Thermally tunable hydrogel crosslinking mediated by temperature sensitive liposome

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Abstract

Hydrogel crosslinking by external stimuli is a versatile strategy to control and modulate hydrogel properties. Besides photonic energy, thermal energy is one of the most accessible external stimuli and widely applicable for many biomedical applications. However, conventional thermal crosslinking systems require a relatively high temperature (over 100 °C) to initiate covalent bond formation. To our knowledge, there has not been a thermally tunable hydrogel crosslinking system suitable for biological applications. This work demonstrates a unique approach to utilize temperature sensitive liposomes to control and modulate hydrogel crosslinking over mild temperature range (below 50 °C). Temperature sensitive liposomes were used to control the release of chemical crosslinkers by moderate temperature changes. The thermally controlled crosslinker release resulted in tunable mechanical and transport properties of the hydrogel. No significant inflammable response observed in the histology results ensured the biocompatibility of the liposome-mediated crosslinkable hydrogel. This work opens new opportunities to implement thermal energy system for control and modulate hydrogel properties.

1. Introduction

Hydrogels have drawn significant attention for use in a wide range of applications such as tissue engineering, regenerative medicine, drug delivery, diagnostics, biosensing, cellular immobilization, wound dressing, bioseparation, etc [1–8]. Hydrogels consist of a crosslinked network of hydrophilic polymers in water [9]. The degree of polymer network crosslinking can significantly influence chemical/physical properties and the functionalities of hydrogel systems. For example, the mechanical properties of hydrogels are generally controlled by the density of crosslinks between polymers [10–12]. Culturing specific cell types within the hydrogel matrix requires the mechanical properties of the gel to be matched with the tissue origin of the specific cell types [13, 14]. Mechanical properties can also influence stem cell differentiation within the gel [15–17]. The density of crosslink between the polymers in the gel is important when used in controlled molecule release applications. The steric interaction between the entrapped molecules and the polymer matrix and the diffusion of molecules both are regulated by the mesh size of the matrix [14]. Denser crosslinks result in hydrogels with smaller mesh sizes, consequently reducing the pathway for molecular diffusion and allowing the extended release of the molecules. Along with molecular transport, storage modulus and swelling ratio are also influenced by the crosslink density of the hydrogel matrix [9]. Controlling the density of crosslinked polymers in the gel can lead to tunable properties of this soft material.

Chemical crosslinking is one of the basic crosslinking strategies. Chemical crosslinking creates permanent covalent bonds among polymer chains. The formation of the covalent bonds involves the addition of crosslinker molecules, polymer–polymer condensation reaction, photosensitive agents, and enzyme-mediated reaction, etc [9, 18–20]. For a given hydrogel system, tunable crosslinking density can be
achieved by controlling the covalent bond formation. Applying external stimuli is one of the most versatile strategies to control this bond formation and modulate hydrogel properties.

The external stimuli may include thermal, electrical, magnetic, UV/visible light, or combinations thereof [21]. Some unsaturated polymers such as poly(vinyl alcohol), poly(ethylene oxide), and poly(acrylic acid) are well known to be crosslinked by gamma and electron beam radiation [22–26]. While this radiation-induced hydrogel formation has the advantage of mild reaction conditions (room temperature and physiological pH), the requirement of a high energy source, the potential damages of biological species within the hydrogel system, and unreacted radicals prepared in this process are some major drawbacks of radiation-induced polymer cross-linking when performed in the presence of cells and biomolecules [27, 28]. Photoactivated hydrogels represent another major class of external stimuli-controlled hydrogel systems. Photoactivated hydrogels with UV and visible light have demonstrated excellent biocompatibility and tunable properties [17, 29–32]. However, photoactivated hydrogels often require the synthesis of specific photosensitive chemical groups on the polymer chains. And photonic energy has a limited depth of penetration to the tissues.

Thermal energy could be a vital strategy to control and modulate hydrogel cross-linking processes. This strategy has an additional advantage for in vivo application because focused thermal energy can penetrate deeply into the tissue [33, 34]. For example, non-invasive hyperthermia is becoming a great tool for cancer treatment [35]. Although temperature-dependent crosslinking of polymer chains in the presence of oxygen molecules has been reported previously [36, 37], temperature over 100 °C is required to reach the activation energy of those reactions, which presents the same challenges as the radiation-induced hydrogel formation and also not feasible for biological applications. To our knowledge, there has not been a thermally tunable hydrogel crosslinking system suitable for biological applications. This work focuses on designing a general hydrogel crosslinking system that can be modulated by mild temperatures.

Instead of conventional methods to control and modulate crosslinking density, we use a temperature-sensitive liposome (TSL) incorporated hydrogel system that can be modulated by mild temperature change above body temperature. TSLs can act as controlled release carriers of a system in response to temperature change. These liposomes have been studied extensively for temperature-triggered drug release [38–41]. However, studying these liposomes for other biomedical applications has been reported insignificantly. Stowers et al showed TSL controlled calcium release to achieve dynamic stiffening of ionically crosslinked hydrogel [42]. To use the near-infrared light as the external source, they encapsulated gold nanorods along with calcium ions into the liposomes and showed liposome-controlled ion release in alginate gelation. We demonstrate an external heat-triggered liposome modulated covalent cross-linkable hydrogel by using TSL in an enzyme-mediated polymer crosslinking system. Liposomes are the thermosensitive enzyme carriers in our system. Enzymes, such as peroxidase, offer a very fast oxidative reaction in the presence of a peroxide [43–46]. A wide variety of functional groups can be crosslinked using this peroxidase-catalyzed reaction [18, 47–49]. Liposomal delivery of peroxidase enzyme combined with cross-linkable polymer has been recently reported by Ren et al to form injectable hydrogel at body temperature [50]. We present the temperature tunability of the hydrogel system above body temperature by engineering the lipid vesicles such that they minimize the enzyme release from liposomes below and at 37 °C but maximize the release at 45 °C, therefore control the density of the crosslinks at temperatures below and above physiological temperature.

We demonstrate the system by combining tyramine-conjugated hyaluronic acid polymer (HA-tyramine) and horseradish peroxidase (HRP) carrying liposomes, shown in figure 1(a). The modulation of HA-tyramine hydrogel crosslink is achieved by embedding the HRP carrying liposomes within the HA-tyramine solution and applying heat at 25 °C, 37 °C, and 45 °C. We used two saturated phospholipids to formulate the liposomes such that they released different amounts of HRP enzymes and altered the hydrogel crosslinking density at 25 °C, 37 °C, and 45 °C. We have evaluated the viscoelastic properties of the crosslinked hydrogel to demonstrate the difference in crosslinking density. In addition to viscoelastic properties, we also measured protein release from the different temperature heated samples and observed temperature tunable protein release kinetics. Lastly, the biocompatibility of the hydrogel system is evaluated by histology and in vitro cytotoxicity which showed no significant inflammatory or toxic response. This work represents a new paradigm for designing a thermally tunable hydrogel system and opens new opportunities for dynamic hydrogel modulation with thermal energy.

2. Materials and methods

HA sodium salt from streptococcus equip was purchased from Sigma–Aldrich and stored at −20 °C until it was used. tyramine hydrochloride, N-hydroxyxysuccinimide (NHS), N-hydroxysulfsuccinimide sodium (Sulfo-NHS), Hydrogen peroxide (H$_2$O$_2$), Peroxidase from horseradish type II (HRP), albumin–fluorescein isothiocyanate (A-FITC), N-(3-dimethylaminopropyl)-N’-ethyl carbodiimide hydrochloride (EDC),
Figure 1. (a) Temperature controlled HA-tyramine hydrogel crosslinking mediated by thermosensitive liposomes. (b) Tyramine conjugation reaction catalysed by EDC, NHS or Sulfo-NHS. (c) Characterization of functionalized HA-tyramine by UV–Vis spectra. 1 mg ml$^{-1}$ HA-tyramine dissolved in PBS to determine degree of tyramine conjugation with Sulfo-NHS and NHS. (d) Evolution of rheological properties of HA-tyramine conjugated with Sulfo-NHS (orange) and NHS (grey) where storage modulus ($G'$), loss modulus ($G''$) and phase angles are shown by dashed, solid and dotted line, respectively. The mixture contained 6.64 mg ml$^{-1}$ of HA-tyramine, 5 mM H$_2$O$_2$, 0.13 units ml$^{-1}$ type II HRP.

2,2$'$-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), 1× phosphate buffer saline (PBS), were all purchased from Sigma–Aldrich. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased in chloroform from Avanti polar lipids, Inc. Chemicals were used without further purification unless specifically stated.

2.1. Synthesis of HA-tyramine conjugates
We converted the HA from Sigma–Aldrich to a functionalized biopolymer that can be covalently crosslinked in the presence of an enzyme catalyst and a peroxide described by Lee et al [51]. HA is a biocompatible, naturally abundant linear polysaccharide in human tissue [52]. We conjugated tyramine molecules to the carboxylic groups of the linear HA polymers in presence of carbodiimide.
catalyst. We used water-soluble carbodiimide and Sulfo-NHS or less soluble NHS to perform the conjugation reaction shown in figure 1(b). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide activated the carboxyl group to form an active intermediate that was unstable and readily hydrolyzed by water [53–55]. NHS or its water-soluble analogue (Sulfo-NHS) was then added to prevent the rapid hydrolysis of the intermediate and to form a more stable NHS-ester intermediate that reacted slowly with tyramines to form stable amide bonds [56]. The reaction composition and condition were chosen, and the purification of tyramine-conjugated HA from the reaction mixture was performed according to Lee et al [51]. After purification, the functionalized HA was lyophilized and stored at 4 °C. Tyramine conjugation to the linear HA polymer chain was determined by Lamda 25 Perkin Elmer UV–Vis spectrometer at 275 nm.

2.2. Rheologicalevaluationofliposomehydrogel system

The viscoelastic properties of the crosslinked tyramine-functionalized polymer were measured by a TA Instruments Discovery HR-2 rheometer accompanied by a 40 mm diameter and 2.009° angle cone and plate geometry. We measured rheological properties of (a) tyramine-modified HA mixed with HRP and H2O2 at room temperature, (b) tyramine-modified HA mixed with H2O2 at three different temperatures, and (c) tyramine-modified HA mixed with the HRP-encapsulated liposomes, and H2O2 at three different temperatures. The polymer was dissolved in 1× PBS at a concentration of 6.6 mg ml⁻¹, then mixed with 5 mM H2O2 for both cases. The crosslinker was added right before loading the mixture on the rheometer plate at a concentration of 0.13 units ml⁻¹ HRP for case (a), and 50 µl ml⁻¹ HRP-encapsulated liposomes for case (c). The rheometer was in the dynamic oscillatory mode with 1% strain and a frequency of 1 Hz in all cases, and for cases (b) and (c), the thermal crosslinking was determined by keeping the plate of the rheometer at 25 °C, 37 °C, and 45 °C.

2.3. SynthesisofHRP-encapsulatedliposomes

For liposome preparation, we followed the dehydration rehydration vesicle (DRV) method described in Ren’s paper [50]. DPPC and DSPC lipids were mixed at 80:20 molar ratios. The mixture of lipids in chloroform was kept in a desiccator overnight to evaporate all organic solvents. The dried lipid film was then hydrated with DI water with stirring at 60 °C for 1 h. This hydration step was followed by a sonication step at 70 °C with a probe-tip sonicator until it was optically transparent. We added an equal volume of HRP solution to the sonicated suspension after the suspension was cooled down to room temperature. The liposome-enzyme mixture was then frozen at −196 °C in liquid nitrogen and lyophilized. In this step, the liposomes were deflated which allow them to merge with neighbors to form lipid sheets, between which HRP was sandwiched. Upon rehydration with 1× PBS, the lyophilized lipid bilayer reformed to vesicle shapes, encapsulating HRP within the new vesicles. Unencapsulated HRP was removed by repeated centrifugation at 2500 g for 3 min. We characterized the sizes of the liposomes by dynamic light scattering (DLS) and scanning electron microscopy (SEM).

2.4. ThethermalreleaseofHRPfromliposomes

The 2nd hydration of the lyophilized lipid sheet was performed in 1× PBS at a concentration of 40 mg ml⁻¹ at room temperature. To compare the release kinetics of HRP from newly formed liposomes, three HRP-liposome samples were heated at three different temperatures (room temperatures: 37 °C and 45 °C). We determined the thermal release of HRP from liposomes based on the enzymatic activity of released HRP at different temperatures. It was performed by an HRP catalyzed reaction with ABTS assay. ABTS is a water-soluble and lipid-impermeable HRP substrate that can create a green end product upon reaction (absorbance at 414 nm) in presence of H2O2 [57]. Released HRP was separated from liposomes by centrifuging at 10 000 g for 1 min. Supernatant was removed from centrifuged liposomes to analyze with substrate ABTS with [ABTS⁻²] = 1.0 mM, and [H2O2] = 0.2 mM. The conversion of ABTS⁻² to the radical cation (ABTS⁺) in presence of HRP and H2O2 was measured as the increase in A₄₁₄ with time using a BioTek Synergy HT microplate reader.

2.5. Protein releasestudyfromliposome-hydrogel system

Albumin (Mw: 66 000 Da) was chosen to study the comparative release of therapeutic from the hydrogel matrix crosslinked at different temperatures. In vitro A-FITC release was carried out by first dissolving 1 mg A-FITC in 8 mg ml⁻¹ uncrosslinked polymer solution in flat-bottom vials. Then the two crosslinkers, H2O2, and enzyme carrying liposomes were added at a concentration of 5 mM and 50 µl ml⁻¹, respectively. This sample was prepared in triplicate. The three uncrosslinked samples in the vials were wrapped in tin foil and kept at room temperature, 37 °C, and 45 °C water baths for 20 min to initiate the crosslinker release and crosslinking. The 20 min of heating was followed by in vitro protein diffusion in 5 ml fresh PBS. The albumin loaded hydrogel samples were slowly stirred at 37 °C during the 5 d protein release experiment. One milliliter aliquots were withdrawn from the release medium and replaced by an equal volume of fresh PBS at each sampling time. The aliquots were diluted in fresh PBS before reading absorbance at 470 nm using a BioTek Synergy HT microplate reader.
2.6. In vivo implantation and in vitro cytotoxicity evaluation
The in vivo animal study was approved by the ethic committee and conducted in compliance with the guidelines of University of Utah Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 20-010030). All in vivo procedures were conducted using adult male C57BL/6 mice (n = 5/timepoint; 10–12 weeks of age). Mice were anesthetized with isoflurane before shaving the skin between the shoulders. The surgical area was cleaned with alcohol and betadine, a subcutaneous pocket was created for the hydrogel sample. The HA-tyramine-liposome hydrogel crosslinked at 45 °C was placed within the pocket, and the skin was then closed with 6–0 Vicryl suture (Ethicon, Johnson, and Johnson, NJ). One-week and 1 month post-implantation, the animals were euthanized, the device and surrounding tissue were removed, and fixed in formalin. We embedded the samples in paraffin, followed by sectioned and stained with hematoxylin and eosin. The hydrogel samples were then evaluated histologically for cellular response to the hydrogel. In vitro cytotoxicity test was performed culturing NG 108 cell line with a sterile piece of hydrogel in the same petri dish for few days and evaluating cell viability with immunofluorescence imaging. The LIVE/DEAD® Reduced Biohazard Viability/Cytotoxicity Kit #1 and their protocol were used to prepare the NG 108 cells for immunofluorescence imaging.

3. Results and discussion
3.1. Synthesis and characterization of functionalized HA
Our objective for this study is to demonstrate that TSL can control and modulate the crosslinking density of a chemically crosslinked hydrogel system. We used enzymatic cross-linkable hyaluronic hydrogel due to the wide applicability and biocompatibility. To synthesize an enzymatic crosslinked hydrogel system, the linear HA polymer was functionalized by tyramine molecules in the presence of carbodiimide and hydroxy succinimide catalysts. The phenol in the tyramine molecules crosslinked two nearby functionalized-HA polymer chains by an oxidative coupling reaction of H₂O₂ and HRP released from the liposomes. We first investigated two different tyramine conjugation methods. The tyramine conjugation was carried out by adding NHS or its water-soluble analog Sulfo-NHS to activate the carboxyl group on the HA backbone (figure 1(b)). After synthesis, lyophilized HA-tyramine was dissolved in PBS to characterize the tyramine conjugation by UV–Vis spectroscopy. Standard free tyramine solution showed a peak absorbance at 275 nm (figures 1(c) and S1(a)(available online at stacks.iop.org/BMM/16/065026/mmedia)) and unfunctionalized HA (HA without tyramine) showed no peak at this wavelength as shown in figure S1(a) with dotted line. We created a calibration plot using the maximum UV absorbance of unfunctionalized HA and five known concentrations of tyramine solutions at 275 nm wavelength (figure S1(b)). The concentration of tyramine in the functionalized polymer solution was estimated using the calibration plot. The estimated concentration was converted to the number of tyramine molecules. The degree of conjugation was defined by the number of tyramine molecules present in 100 repeated monomers of functionalized HA. Our determined degree of tyramine conjugation in HA-tyramine synthesized with EDC and Sulfo-NHS was 11. We observed the degree of tyramine conjugation as seven when NHS was used in this reaction instead of Sulfo-NHS. The lesser tyramine conjugation from NHS-intermediate presented in figure 1(c) indicated lesser reactivity of the intermediate towards tyramine molecules. This phenomenon can be explained by the reduced water solubility of the NHS activated intermediate in the reaction due to its hydrophobic nature [56, 58]. The rheological properties of the crosslinked hydrogel synthesized by both Sulfo-NHS and NHS were measured by the plate and cone rheometer at room temperature. The hydrogel was observed to have a higher storage modulus (figure 1(d)) if the gel precursor was synthesized using Sulfo-NHS instead of NHS. The higher mechanical strength of the cross-linked gel resulted from the enhanced tyramine reaction sites achieved by Sulfo-NHS activation.

3.2. Synthesis and characterization of enzyme-encapsulated TSLs
Once we have synthesized the enzymatic crosslinkable HA-tyramine conjugates, the strategy was to control the crosslink reaction by enclosing the enzymes within the TSL capsules until the system reached the desired temperature. TSLs have been studied significantly for temperature-triggered drug release [38–41]. Saturated phosphatidylcholines with two hydrocarbon chains length of 14 or longer are the most commonly used lipids in this purpose due to their main phase transition temperature (T_m) close to physiological temperature [38, 59]. We chose two saturated lipids DPPC and DSPC, to formulate our enzyme encapsulated liposomes. These two phosphatidylcholines have the same polar head but different hydrocarbon chain lengths [60]. DPPC undergoes a change of physical state at around 41 °C, whereas DSPC having two carbon longer chain lengths than DPPC undergoes a similar change at 55 °C [61]. An overall transition temperature of 45 °C was achieved by mixing these two lipids at an 80:20 molar ratio of DPPC and DSPC, respectively.

During liposome formation, the dried lipids were hydrated in their fluid state, meaning above their transition temperatures (60 °C–70 °C) following the previously reported protocols [62, 63]. The DRV method, developed by Gregoriadis et al [64], was used for HRP encapsulated liposome synthesis to avoid the
enzymes being exposed to these high temperatures. In this method (figure S2), the 1st hydration step made blank multilamellar vesicles (MLVs) shown in figure S3. The following probe sonication step downsized these MLVs to small unilamellar vesicles (SUVs). The enzymes were mixed with blank preformed liposomes at room temperature before lyophilization. During lyophilization, the water is removed, and these SUVs were quickly deflated, which allowed them to merge with neighbors to form lipid bilayer sheets [65].

The enzyme HRP was squeezed between these sheets during the drying step. Controlled rehydration of these freeze-dried lipid sheets with HRP in between induced vesicle formation while entrapping some HRP molecules within the new vesicles. The concentrated rehydration was performed at room temperature and resulted in micron size liposomes with tighter size distribution (figure 2(a)) than the empty MLVs formulated in the 1st hydration step shown in figure S3. The average size and zeta potential of rehydrated liposomes were observed to be 825 nm and −2.5 mV, respectively, by DLS. The SEM images in figure 2(b) also verified the average size of the liposomes determined by DLS.

The temperature-controlled enzyme release from the liposomes was characterized by measuring the enzyme activity in a colorimetric assay. The assay contained ABTS and H$_2$O$_2$ that reacted with the HRP released from three liposome samples heated at 25 °C, 37 °C, and 45 °C, and absorbed maximum light of 414 nm. The activity of HRP released from the liposomes heated to 45 °C was observed to be higher than the other two samples shown in figure 2(c). As the rate of absorbance change was proportional to the HRP concentration in the assay, this plot proved a higher amount of HRP was released from liposomes when heated to 45 °C than 25 °C and 37 °C.

We engineered the liposomes such that we get maximum release of HRP at 45 °C. The liposomes were synthesized by mixing DPPC and DSPC at 80:20 mol% ratio. At temperatures below 41 °C, this mixture was in the gel phase in the phase diagram of the binary DPPC–DSPC mixture shown in figure 2(d), adapted from Dreelle [61]. This phase referred to the ordered packing of adjacent lipid molecules in the liposome membrane and lesser permeability to encapsulated molecules [41]. At temperatures above 44 °C, the mixture located just above the fluid line. The membrane became highly permeable at this temperature due to the transition from an ordered gel phase to a disordered liquid phase [41, 66]. The dramatically increased permeability resulted in the higher release of HRP from liposomes when heated to 45 °C. The average HRP concentration released from 50 µl DPPC–DSPC liposome suspension heated at 45 °C for 10 min was 0.26 µg ml$^{-1}$. The total amount of HRP released from 50 µl liposomes heated to 25 °C, 37 °C and 45 °C for 10 min was 0.045 µg, 0.05 µg and 0.077 µg, respectively.

As HRP was reported to show different enzyme catalytic activity at different temperatures [67] and can be denatured at high temperatures [68–70], it was important to know the effect of the temperatures used in this work on the enzyme activity of HRP. Therefore, to compare the catalytic activity of HRP at different temperatures, five samples of a known concentration of HRP (0.1 µg ml$^{-1}$) were heated at five temperatures ranging from 25 °C to 65 °C for the same length of time and mixed with ABTS assay.

The rate of absorbance changes in the ABTS assay, measured at 414 nm, was observed to drop when the HRP solution was heated at temperatures above 45 °C, shown in figure 2(e). No significant difference of ∆A$_{414}/$∆t values for samples heated at 25 °C, 37 °C, and 45 °C provided evidence of no significant loss of HRP activity until heated to 45 °C.

3.3. Thermosensitive hydrogel crosslinking of functionalized HA-tyramine

Following the synthesis of the functionalized polymer and thermally controlled enzyme carriers, we characterized the temperature-modulated hydrogel formation. In the enzyme mediated crosslinking reaction, the enzyme being oxidized by H$_2$O$_2$ transformed the conjugated tyramine molecules to tyramine radicals. These radicals attached to the HA polymers then participated in a coupling reaction and formed C–C or C–O bond between them. Coupled tyramines of adjacent polymers thus create crosslink between the polymer chains and give rise to a hydrogel network [43, 51]. The HRP encapsulating liposomes introduced tunability into this crosslinking reaction by controlling the presence of enzymes in the reaction site at 25 °C, 37 °C and 45 °C as shown in figure 1(a). Maximum release of HRP from the liposomes when heated to 45 °C resulted in faster and denser crosslinks between polymers. This temperature modulated crosslinking of the HA-tyramine polymers was characterized by measuring the rheological properties of the polymer and liposome formulations heated at three different temperatures. The example of the rheological measurement is shown in figures 3(a) and (b). The faster increase of storage modulus was recorded when the mixture was heated at 45 °C, suggesting that more HRP was released from the liposomes at this temperature and reacted with more functional groups to crosslink the HA polymers. The gel point corresponding to the time when the storage modulus crossed over the loss modulus was reached in ~2 min, and the phase angles were close to 0° after 25 min of time progression. The uncross-linked viscous material started to show viscoelastic solid material properties after the mixture was heated for 25 min at 45 °C, shown in the frequency response plot in figure S4.

A very slow increase in storage modulus was observed when the sample was heated at 37 °C and 25 °C. This slow crosslinking might be caused by a
very low release of HRP during the pre-transition of
the lipid phase at temperatures below $T_m$ [71, 72].
This lesser release of molecules at these two temper-
atures was also verified by the thermal release pro-
file of HRP in figure 2(c). Figure 3(c) displayed the
before and after heating the polymer-liposome mix-
ture for 15 min at 45 °C. The endpoint storage and
loss modulus values of the polymer-liposome mix-
ture kept at the three temperatures for 25 min are
presented in figure 3(d). These values were recor-
ded from two experiments performed on two differ-
ent days using HRP containing liposomes from the
same batch. The higher storage modulus in a 45 °C
heating environment than the other two temperature
settings in repeated experiments suggested a higher
degree of crosslinking in response to the increased
enzyme release from liposomes at this temperat-
ure. The permeability of the liposomal membrane at
temperatures below 45 °C was not enough for the trapped enzymes to escape and participate extensively in the crosslink reaction. Upon heating at 45 °C, the permeability enhanced significantly to release more enzymes and increase crosslink density between HA-tyramine polymer chains.

In figure S5 the storage modulus values of the polymer-liposome mixture were compared with the storage modulus of the polymer (HA-tyramine) without liposomes. Both the sample (polymer-liposome mixture) and control (polymer without liposomes) were heated to 25 °C, 37 °C and 45 °C for same time during measurement. The storage modulus of the control did not show any temperature effect after heating and the values were significantly smaller than the values observed with the polymer-liposomes mixtures. This result further clarified that the crosslinking of polymers, therefore, the change of modulus resulted from the different liposomal release of HRP when heated to different temperatures. Using the liposomes, we achieved additional control over the crosslinking of the polymer chains by mildly increasing the system temperature above physiological temperature. This additional temperature tunability above body temperature of this hydrogel can be utilized for focused heating-controlled site-specific gel implantation as well as for temperature modulated therapeutics delivery when loaded with drugs.

3.4. Protein release and in vitro biocompatibility study from thermostensitive hydrogel

To demonstrate the temperature modulated therapeutic release from our liposome-mediated hydrogel crosslinking system, we embedded albumin proteins into the hydrogel/liposome precursor and crosslinking at different temperatures. A-FITC was chosen consciously to study the comparative release of a high molecular weight molecule from the hydrogel crosslinked at different temperatures. The diffusion of high molecular weight molecules was reported to be controlled by the crosslinking density of the gel matrix [14], which was also observed in our study, as presented in figure 4(a). The release of A-FITC from the hydrogel matrix crosslinked at all three temperatures exhibited a burst release at the beginning, which was likely due to the protein concentration gradient inside and outside of the hydrogel matrix. This initial burst release was then followed by a 2nd slower phase of protein release from the core of the matrix. However, 90% of A-FITC was released in the 1st 2 h from the 25 °C crosslinked hydrogel. A marked decrease in the amount of released protein during the initial burst and subsequent slower phase was observed when the liposomal gel was crosslinked at temperatures above room temperature. The 37 °C crosslinked gel underwent 80% of albumin release in the 1st 2 h. The gel crosslinked at 45 °C showed less than a 35% release in the 1st 2 h and sustained release for up to 120 h. The
Figure 4. (a) Cumulative release of A-FITC from HA-tyramine-liposome hydrogels. 1 mg A-FITC was dissolved in 8 mg ml$^{-1}$ HA-tyramine before crosslinking, and 5 mM H$_2$O$_2$, 50 µl ml$^{-1}$ liposomes were added to the precursor and heated at 25 °C (•), 37 °C (♦) and 45 °C (▲). (b) Immunofluorescent image of NG-108 cultured around the HA-tyramine hydrogel in the same petri dish; live cells were stained with green dye and dead cells were stained red.

sustained release of albumin from this gel suggests the slower diffusion of albumin due to the denser crosslinked gel matrix. Approximately 75% of the loaded protein was liberated from the gel in 120 h of the experiment. The rest of the proteins remaining in the gel even after 120 h also suggests high crosslinking density in this hydrogel. The degradation of the matrix network may ensure the complete release of the proteins from this matrix. On this basis of the A-FITC release data, it can be claimed that the temperature modulated high molecular weight therapeutic release is achievable from this TSL-mediated enzyme crosslinkable hydrogel.

Once we have demonstrated the controlled release of protein in vitro, we conducted in vitro cytotoxicity test to demonstrate the biocompatibility of the hydrogel. The in vitro biocompatibility test serves as a pre-screening test for our in vivo histology test. NG108 cell line was selected instead of a more genetic cell line, such as HeLa cells, because the NG108 cell line can be differentiated in the cell culture. The differentiation process allowed the cells to be viable in the culture without proliferation. This process allowed us to observe the cytotoxicity over a longer period without the need for cell passaging. The cells stained with green dye (LIVE) and red dye (DEAD) during the immunofluorescence imaging are shown in figure 4(b). Most of the cells were alive and some cells were interconnected near the hydrogel. A good number of adhered, interconnected cells in the same petri dish with the hydrogel proved that the hydrogel caused minimal cytotoxicity to this cell line.

3.5. In vivo histology study of liposome-hydrogel formulation

HA has been clinically used as a safe and biocompatible dermal filler in mesotherapy. A good number of studies also demonstrated the biocompatibility and efficiency of crosslinked HA derivatives in medical industry [73, 74]. Since the non-crosslinked and non-liposome based HA substrates have already been studied before, we here evaluated the biocompatibility of our liposome-based HA hydrogel while implanted in adult mice body. We implanted the liposome/hydrogel formation in a standard rodent model and evaluated the biocompatibility of the gel formation by histology (figure 5). At 1 week post in vivo subcutaneous implantation, the hydrogel loosely adhered to the surrounding tissues. The hydrogel and surrounding tissue were harvested and processed for histological analysis. The hydrogel appeared to maintain its structural integrity during the 1 week implantation as observed by gross inspection and within the histological slides. Adjacent to the hydrogel was a thin fibrous tissue layer with some inflammatory cells, including neutrophils, lymphocytes, and macrophages (figure 5(a)). The hydrogel was still present 1 month post-implantation, as was the fibrous layer surrounding the sample. Histologically there was a dense fibrous cellular layer adjacent to the hydrogel (figure 5(b)). As with most implantable devices, there was an immune response to the biomaterials and fibrous encapsulation of the device, although the fibrous encapsulation was limited when implanted for 1 month.
4. Conclusion

In this work, a biocompatible hydrogel with temperature modulated properties has been developed by incorporating the enzyme containing temperature-responsive liposomes into the biocompatible functionalized HA solution and heating at desired temperatures. The storage modulus of the hydrogel crosslinked at 45 °C was observed to be almost five times or higher than that of the hydrogel crosslinked at temperatures lower than 45 °C. Maximum gelation occurred at the phase transition temperature of the liposomes present in the mixture. An external temperature source can induce the release of the enzymatic crosslinkers from these liposomes to initiate the rapid gelation at the targeted site or halt the gelation until the solution reaches the desired site. This feature can be used with other chemically cross-linkable gels for temperature tunable therapeutic delivery or externally controlled site-specific soft gel implantation. Extended protein delivery (70% release of protein in 5 d) was achieved by loading the protein molecules with the polymer-liposome gel precursor and heating it above body temperature. The temperature tunable crosslink has made this biocompatible, biodegradable system a promising material for various localized biomedical applications.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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