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A stimuli responsive liposome loaded hydrogel provides flexible on-demand release of therapeutic agents

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ABSTRACT

Lysolipid-based thermosensitive liposomes (LTSL) embedded in a chitosan-based thermoresponsive hydrogel matrix (denoted Lipogel) represents a novel approach for the spatiotemporal release of therapeutic agents. The entrapment of drug-loaded liposomes in an injectable hydrogel permits local liposome retention, thus providing a prolonged release in target tissues. Moreover, release can be controlled through the use of a minimally invasive external hyperthermic stimulus. Temporal control of release is particularly important for complex multi-step physiological processes, such as angiogenesis, in which different signals are required at different times in order to produce a robust vasculature. In the present work, we demonstrate the ability of Lipogel to provide a flexible, easily modifiable release platform. It is possible to tune the release kinetics of different drugs providing a passive release of one therapeutic agent loaded within the gel and activating the release of a second LTSL encapsulated agent via a hyperthermic stimulus. In addition, it was possible to modify the drug dosage within Lipogel by varying the duration of hyperthermia. This can allow for adaption of drug dosing in real time. As an in vitro proof of concept with this system, we investigated Lipogels ability to recruit stem cells and then elevate their production of vascular endothelial growth factor (VEGF) by controlling the release of a pro-angiogenic drug, desferroxamine (DFO) with an external hyperthermic stimulus. Initial cell recruitment was accomplished by the passive release of hepatocyte growth factor (HGF) from the hydrogel, inducing a migratory response in cells, followed by the delayed release of DFO from thermosensitive liposomes, resulting in a significant increase in VEGF expression. This delayed release could be controlled up to 14 days. Moreover, by changing the duration of the hyperthermic pulse, a fine control over the amount of DFO released was achieved. The ability to trigger the release of therapeutic agents at a specific timepoint and control dosing level through changes in duration of hyperthermia enables sequential multi-dose profiles.

Statement of Significance

This paper details the development of a heat responsive liposome loaded hydrogel for the controlled release of pro-angiogenic therapeutics. Lysolipid-based thermosensitive liposomes (LTSLs) embedded in a chitosan-based thermoresponsive hydrogel matrix represents a novel approach for the spatiotemporal release of therapeutic agents. This hydrogel platform demonstrates remarkable flexibility in terms of drug scheduling and sequencing, enabling the release of multiple agents and the ability to control drug dosing in a minimally invasive fashion. The possibility to tune the release kinetics of different drugs independently represents an innovative platform to utilise for a variety of treatments. This approach allows a

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significant degree of flexibility in achieving a desired release profile via a minimally invasive stimulus, enabling treatments to be tuned in response to changing symptoms and complications.

1. Introduction

The use of single factor delivery to treat various pathological and disease states thus far has proven insufficient from a clinical standpoint. This is hardly surprising given the complexity of many disease states healing processes. It is apparent that biological phenomena such as angiogenesis, inflammation and tissue remodelling are complex and interlinked processes that are controlled in a spatiotemporal manner [1–6]. It is evident, therefore, that there is a clear need to engineer biomaterials that can control the release of factors over time. Materials capable of controlling the release of multiple factors such that there is a differential release of specific factors over time can mimic the spatiotemporal nature of biological processes. In addition, numerous pathological conditions require dosing of either a biologic or small molecule which can be difficult to predict a priori and may need to be actively managed. The ability to control in real-time may allow for the 'dialling in' of the dose needed at each instance in time.

Lysolipid-based thermosensitive liposomes (LTSLs) can release an encapsulated drug in response to a locally applied mild hyperthermic stimulus, and have been shown to enhance efficacy when compared to equivalent non-responsive traditional delivery systems [7,8]. The combination of LTSLs within an *in situ* gelling hydrogel based on chitosan has previously been shown to allow controllable and localised release of chemotherapeutics, positively influencing *in vitro* efficacy over an extended period [9]. We sought to expand on this system and assess the ability of this combination of LTSLs and chitosan hydrogel (denoted Lipogel), investigating the ability to manipulate the release kinetics of the delivery platform. As a proof of concept, we used therapeutic angiogenesis, which requires a combinational approach that can be modified 'on demand'. One of the main challenges of combined therapy is to control the release behaviour of each drug seperately. As combination therapies with multiple drugs have been routinely used in many cases of clinical treatment to improve outcomes, significant interest has been shown in the investigation of new approaches and systems for differential release of multiple drugs from a single on-demand delivery system [10,11]. A number of growth factor combinations have been attempted previously demonstrating enhanced functionality compared to single therapeutic treatment [12–16]. Previous studies have demonstrated that combining hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) *in vivo* can induce a stronger angiogenic response then either agent alone [17–20]. We elected to deliver free loaded HGF in combination with on-demand deferroxamine (DFO) release to facilitate increased VEGF response in MSCs to demonstrate the potential of Lipogel to permit dual release kinetics as a pro-angiogenic therapeutic platform.

Most traditional drug delivery systems are engineered to yield a sustained release of bioactive compounds [21]. Emergent clinical situations may create a need for more advanced delivery systems, which require significantly different drug doses over short periods, and in some cases, circadian drug timing to increase overall effectiveness [22,23]. The majority of "smart" materials that are activated by external stimuli (e.g. magnetic fields, radio waves or light) potentially offer this flexibility [24–27]. In particular, ultrasound has been used to trigger release from liposomes surrounded by a hydrogel matrix [28]. We hypothesized that altering the timing of hyperthermia (short duration to long duration) exposure of thermosensitive liposomes embedded in chitosan hydrogels would result in multiple drug doses. Mild hyperthermia would preserve the hydrogel structure while permitting multi-dose release kinetics.

2. Materials and methods

2.1. Preparation and rheological testing of chitosan/β-GP gels

Ultra-pure chitosan with a degree of deacetylation >95% (UP CL214 from Pronova Biomedical, Oslo, Norway) was used for the preparation of the different gels. For a 2% w/v chitosan/7% w/v β-glycerophosphate (β-GP) gel, 100 mg of chitosan was dispersed in 4.5 mL distilled H_2O at pH 8–9 and 350 mg of β-GP (Sigma Aldrich, Ireland) was dissolved in 0.5 mL distilled H_2O, also at pH 8–9, and chilled. The β-GP solution was then added drop by drop to the chitosan solution with stirring on ice to achieve a homogenous gel. Gels were stored on ice until use soon after. Gels containing free-loaded DFO were prepared by dissolving DFO (Sigma, Ireland) to the correct concentration in the constituent water prior to addition of chitosan or β-GP.

The rheological properties of chitosan/β-GP gels were assessed using oscillatory measurements on an AR-1000 parallel plate rheometer (TA Instruments). The thermoresponsiveness of the gels was assessed as a function of temperature and over time at a constant temperature of 37 °C, with storage modulus G′ being used as an indicator of gel stiffness. The temperature was increased by 1 °C/min from 20 to 50 °C using a temperature sweep mode.

2.2. Fabrication and characterisation of DFO-loaded thermosensitive liposomes

Thermosensitive liposomes were prepared as described by Negussie et al., [29,30] with slight changes. Liposomes were composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (DSPE-PEG) and monostearoylphosphatidyl choline (MSPC). Briefly, DPPC, MSPC and DSPE-PEG2000 (Avanti Polar Lipids, USA) in a molar ratio of 85.3:9.7:5.0 were dissolved in chloroform/methanol and a lipid film was formed in a rotary evaporator at 60 °C. The film was exposed to a N_2 gas flow in order to remove solvent residues. DFO-loaded liposomes were prepared by hydrating the lipid film with a DFO solution in imidazole buffer (220 mM DFO, 50 mM imidazole, pH 7.4) at 60 °C, aiming for a final lipid concentration of 50 mg/mL. For liposomes <200 nm, multilamellar liposomes were initially sized by extrusion (5 times) with a LIPEX™ Extruder (Northern Lipids Inc., Canada) through two stacked Nuclepore® polycarbonate membrane filters (Whatman plc, United Kingdom) with a pore size of 800 nm and then sized by extrusion (10 times) with a pore size of 200 nm.

Differential scanning calorimetry (DSC) was utilised to determine the gel-to-liquid transition temperature (T_m) of liposomes on a TA Instruments DSC Q2000 apparatus. Samples of ~10 mg in closed aluminium pans were heated from room temperature to 60 °C with a heating rate of 2 °C min^{-1}. Drug release over the Tm was confirmed by incubating liposome dispersion in PBS at 37 °C or 42 °C, for up to 60 min. Samples were then ultracentrifuged (45,000 rpm, 10 min, 4 °C), and the supernatant was analysed for DFO content via high-performance liquid chromatography

(HPLC) on an Agilent 1120 Compact LC with a Phenomenex Gemini 5u C18 column, mobile phase acetonitrile: phosphate buffer (10%:90% v/v), containing 20 mM ethylenediaminetetraacetic acid (EDTA), pH adjusted to 6.5 and UV detection at 440 nm with an injection volume of 5 μl. All samples were filtered with 0.45 μM Durapore PVDF filters (Millipore, Ireland) and combined with 4 mM iron (lll) chloride (Sigma Aldrich, Ireland) in a 1:1 ratio immediately before analysis.

2.3. Preparation of DFO/HGF loaded chitosan/β-GP gels

Gels were prepared and characterised as described above. For every 5 g of gel solution, 208 μl of liposome dispersion was added and gently mixed, corresponding to a final liposome encapsulated DFO concentration of 100 μM/g gel. Gels containing free DFO were prepared by dissolving DFO in the constituent distilled H_2O, prior to gel preparation, to give a final concentration of free DFO of 100 μM/g gel. For dual release samples, DFO was added as described above. HGF (R&D Systems) was dissolved in PBS at a concentration of 10 μg/ml. 100 μl of HGF was added and gently mixed, corresponding to a final HGF concentration of 1 μg/g gel.

2.4. Assessment of HGF/DFO release from chitosan/β-GP gels

To measure DFO release, 1 g of chitosan/β-GP containing free or liposomal DFO was added to a glass vial and allowed to gel in a water bath for one hour at 37 °C. 1 mL PBS was added and gels were allowed to incubate at 37 °C whilst shaking at 100 rpm. PBS was removed daily up to 2 weeks and replaced with fresh pre-warmed (37 °C) PBS. After removing the release media at days 2, 6 and 10, PBS at 42 °C was added to a set of samples, which were then incubated at 42 °C (hyperthermic pulse) for one hour and subsequently incubated at 37 °C for the rest of the study.

For assessment of release from samples that were exposed to multiple hyperthermic pulses, 100 μM DFO Lipogel samples were prepared, 1 ml PBS was added and gels were allowed to incubate at 42 °C or 37 °C with a 1, 2, 5, 10 or 30 min 42 °C hyperthermic pulse whilst shaking at 100 rpm for 24 h.

To investigate the ability to release multiple doses of DFO, gels were prepared as above. At days 1, 2, 3 and 4 the release media were removed and PBS at 42 °C was added. Samples were then incubated at 42 °C for a mixed sequence of 1,3,3 and 3 min on 4 consecutive days or 1,3,10 and 30 min on 4 consecutive days and later incubated at 37 °C for the rest of the study.

All release media samples were ultracentrifuged (45,000 rpm, 10 min, 4 °C), and the supernatant was analysed for DFO content via HPLC as described above. HGF release was assessed by ELISA according to the manufacturer's instructions (Quantikine, R&D systems). 50 μl samples were tested in duplicate and compared to a standard curve. Growth factor concentration in the release buffer could then be calculated

2.5. Hydrogel temperature mapping by MRI

All MRI experiments were performed on a clinical 1.5-Tesla MR scanner (Achieva; Philips Health care, Best, The Netherlands) with an 8 elements head coil. A 50 mL Falcon tube was filled with a 2% chitosan/7% β-GP hydrogel and placed parallel to the main magnetic field (B0) in the middle of the head coil inside a water bath. The water of the water bath can be heated via a tubing system and a heater/pump outside the Faraday cage of the MRI. Parallel to the chitosan/β-GP hydrogel tube a 50 mL Falcon tube filled with 2% agar gel was placed. The agar gel tube was thermally insulated from the water bath and served as a constant temperature reference.

The chitosan/β-GP hydrogel heating was monitored with the MR thermometry using the proton resonance frequency (PRF) shift method. Time-resolved MR temperature imaging was performed using a gradient echo sequence. Relevant scan parameters were: TR = 37.6 ms; TE = 19.5 ms; FA = 19.5°; EPI-factor = 11; Field Of View = 400 × 250 × 56; acquisition matrix size, 160 × 158; number of slices = 8; reconstructed voxel size, 2.5 × 2.5 × 7 mm^3; dynamic scan duration = 3.9 s.

MRI temperature maps were calculated using the phase information in the gradient echo images. Potential drift of the temperature (caused by drift in time of the phase of the MRI signal not related to temperature) was compensated by subtracting the average temperature in a reference region of interest (ROI) selected within the non-heated agar gel.

Calibrated fiber optic temperature probes (Luxtron) were inserted in the water bath and in the chitosan gel.

2.6. Assessment of DFO bioactivity

hMSCs were derived from bone marrow aspirates obtained from human volunteers, with informed consent at the Regenerative Medicine Institute, the National University of Ireland, Galway. All procedures were performed with ethical consent from the Clinical Research Ethical Committee at University College Hospital, Galway. The hMSCs were isolated using standard protocols and stringent analysis of cell phenotype (tri-lineage differentiation and a full panel of cell surface markers), as published in Duffy et al. [31].

In order to assess the bioactivity of the DFO/HGF gel formulations, hMSC monolayers were cultured in adherent 6-well plates at a density of 4×10^4 cells/well in fully supplemented hMSC growth media, under normal culture conditions. PBS release media from Lipogel with and without a hyperthermic pulse were first analysed by HPLC to determine relative DFO levels. Media were removed from both pulsed and unpulsed gels 24 h after the application of a 1 h hyperthermic pulse in the pulsed gels. hMSCs were treated with Lipogel release media containing 6, 4 and 3 μg DFO/ml from day 2, 6 and 10 pulsed Lipogel, respectively. Wells containing only PBS acted as controls. hMSC media were removed at 72 h and frozen until analysis. VEGF levels in cell culture supernatants were determined using quantitative ELISA (R&D systems) as a measure of DFO activity.

2.7. Assessment of HGF bioactivity

In order to assess the bioactivity of the HGF released from Lipogel, MSCs (MSCs) were harvested and washed twice with serum-free growth media. Cells were seeded at a density of 7×10^4 -cells/cm^2 in a hanging well cell culture insert with a pore size of 8 μm, suitable for insertion into a 12-well plate (Millipore, Ireland). Next, 2 mL of serum-free hMSC growth media was added basolaterally and 0.5 mL was added apically. Cells were allowed to incubate in serum free media and were deprived of serum for a total of 2 h, at which point basolateral media were replaced with either serum free growth media supplemented with 20 ng/mL HGF derived from Lipogel release media, serum free growth media (negative control) or growth media supplemented with 20% serum (positive control). Samples were harvested at 24 h. The inserts were immersed in 4% formalin for 10 min to fix adherent cells and stained with haematoxylin (Sigma, Ireland) for a further 10 min. Following a final rinse with PBS the membranes were removed with a scalpel blade. Membranes were mounted on a glass slide, bottom side down. Cell migration was quantified by counting the cells from five random fields on the underside of the membrane at 10x magnification (Nikon Eclipse 90i).

2.8. Statistical analysis

One-way and two-way ANOVA were performed followed by pairwise multiple comparison procedures (Tukey test). 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.

3. Results

3.1. Free and Liposome encapsulated DFO release within a chitosan/β-GP gel

Chitosan/β-GP hydrogels were prepared containing a 100 μM concentration of free-loaded or LTSL-encapsulated DFO (denoted Lipogel). Release of DFO from the hydrogel into PBS at 37 °C over the course of 10 days was measured via HPLC, to assess the ability of the gel to sustain the release of DFO for an extended period. Results indicate that the free-loaded hydrogel produced an initial burst release with 40% DFO released within the first 4 h. By 72 h, over 90% of the DFO had been released from the gel. Release of DFO from Lipogel was slower than free-loaded drug at 37 °C for the duration of the study, with 50% DFO release at the same timepoint (Fig. 1).

3.2. Controllable release of DFO through alteration of the time of initiation of a hyperthermic pulse

The potential to spatiotemporally control the release of DFO from Lipogel by delaying the application of a hyperthermic pulse was assessed. Lipogel containing 100 μM LTSL-encapsulated DFO was incubated at 37 °C and underwent a 1 h hyperthermic pulse (42 °C) on day 2, 6 or 10. Lipogel produced an initial elevated release for the first 24 h due to passive DFO diffusion from the LTSLs. Hydrogels which were unpulsed continued to release diminishing levels of DFO (as seen in Fig 1). Once a hyperthermic pulse was applied, hydrogels successfully produced a second burst release of DFO at all assayed timepoints. DFO release sustained for three further days post-pulse, compared to unpulsed gels. Initial DFO release 24 h after the application of a hyperthermic pulse at 42 °C released 30 ± 2% on day 3, 24 ± 1% on day 7 and 15 ± 1% on day 11 of total DFO encapsulated in each gel bolus (Fig. 2A/B).

3.3. Dual DFO dose release from Lipogel

The potential to combine the rapid burst release effect observed with free loaded DFO in the hydrogel matrix and the delayed, controlled release of LTSL encapsulated DFO was assessed. Lipogel which contained a 1:1 ratio of free loaded DFO and LTSL encapsulated DFO (200 μM total) was subjected to an eight day release study. Lipogel was held at 37 °C, and underwent a hyperthermic pulse on day 6 or remained at 37 °C for the duration of the experiment. Lipogel produced an initial burst release of DFO as a result of the quick diffusion of the unencapsulated drug. Over 30% of the total amount of DFO loaded within the Lipogel was released within the first 48 h. Once both hydrogel groups had reached a plateau (day 6), a hyperthermic pulse of one hour was applied to one of them. Hydrogels which were pulsed successfully produced a second elevated level of drug release, increasing DFO levels by 40.0 ± 2.4% relative to unpulsed controls (Fig. 3A/B).

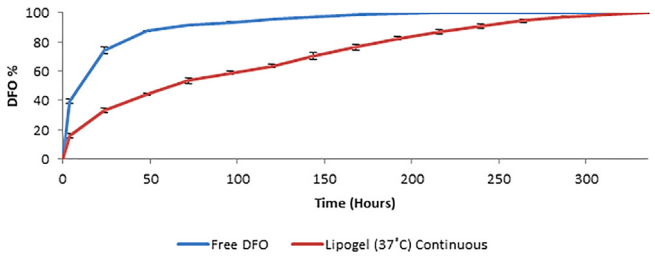

Fig. 1. Release of DFO from chitosan/β-GP gels containing free DFO (100 μM). Gels maintained at 37 °C demonstrated a burst release of DFO, with 90% of the total encapsulated drug released at day 3. Release from Lipogel at 37 °C was slower and sustained over 14 days.

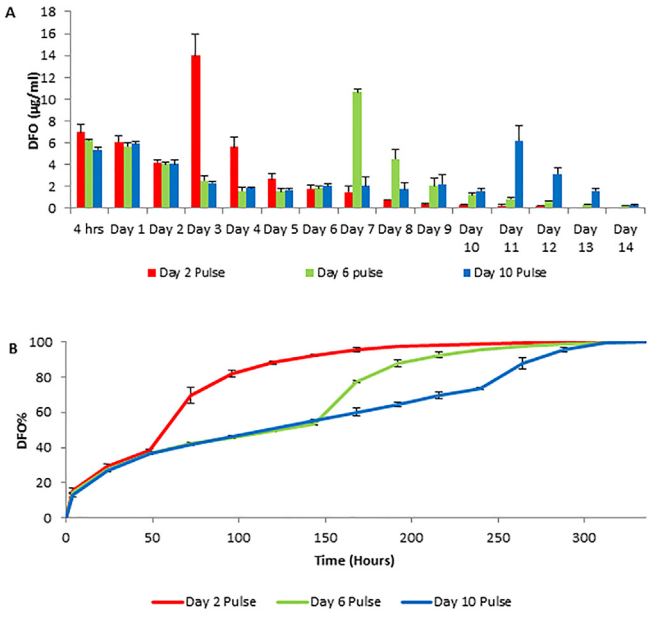

Fig. 2. (A) Release of DFO (100 μM) from chitosan/β-GP gels containing liposomal DFO held at 37 °C after a 1 h hyperthermic pulse at 42 °C on day 2, 6 and 10 for 14 days. A 42 °C thermal pulse produced a significant increase in DFO release at all of the pulse time points. This increase remained significant for a further three days. (B) Release of cumulative DFO (100 μM) from chitosan/β-GP gels containing liposomal DFO at 37 °C after a 1 h pulsation at 42 °C on day 2, 6, or 10. DFO release 24 h after the application of a hyperthermic pulse at 42 °C was increased by 30 ± 2% on day 3, 24 ± 1% on day 7 and 15 ± 1% on day 11 (mean ± SD, n = 3).

3.4. Dual release of DFO and HGF

The potential to combine a rapid burst release effect achievable with a free loaded agent in the hydrogel matrix and the delayed, controlled release of LTSL-encapsulated DFO in a time dependent manner was assessed. Lipogel containing 1 μg/mL HGF and 100 μM LTSL-encapsulated DFO was used in a six day release study to determine the tenability of the system. Lipogel was held at 37 °C, and underwent a hyperthermic pulse (1 h at 42 °C) on day 4. The free loaded HGF showed an initial burst release up until day 6 when release plateaued. DFO release presented a reduced rate prior to the application of a hyperthermic pulse. Once the hyperthermic pulse was applied to the Lipogel on day 4, there was a 57% increase in DFO on day 5 (Fig. 4).

3.5. Bioactivity of Released DFO and HGF

Bioactivity of the released DFO was assessed through measurements of VEGF expression in MSCs after exposure of the cells to Lipogel release media. Prior to that, DFO concentration was assessed by HPLC. MSCs were treated with 6 μg/mL of DFO from Lipogel release media after a 1 h hyperthermic pulse on day 2, 4 μg/mL of DFO from Lipogel release media after a 1 h hyperthermic pulse on day 6 and 3 μg/mL of DFO from Lipogel release media

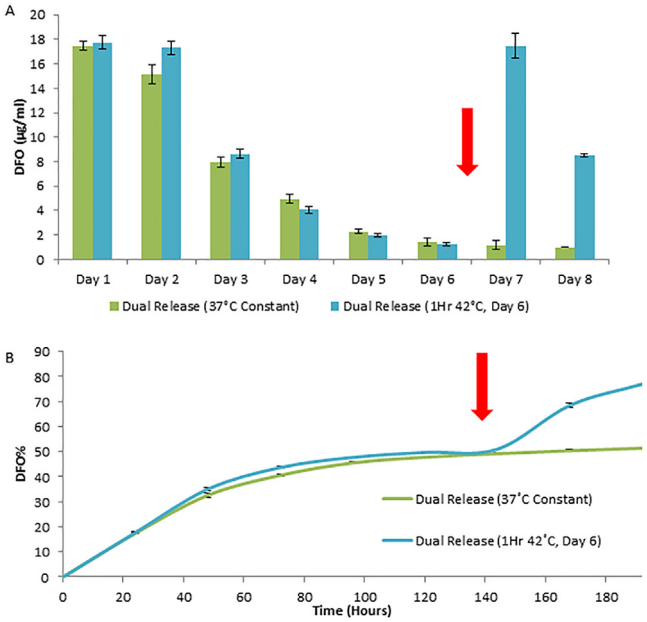

Fig. 3. (A) Absolute and (B) cumulative DFO release profiles from dual loaded Lipogel at 37 °C with and without a one hour pulse at 42 °C on day 6 (red arrow). Both groups displayed a significant initial burst of free DFO and induction of hyperthermia for a period of one hour on day 6 was sufficient to release a second significant dose of DFO from the hydrogel. The application of a hyperthermic pulse results in a 40 ± 2.4% increase in DFO release compared to an unpulsed, control sample (mean ± SD, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

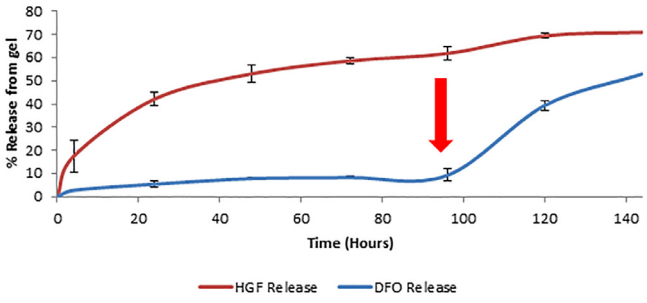

Fig. 4. Cumulative Release of HGF (1 µg/mL) and DFO (100 µM) from Lipogel incubated at 37 °C with a 1 h hyperthermic pulse at 42 °C on day 4. HGF release showed an initial burst release for the first 24 h followed by a more sustained release. A 42 °C thermal pulse on day 4 produced a 57% increase in DFO release (mean ± SD, n = 3).

after a 1 h hyperthermic pulse on day 10. MSCs displayed a significant increase in VEGF release after 72 h in culture with the pulsed Lipogel release media at all timepoints compared to the unpulsed Lipogel and untreated cells alone, as a control Free DFO was included to assess degradation over time (Fig. 5).

To assess the bioactivity of HGF release from Lipogel, MSCs were serum-starved and assessed with regard to ability to migrate through a transwell insert in response to HGF release media, relative to High Serum, Free HGF and No Serum controls. Since HGF is involved in cellular migration, bioactivity can be quantified by its ability to induce cellular migration. MSCs successfully migrated through the transwell insert and the level of migration was significantly increased relative to the No Serum control (Fig. 6).

3.6. Multi-dose release of DFO from Lipogel by varying the duration of hyperthermia

The release of DFO from Lipogel was assessed at 37 °C, with two one hour 42 °C hyperthermic pulses 96 h apart. Hydrogels that received their first hyperthermic pulse at day 2, 4, 6 and 8 successfully produced an elevated release of DFO and increased DFO release for an additional three days compared to unpulsed hydrogels held at 37 °C. A second one hour hyperthermic pulse (42 °C) was applied 96 h after the application of the first hyperthermic pulse which resulted in no additional elevated release of DFO. DFO levels remained the same after a second one hour hyperthermic pulse as control groups that only received one hyperthermic pulse (Fig. 7).

3.7. Applying multiple doses of hyperthermia to permit multiple doses of DFO

Having determined that the Lipogel system could not produce a second dose of DFO in response to a second application of the same duration hyperthermia, we next sought to determine if it was possible to fine tune DFO release by varying the duration of hyperthermia that hydrogels were exposed to. To visualize the spatiotemporal heating pattern of the Lipogel MR thermometry was performed during water bath heating of the gel. (Fig. 8A & B). Over a period of 24 min the water bath was heated from 20 to 35 °C and coronal MR temperature maps were recorded by MRI. Temperature maps acquired at 3 min increments demonstrate the heat transfer in time from the periphery to the core of the LipoGel (Fig. 8C).

Once Lipogels temperature mapping was established, the ability of Lipogel to permit multiple doses by modifying the duration of hyperthermia was assessed. Lipogel underwent a 1, 2, 5, 10 and 30 min 42 °C hyperthermic pulse and was left at 37 °C for 24 h prior to analysis to permit for DFO release. As controls, hydrogels were either left at 37 °C (minimum release) or 42 °C (maximum release) for 24 h. Hydrogels which were pulsed for 1 min released the same levels of DFO as unpulsed hydrogels. Extending the duration of hyperthermia (2–10 min) increased the levels of released DFO. By 30 min the same level of DFO was released as the continuously pulsed hydrogels (Fig. 9). This demonstrates that the ability to produce a tuneable release is determined by the duration of hyperthermia and that maximal release occurs after 30 min of exposure to a hyperthermic pulse.

3.8. Combination of altered duration and timing of a hyperthermic pulse to create a flexible multidose release profile

Having determined that DFO release from the Lipogel system could be significantly controlled through alteration of duration or time of initiation of a hyperthermic pulse we next sought to combine these factors to create a flexible multidose effect. Lipogel was subjected to increased durations of hyperthermia at multiple timepoints. All samples were left at 37 °C for 24 h to equilibrate (Day 0). Following this, groups received either the same duration of hyperthermia (1, 3, 10 and 30 min) every 24 h or were subjected to a step-wise increased duration of hyperthermia every 24 h (1, 3, 10 and 30 min). Samples that received the same duration of hyperthermia daily all showed an initial burst release compared to unpulsed controls on day 1 (Fig. 10A). DFO levels decreased at each subsequent timepoint after the application of a hyperthermic pulse. Samples which received progressively increased levels of hyperthermia showed a higher level of DFO release compared to samples that repetitively received the same levels of hyperthermia.

To further demonstrate this multidose effect, Lipogels that received increasing levels of hyperthermia for the first 48 h (1 and 3 min) and then subsequent daily pulses of 3 min for the next 48 h (3 and 3 min) were directly compared to Lipogels that received consecutively elevated levels of hyperthermia (1, 3, 10 and 30 min) for the duration of the release study. As the duration of hyperthermia increases, it is hypothesized that the depth of heat

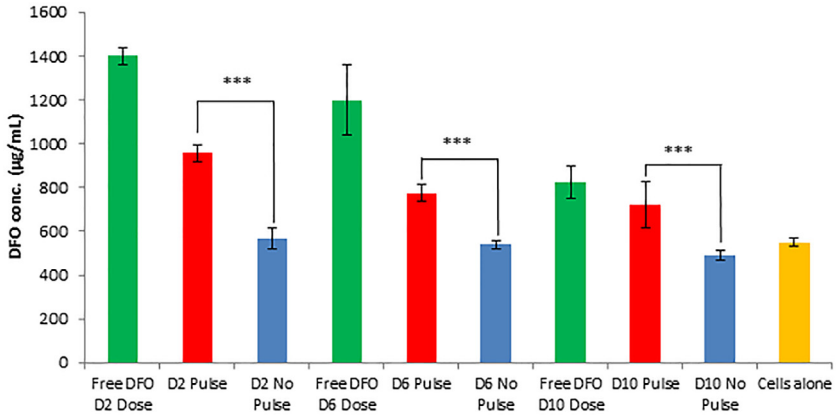

Fig. 5. VEGF expression in MSCs following exposure to Lipogel release media. Cells showed significantly increased VEGF expression in the pulsed Lipogel groups when compared to unpulsed or untreated cells after 72 h. Free DFo was used as a control to assess degradation over time (mean ± SD, n = 3) Note: *** denotes $p < 0.0001$.

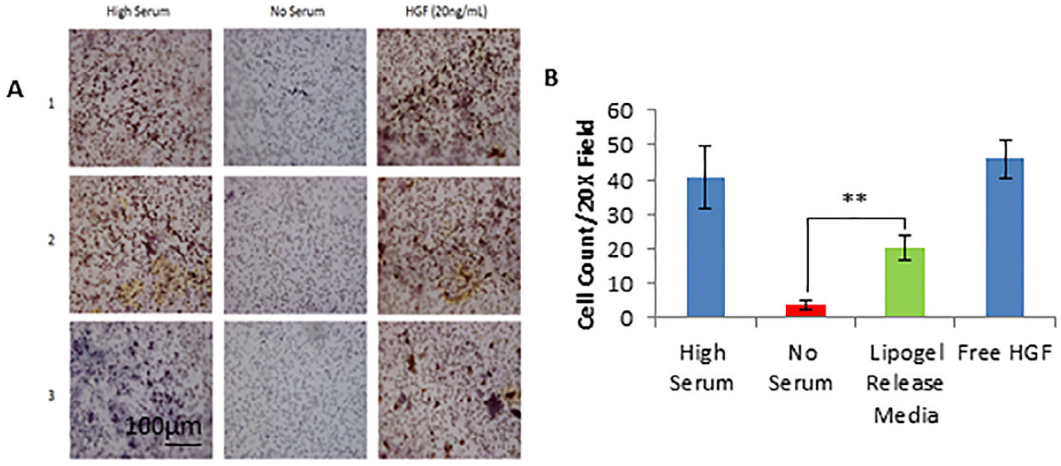

Fig. 6. A measurement of cellular migration of MSCs through a porous transwell membrane. Cells were serum starved and then permitted to migrate through the membrane in response to a serum/no serum/HGF release/Free HGF media stimulus. (A) Cells were collected on the membrane of a 12-well transwell cell culture insert which was stained with haematoxylin. (B) Cells were counted at 5 random fields of view at 10× magnification per membrane. HGF release media displayed significantly increased transwell migration after 48 h relative to no serum stimulus controls (mean ± SD, n = 3) Note: ** denotes $p < 0.001$.

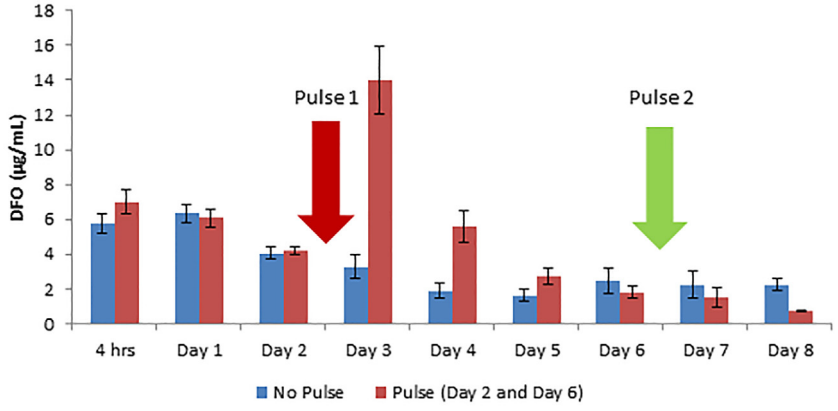

Fig. 7. An analysis of DFO (100 µM) release from chitosan/β-GP gels containing liposomal DFO at 37 °C after application of a 1 h hyperthermic pulse at 42 °C on day 2, 4, 6 and 8 (red arrow), followed by a second hyperthermic pulse at day 6, 8, 10 and 12 (green arrow) respectively. Fold increase in DFO after the initial hyperthermic pulse is 3.4 on day 2 (A), 7.8 on day 4 (B), 5.8 on day 6 (C) and 3.75 on day 8 (D). No further increase in DFO released from the Lipogel was observed after the application of a second hyperthermic pulse on days 6,8,10 and 12 (mean ± SD, n = 3). Note: *** denotes $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

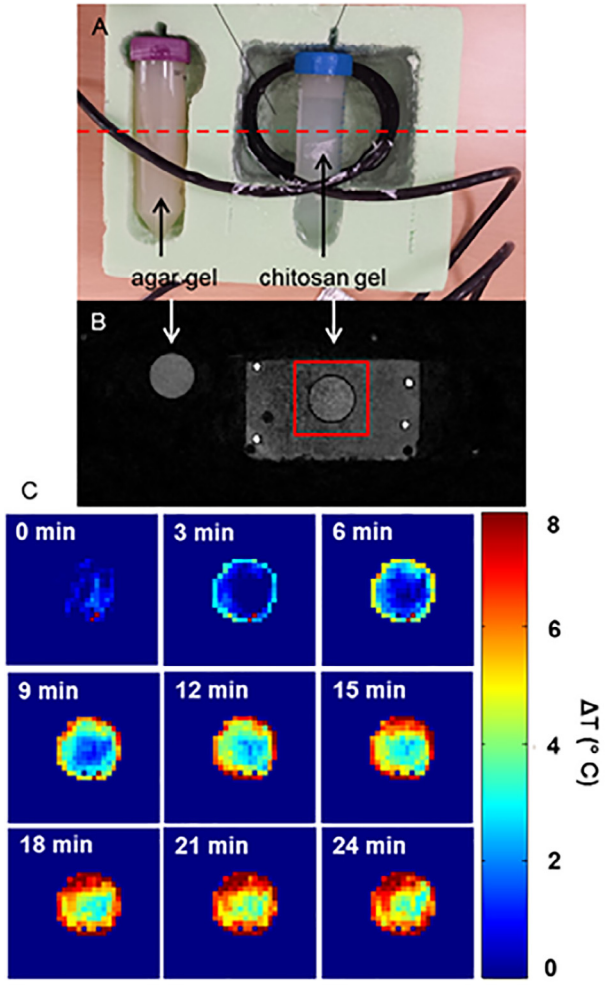

Fig. 8. (A) Picture of heating set-up showing the chitosan hydrogel in the water bath and agar gel thermally insulated. The red dotted line indicates the position of the coronal MR images shown in B and C. (B) Coronal magnitude MR image showing the chitosan gel in water bath and the agar gel. (C) Zoom (indicated by red rectangle in figure B of coronal temperature maps showing the temperature changes in the chitosan gel at 3 min increments. The temperature changes outside the chitosan gel were automatically set to 0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

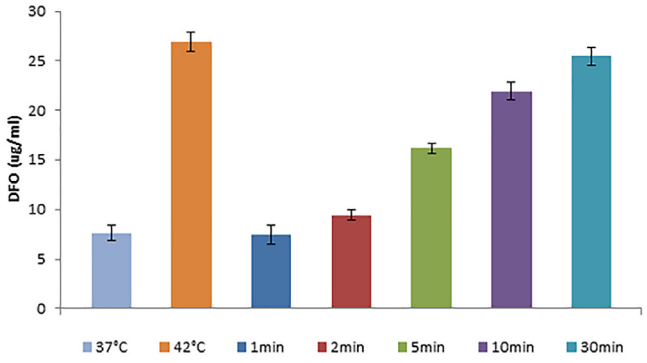

Fig. 9. Absolute DFO release from Lipogel in response to increased durations of hyperthermia. DFO release increases in response to extended durations of hyperthermia. Induction of hyperthermia for 30 min was sufficient to release the same amount as hydrogels held at 42 °C for the duration of the release study, which was 24 h (mean ± SD, n = 3).

penetration through the gel increases, thereby activating release from a higher number of thermosensitive liposomes. Hydrogels exposed to progressively elevated durations of hyperthermia over the course of the study showed a comparatively higher level of DFO release at later timepoints (Fig. 10B/C). By altering the timing and duration of hyperthermia it was possible to produce a wide variety of release phenomena, demonstrating the flexibility of the Lipogel system.

4. Discussion

Polymer-based delivery systems are typically used for attaining localised, sustained release of bioactive molecules. However, a lack of tight control over the temporal administration of drugs tends to require larger doses in order to achieve the desired effect within the therapeutic window, this can inadvertently lead to increased toxicity and other undesirable side effects [25]. In addition, numerous drug treatment regimens require frequent or continuous administration based on the patients response over time to the treatment, which can compromise both patient compliance and comfort. In order to address these concerns, a reservoir-based drug delivery system that can release multiple doses of single or combinatorial drug therapeutics in response to an externally applied stimulus would represent a powerful delivery platform [32]. This system would allow for a highly localised concentration of drug, delivered directly to the target site upon implantation, while allowing for flexible scheduling of dosing in a minimally invasive manner by a healthcare professional.

The combination of LTSLs in a thermoresponsive hydrogel for tuneable drug delivery remains an understudied concept. Han et al. incorporated poly(N-isopropylacrylamide) (PNIPAAm)-modified thermosensitive liposomes into a thermoresponsive PNIPAAm gel and investigated the release of liposome-encapsulated calcein without the use of hyperthermic pulses [33]. Ullrich et al. encapsulated carboxyfluorescein-loaded thermosensitive liposomes in alginate beads, and report pulsatile release over a period of 140 min [34]. Lopez-Noriega et al. have recently demonstrated that LTSLs encapsulated within a thermoresponsive hydrogel could provide a controllable platform for doxorubicin release over a period of 7 days after a single pulse on day 2 (76).

The release of DFO from Lipogel in response to a delayed hyperthermic pulse was examined over the space of 14 days under a range of conditions. Free drug was released from the chitosan/β-GP hydrogel very rapidly, demonstrating the challenges in producing a long term release of hydrophilic drugs from hydrogel matrices (Fig. 1). By embedding the LTSLs within a hydrogel matrix it became possible to overcome the rapid release observed with the free loaded DFO within the gel. Thus, Lipogel samples held at 37 °C for the entire 14 days produced a more prolonged sustained release, due to the barrier imposed to drug permeation by the lipid bilayer (Fig. 1). This passive release demonstrates the ability of the Lipogel system to exploit a current disadvantage of LTSLs when administered systemically, namely, their tendency to leak a portion of their payload during short periods at 37 °C [35–37]. LTSLs in combination with mild hyperthermia represent an attractive therapeutic treatment option but room for improvement is still required to prevent premature drug release and the subsequent toxicity issues that are related to this. The LTSLs can be preserved within the hydrogel matrix until such time that the application of a hyperthermic pulse is required to release the required drug dosage. The hydrogel, as well as providing a support framework for the incorporated LTSLs also permits diffusion of the released drug molecule through the gel. A significant increase in DFO release was achievable upon application of transient hyperthermia for one hour at days 2, 4, 6, 8, 10 and 12 demonstrating that LTSL thermosensitivity was preserved in the Lipogel environment over this period (Fig. 2A/B). DFO release was increased by 30–15% after induction of hyperthermia, compared to samples held at 37 °C.

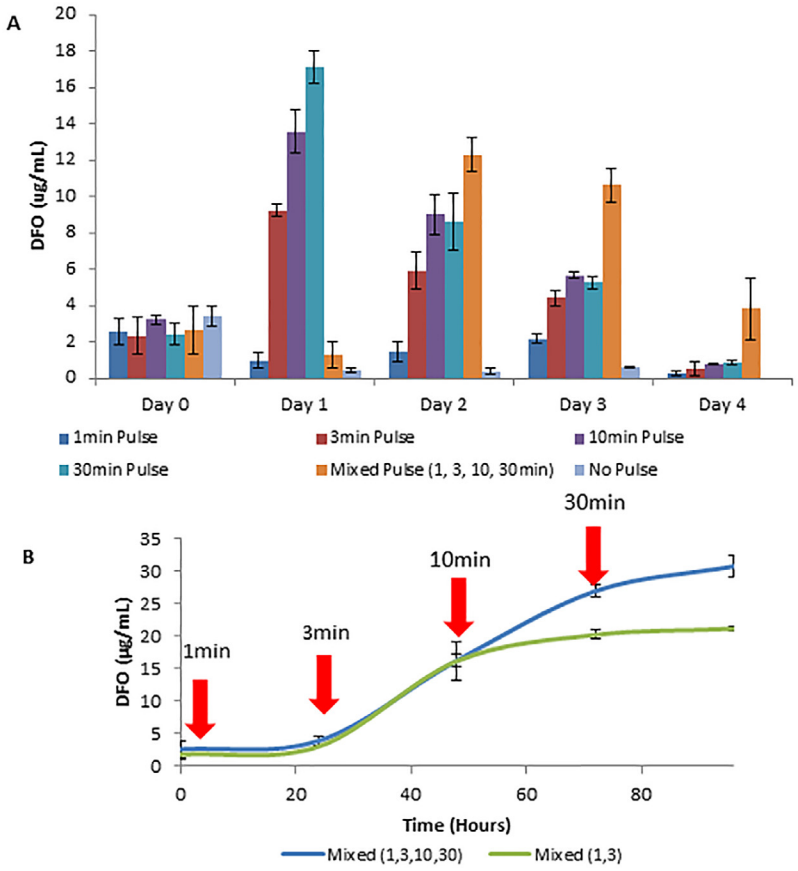

Fig. 10. (A) DFO release (100 µM) from chitosan/β-GP gels containing liposomal DFO at 37 °C after a repeated hyperthermic pulse of 1, 3, 10 or 30 min, daily. Each group received the same duration of hyperthermia (1–30 min) every 24 h. Mixed Pulse group received step-wise increased duration of hyperthermia every 24 h (1, 3, 10 and 30 min). An initial short duration of hyperthermia (1 min) resulted in a higher amount of DFO release relative to the unpulsed control. Longer durations of hyperthermia resulted in greater levels of DFO release from Lipogel as the heat transfer penetrated deeper towards the core of the gel, thereby activating release from a higher number of thermosensitive liposomes. The application of a mixed pulse resulted in a multidose effect, significantly increasing DFO release compared to groups that received the same duration of hyperthermia at each timepoint. (B) Mixed pulse Lipogel permitted multiple DFO doses by increasing the duration of hyperthermia every 24 h (red arrow) (1, 3, 10, 30 min). Cessation of increased levels of hyperthermia after the first 48 h ceased this multi dose effect (mean ± SD, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dual drug delivery systems, which are capable of controlling the release behaviour of multiple drug combinations, are attractive for the administration and optimization of a variety of therapeutic effects. Given the powerful effects that drug combinations provide when applied synergistically [38–41], delivery system based on crosslinked hydrogels with embedded particles [42,43] represent a promising treatment platform. An agent required for an early stage can be incorporated within the gel and be released within the first few days. In the case of Lipogel, liposomal release can then be triggered by hyperthermia to deliver a second encapsulated agent. Prior to loading multiple therapeutic agents in Lipogel, a 1:1 ratio of free DFO (to act as Agent 1) and liposomal DFO (to act as Agent 2) was tested. An initial large burst release was observed due to free DFO diffusing through the hydrogel matrix. A hyperthermic pulse was applied for 1 h on day 6 after the initial burst release had subsided. This resulted in a 40 ± 2.4% fold increase in DFO release compared to unpulsed samples at the same timepoint (Fig. 3A/B).

Having demonstrated that the Lipogel system was capable of releasing two doses of drug separately, we next applied this release scheduling to two distinct pro-angiogenic agents. Delivery of multiple pro-angiogenic agents has been attempted previously. Although the administration of single agents has been shown to support angiogenesis in animal models, there are still problems associated with vessel stability and maturity [44,45]. The complex process of cell migration, differentiation and proliferation requires specific growth factors, which are both time-dependent and spatially distributed. For example, delivery of free loaded VEGF, involved in the initiation of angiogenesis, followed by the delivery of encapsulated platelet-derived growth factor (PDGF)-BB has been shown to stabilize newly formed blood vessels [46–48]. In a more recent study, the burst release of VEGF and angiopoietin (Ang)-2, followed by the delayed release of PDGF and Ang-1 *in vivo* produced a dramatic increase in vessel formation and promotion of vessel maturation, demonstrating the importance of temporal control of release [49]. We elected to deliver free loaded HGF in combination with on-demand DFO, which facilitates increased VEGF response, to demonstrate the potential of Lipogel to permit dual release kinetics as a pro-angiogenic therapeutic platform. The release profile of HGF was similar to the one obtained for free loaded DFO (Fig. 3), consisting of a first burst release followed by a more sustained release up to 5 days. Liposomal release could then be triggered by hyperthermia to allow the release of a second encapsulated agent (DFO) (Fig. 4). Lipogel can allow the induction of multiple pro-angiogenic agents in a spatio-temporal manner to facilitate vessel development and subsequent maturation [50–52]. We have demonstrated the potential of this system as a pro-angiogenic platform, however the ability to tune the release of different therapeutic agents without the potential compounded off-target effects of bolus combination therapies could facilitate

synergistic regenerative treatment options and could also be of utility in the treatment of other disease states, such as cancer therapy and pain management [11].

In order to assess the bioactivity of DFO released, Human Mesenchymal Stem Cells were exposed to the Lipogel release media after the application of a hyperthermic pulse. Pulsed groups showed statistically significant levels of VEGF at all timepoints compared to unpulsed groups and cells alone controls (Fig. 5). This illustrates that DFO released from the liposome loaded hydrogel after the application of a hyperthermic pulse remained bioactive for even as late as day 10. This shows enormous potential for the late stage delivery of therapeutics that can remain protected within the LTSLs. In addition released HGF was sufficient to induce a migratory response in MSCs cultured on a transwell insert, demonstrating its bioactivity too (Fig. 6). A growing body of evidence is accumulating which suggests that the delivery of multiple pro-angiogenic agents in a sustained fashion provides superior efficacy to the administration of single agents or bolus delivery. The Lipogel system described here allows for the ability to release multiple agents in a controlled fashion.

Having investigated the dual delivery potential and bioactivity of released agents from the Lipogel system, we next sought to better characterise the flexibility of the LTSL-sequestered drug portion of the system to produce a variety of release phenomena by altering both duration and time of initiation of hyperthermia. Lipogel was incapable of permitting a second dose of DFO when the duration of hyperthermia was the same as the first application of hyperthermia on the Lipogel bolus (1 h) (Fig. 7). For this reason, we investigated alternating durations of hyperthermia to permit release of multiple doses of DFO. We hypothesized that by applying a short duration of hyperthermia, the thermal distribution around the hydrogel bolus would be limited to the surface and thus fewer LTSLs would become activated, releasing smaller doses of drug. As the duration of hyperthermia increases, heat penetration increases resulting in more LTSLs activated, whereby higher doses of drug are released. We tested this hypothesis by MR temperature mapping of the Lipogel during water bath heating. Over a period of 24 min, it is clear that heat penetrates from the surface to the core of the gel over time (Fig. 8). Macroscale drug-delivery systems have been developed that respond to a variety of external cues, including magnetic and electric fields, ultrasound and wireless signals (Ref). Ultrasound responsive materials are have previously been used to accelerate the degradation of the drug carrier by producing cavitation, thereby accelerating release during the 'on' phase. When the stimulus is removed, release rates can remain elevated or return to baseline levels. This results in an on-demand degradation controlled release. The Lipogel platform uses convection to trigger release of the drug carrier so does not affect the material properties of the carrier when utilising the external stimulus which could promote a more advantageous drug delivery system going forward.

Applying this concept to the drug loaded hydrogel, it was possible to create a multi-dose release of DFO by escalating the duration of hyperthermia for each successive hyperthermic episode. By dispersing liposomes within a hydrogel, it became possible to optimize the dose of DFO released by optimizing the duration of hyperthermia (Fig. 9). Maximal LTSL release occurred after 30 min hyperthermic exposure with the assayed size of Lipogel bolus. This 'onion layer' effect is exemplified in Fig. 10, where Lipogel receiving the same duration of hyperthermia at each subsequent hyperthermic pulse, releases much lower levels of DFO compared to samples receiving a mixed duration of hyperthermia (i.e. longer durations of hyperthermia at each subsequent timepoint). This approach combined alterations in the time of hyperthermic initiation and in the duration of each individual pulse to create a desired release profile. The described multi-dose effect represents a superior control on the performance of a drug delivery system. Through judicious application of hyperthermia it becomes possible to release sequential doses of DFO. By applying greater durations of hyperthermia, deeper heat penetration through the gel occurs and results in the next "on demand" drug release. This represents an innovative way to administer drugs, and permits for a significant spatiotemporal control, since local release is assured due to local hydrogel retention, and dosing is externally and minimally invasively controllable through LTSL activation. Liposomal release could be activated externally through minimally invasive application of hyperthermia treatments using radiofrequency microwaves or high intensity focused ultrasound (HIFU). These approaches have been used previously for the delivery of chemotherapeutic and antibiotic drugs [53,54].

Future work will investigate the use of HIFU as a means to activate the LTSLs in a clinical setting. Indeed, future work could examine dosing regimens which could be adjusted on-demand based on a patient's physiological response and clinical needs after implantation of the hydrogel. In the current work we demonstrate the utility of our multi-dose platform using DFO as a model small molecule drug. However, each of the comprising components of Lipogel (i.e. the small molecule drug, growth factors, the stimuli-responsive trigger, the liposome and the hydrogel) can be easily replaced to tune our platform for practically any biological application of interest, thus offering immense potential for advancing drug delivery systems.

5. Conclusion

By combining lysolipid-based thermosensitive liposomes and an injectable chitosan hydrogel matrix, Lipogel allows a localised, tunable and time dependent delivery of multiple therapeutics. It was possible to tune the release of multiple agents while still maintaining a bioactive effect. The possibility to tune the release kinetics of multiple drugs represents an innovative platform to utilise for a variety of treatments. Lipogel permits for a significant degree of flexibility in achieving a desired drug scheduling and sequencing, significant control could be placed on the release profile by modifying the duration of the hyperthermic stimulus. Many of which could be implemented once the gel bolus is already *in situ*, enabling treatment to be tuned in response to changing symptoms or complications. The potential to modify dosing schedules (i.e. multi-dose) by altering the external stimuli represents a powerful platform to personalize patient treatments.

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