hz.

**DOX Release from Liposome-C/β-GP Hydrogel Composites:** The lipogel formulation (1 g) at room temperature was transferred into a glass vial and allowed to gel in a water bath for 1 h at 37°C. Next, Roswell Park Memorial Institute (RPMI-1640) media (2 mL, Sigma-Aldrich, Ireland) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich, Ireland) was added and gels were incubated at 37°C while shaking at 100 rpm. Media were removed at given time points (4 h, 1, 2, 3, 5, and 7 d) and replaced with fresh prewarmed media. After removing the medium at 2 d, media at 42°C were added to a set of samples, which were incubated at 42°C for 1 h and later incubated at 37°C for the rest of the study. All release media samples were ultracentrifuged (45 000 rpm, 10 min), and the supernatant was analyzed for DOX content via high-performance liquid chromatography (HPLC) on an Agilent 1120 Compact LC with a Phenomenex Gemini 2.0 μm 5 μm Durapore PVDF filters (Millipore, Ireland) before measurement. The resulting suspension was neutralized to pH 7.4 by adding 0.45 M sodium carbonate buffer. Subsequently, liposomes were loaded with DOX by incubation at 37°C for 1 h before incubation at 42°C for a further 2 d. Inserts containing Lipogel were placed in the wells, and media in the plates were replaced (1 mL) at hourly intervals, 300 μL apically. Cells exposed to an equivalent quantity of DOX diluted in RPMI (1.3 mL), or untreated cells acting as positive and negative controls, respectively. Samples were analyzed at 48 h or at this point underwent a hypertermic pulse (42°C) for 1 h before incubation at 37°C for a further 2 d. Inserts containing Lipogel were transferred to new wells after 48 h, to enable a complete independent examination of the efficacy of the initial and second burst release of DOX from the formulation, with and without a hyperthermic pulse. Live/Dead stain, which stains live cells green and dead cells red, was used to assess cytotoxicity visually, while a PicoGreen dsDNA assay was used to quantitatively assess viable cell numbers (whereby levels of dsDNA are utilized as a surrogate measure for the levels of viable cells per well), both according to the manufacturer’s instructions. All samples were assayed in triplicate.

**Statistical Analysis:** Two-way ANOVA followed by pairwise Holm–Sidak analysis was performed. Error is reported as standard deviation (SD) and significance was determined using a probability value of P < 0.05. A minimum of N = 3 replicates were performed for all experiments.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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