Hyperthermia-induced drug delivery from thermosensitive liposomes encapsulated in an injectable hydrogel for local chemotherapy.

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Citation
Hyperthermia-induced drug delivery from thermosensitive liposomes encapsulated in an injectable hydrogel for local chemotherapy


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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.[1-4] 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.[5, 6] 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.\textsuperscript{[7-9]}

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.\textsuperscript{[10, 11]}

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.\textsuperscript{[12]} 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.\textsuperscript{[13]}

DOX can be efficiently loaded in LTSLs by the pH gradient method,\textsuperscript{[14]} 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 acid pH, such as in the endosomal compartments after cell internalization.\textsuperscript{[15]}

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 crosslinked 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.
Afterwards, to maintain levels of released DOX within an optimal and efficacious concentration window, liposomal release can be activated externally through minimally invasive application of hyperthermia treatments (Scheme 1E) using radiofrequency, microwaves or high-intensity focused ultrasound (HIFU).\textsuperscript{[16-18]} 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 hyperthermic stimulation in the treatment, may enable chemosensitization in the tumor.\textsuperscript{[19, 20]}

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 (supplementary data) 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°C 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 days 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 when applying an external 42°C pulse for 1 hour at day 2, due to the activation of the thermosensitive liposomes (~ 7-fold increase in release compared to 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 37ºC (Figure S5), in contrast with the controlled release achievable with Lipogel.

The cumulative release profile from the samples is displayed in Figure 1D. After 2 days, when DOX levels have reached a plateau, triggered release provides further dosing for 3 days. Additionally, as mentioned above, although the majority of the thermosensitive liposomes is irreversibly trapped in the gel, a certain amount of them diffuses through the large pores of the C/β-GP matrix. This phenomenon has been observed before, 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 days (supplementary data). 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. 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. 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 on the efficacy of intratumoral treatments.

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 and 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 hours incubation, a significant decrease in dsDNA was observed, demonstrating the efficacy of the first passive diffusion of free DOX and drug-loaded liposomes from the gel. Afterwards, the gel-containing inserts were transferred to another well with intact cells to independently evaluate
the activity of DOX released after the external thermal activation of the drug-loaded liposomes irreversibly trapped in the gel. Two days after a one hour hyperthermic pulse at 42°C, the efficacy of the gels was drastically enhanced when compared to non-pulsed samples. In fact, pulsed Lipogel reduced dsDNA to levels comparable to a free DOX control, in which cells were incubated continuously with free DOX for four days. The heat pulse itself did not produce a significant reduction in cell viability. This result demonstrates that efficacy was effectively extended and enhanced through hyperthermia-triggered release and that DOX released from Lipogel maintained 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 drugs 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.\cite{26, 27}

**Experimental Section**

Fabrication of DOX-loaded hyperthermia sensitive liposomes: Thermosensitive liposomes were prepared as described by Negussie *et al*.\cite{28, 29}, 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 mM citrate buffer (pH 4.0) at 60°C, aiming for a final lipid concentration of 50 mg/mL. 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 multiangle goniometer (Malvern Ltd., Malvern, U.K., with a JDS Uniphase 22 mW He-Ne laser operating at 632 nm, an optical fibre-based detector and a digital LV/LSE-5003 generator, measurement angle 90º). 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 mM sodium carbonate buffer. Subsequently, liposomes were loaded with DOX by incubation at 37°C for 1 hour (DOX: lipid 5:100 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 [30]. Briefly, 100 mg of ultra-pure chitosan (UP CL214 from Pronova Biomedical, Norway) was dissolved in 4.5 mL dH₂O at pH 8-9. 350 mg of β-glycerophosphate was dissolved in 0.5 mL dH₂O also at pH 8-9 and added dropwise to the chitosan solution. To each 5 g of gel, 208 µL of liposome dispersion was added and gently mixed, corresponding to a final DOX concentration of 116 µg/g gel and a final lipid concentration of 2.5 mg/g gel. Gels containing free DOX were prepared by dissolving DOX in the constituent dH₂O, prior to gel preparation, to give a final concentration of 116 µg/g gel concentration.

*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 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: 1 g of Lipogel formulation at room temperature was transferred into a glass vial and allowed to gel in a water bath for one hour at 37°C. Next, 2 mL of Roswell Park Memorial Institute (RPMI-1640) media (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 whilst shaking at 100 rpm. Media were removed at given time points (4 hours, 1, 2, 3, 5 and 7 days) and replaced with fresh pre-warmed media. After removing the medium at 2 days, media at 42°C were added to a set of samples, which were incubated at 42°C for one hour 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 5u C18 column, mobile phase (95% KH₂PO₄ 20mM pH=3, 5% acetonitrile):acetonitrile (75%:25% v/v), UV detection at 480 nm. In order to quantify the intact liposomes released from Lipogel, 1 mL of a 0.5% (v/v) Triton X100 solution was added to the pellet remaining after aspiration of centrifuged release media, gently mixed and then analyzed by HPLC. All samples were analyzed in triplicate and filtered with 0.45 μm Durapore PVDF filters (Millipore, Ireland) before measurement.

Assessment of bioactivity of DOX released from Lipogel: A2780 ovarian carcinoma cells were cultured in identical media as that utilized in the DOX release study at 37°C and in a 5% CO₂ environment. To determine the bioactivity of Lipogel-released DOX, cells were seeded into 24-well plates at a density of 100,000 cells per well with the addition of 500 μL of RPMI-1640 media. The cells were incubated overnight to allow adherence to occur. 100μL of Lipogel was added to transwell cell culture inserts with a pore size of 8μm (Millipore) and permitted to thermogelate at 37°C for one hour. Medium was removed from wells containing A2780 cells, inserts containing Lipogel were placed in the wells and media in the wells were replaced (1mL basolaterally, 300μL apically). Cells exposed to an equivalent quantity of
DOX diluted in 1.3 mL RPMI, or untreated cells acted as positive and negative controls, respectively. Samples were analyzed at 48 hours or at this time point underwent a hyperthermic pulse (42°C) for one hour before incubation at 37°C for a further 2 days. Inserts containing Lipogel were transferred to new wells after 48 hours, to enable completely 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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Scheme 1. A) Lipogel is fully syringable and is conceived as a local injectable, consisting of a chitosan/β-GP thermoresponsive gel hosting a suspension of DOX-loaded thermosensitive liposomes. B) Release from the gel in situ is controllable using minimally invasive hyperthermia, achievable using a modality such as high intensity focused ultrasounds. A small portion of drug-loaded liposomes is released from Lipogel maximizing the drug delivery distance from the gel implant. C) The majority of liposomes are locked into the gel upon initiation of crosslinking during thermogelation. D, E) Liposomes sequester the majority of drug at body temperature, but rapidly become more permeable upon mild hyperthermia and release their drug payload.
Figure 1. A) Photographs of Lipogel before (20ºC) and after (37ºC) gelation. B) Storage modulus of a regular chitosan/glycerophosphate (C/β-GP) gel and a DOX-loaded liposome/hydrogel composite (Lipogel) as a function of temperature. Gels containing liposomes began to thermorespond at 33ºC while unaltered gels thermoresponded at 35ºC. C) and D) Non-cumulative and cumulative DOX release profiles from Lipogel with and without a one hour pulse at 42ºC, indicated by the arrow. Given that ~20% of the drug-loaded liposomes are released from the gel over 7 days (supporting information), a significant fraction (around 40%) of DOX remains trapped in the pulsed Lipogel after 7 days, probably due to electrostatic interactions [31].
Figure 2. A) Fluorescent micrographs 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.
A novel drug delivery system, enabling an in situ, thermally triggered drug release is described, consisting of an injectable thermoresponsive chitosan hydrogel containing doxorubicin-loaded thermosensitive liposomes. The design, fabrication, characterization and an assessment of in vitro bioactivity of this formulation is detailed. Combining on-demand drug delivery with in situ gelation has resulted in a promising candidate for local chemotherapy.

Drug delivery systems


Hyperthermia-induced drug delivery from thermosensitive liposomes encapsulated in an injectable hydrogel for local chemotherapy
Supporting Information

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Figure S1. Differential scanning calorimetry (TA Instruments DSC Q2000 apparatus) thermogram of doxorubicin-loaded liposomes showing phase transition temperature at 41°C.
Figure S2. Hydrodynamic size distribution of extruded liposomes by dynamic light scattering (DLS).

Figure S3. An analysis of DOX release from thermosensitive liposomes in PBS. Liposome dispersion was held at 37° C or 42° C for up to 60 minutes. Up to 65% of encapsulated DOX was released within 60 minutes.
Figure S4. Rheological characterization of different hydrogel compositions as a function of time, keeping temperature constant at 37°C. Unmodified gels or gels containing free DOX or liposomal DOX (same DOX concentration) all demonstrated thermoresponsive behaviour. Both free DOX and liposomal DOX produced a reduction in the maximum storage modulus achieved. However, all gels resisted dissolution when incubated in aqueous media and resisted disruption upon handling.
Figure S5. Cumulative DOX release from chitosan/β-GP gels containing free DOX maintained at 37°C or 42°C, or gels containing liposomal DOX (same DOX concentration) maintained at 42°C. Release at 42°C from free DOX and liposomal DOX gels was almost identical, suggesting that liposomes were able to release all encapsulated DOX at this temperature. Interestingly, free DOX gels maintained at 37°C demonstrated a more rapid release of DOX, which is attributable to a lesser degree of polymer network organization at this lower temperature, and the resultant effect on drug diffusion [1]. Changes in gel strength as a function of temperature are represented by the variation of the storage modulus in Figure 1B in the main text.
**Figure S6.** Sustained release of intact DOX liposomes from a chitosan/β-GP gel at 37°C over seven days. In order to quantify the liposomes released from Lipogel, a 0.5% (v/v) Triton X100 solution was added to the pellet remaining after aspiration of centrifuged media obtained in the release studies, gently mixed and then analyzed by HPLC. Release is expressed as a percentage of the total amount of liposomes (total DOX concentration) initially encapsulated within the gel.

**Figure S7.** An analysis of DOX release from thermosensitive liposomes at pH 5, as an approximation of release upon endosomal integration. Liposome dispersion was held at 37°C
in 120 mM ammonium acetate buffer for the duration of the experiment. DOX uptake induced by pH gradients implies its accumulation in the acidic interior of the liposome until

\[
[\Delta H^+]_{\text{in}}/[\Delta H^+]_{\text{out}} = [H^+]_{\text{in}}/[H^+]_{\text{out}} \quad [22]
\]

Therefore, liposomes produced a rapid release of DOX in response to reduced pH, meaning that tumor cells which internalize released liposomes could be exposed to encapsulated DOX upon endocytosis.

**Figure S8.** A comparison of non-cumulative and cumulative DOX release profiles from Lipogel in supplemented cell culture medium and PBS with a one hour pulse at 42°C, indicated by the arrow. Almost identical profiles are found, suggesting that serum proteins do not affect DOX release from the gels.