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PAPER

Thiolated Human Serum Albumin Cross-linked Dextran Hydrogel as a Macroscale Delivery System

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Hydrogels play an important role in macroscale delivery systems by enabling the transport of cells and molecules. Here we present a facile and benign method to prepare a dextran-based hydrogel (Dex-sHSA) using human serum albumin (HSA) as a simultaneous drug carrier and covalent cross-linker. Drug binding affinity of the albumin protein was conserved in the thiolation step using 2-iminothiolane and subsequently, in the *in situ* gelation step. Oscillation rheometry studies confirmed the formation of a three-dimensional viscoelastic network upon reaction of dextran and the HSA protein. The mechanical properties of Dex-sHSA hydrogel can be tuned by the protein concentration, and the degree of thiolation of sHSA. Sustained release of hydrophobic drugs, such as ibuprofen, paclitaxel and dexamethasone, from the Dex-sHSA network was shown over one week. Hence, this albumin-based dextran hydrogel system demonstrates its potential as a macroscale delivery system of hydrophobic therapeutics for a wide range of biomedical applications.

Introduction

Hydrogels are composed of cross-linked hydrophilic polymer networks, resulting in porous, macroscopic materials of high water content. Their unique physicochemical properties enable them to perform as macroscale delivery systems of bioactive agents ranging from cells to molecules.[1, 2] Within such systems, methods to gain spatiotemporal control over release of therapeutic payloads have involved diffusion[3-7], swelling,[8-10] degradation,[11-13] light[14-19] or drug-carrier affinity interactions.[20] More specifically, the latter mechanism facilitates controlled release through the strong and reversible association of the drug molecules using non-covalent interactions such as hydrogen bonding, ionic, van der Waals, and hydrophobic interactions within the macroscale material scaffold. In the context of lipophilic drug delivery, the problem of encapsulating a lipophilic drug within a water-rich environment is resolved through the inclusion of hydrophobic side chains,[21, 22] hydrophobic drug carriers,[23] such as cyclodextrin,[24-29] within the polymer or using a molecular imprinting approach[30, 31] to prepare the polymeric scaffold. Since it is estimated that 40% of new active chemical entities discovered by pharmaceutical companies are poorly water soluble (aqueous solubility less than 10 μM),[32] macroscale drug delivery systems capable of transporting hydrophobic therapeutics are highly relevant.

Inspired by nature, human serum albumin (HSA) is emerging as an attractive drug carrier in biomedical materials.[33] HSA is the most abundant plasma protein in human (35-50 g L^{-1}) with a molecular weight of 66.5 kDa and has been shown to be biocompatible, biodegradable, non-immunogenic and non-toxic through its *in vivo* metabolism by proteases into innocuous degradation products. Naturally, HSA acts as a transport vehicle in the blood for a variety of molecules, ranging from metal ions, fatty acids, amino acids to numerous drug compounds. Furthermore, HSA has 6 potential binding sites including 2 major hydrophobic cavities[34] resulting in numerous possibilities for specific non-covalent interactions with drug compounds. Thus far, various approaches have been explored to develop albumin-based hydrogels for drug delivery, involving albumin cross-linking by coupling agents,[35-37] free radical polymerization,[38] UV[39, 40] or γ-ray irradiation.[41] Despite successful implementation of albumin in these drug delivery systems, the biocompatibility of the reported gelation procedures and their effect on the albumin protein remains unclear.

We here report, a facile method to simultaneously use HSA as a cross-linker and drug carrier within a dextran polymeric hydrogel system (**Fig. 1**). Dextran is a highly water-soluble and biocompatible natural polymer. Native HSA was modified with thiol groups at lysine residues of the protein under physiological conditions. Finally, a covalently cross-linked hydrogel network was prepared using a Michael addition reaction between maleimide functionalized dextran (Dex-Mal) and thiolated albumin (sHSA) in aqueous solution. The conformation of albumin was observed to be retained in circular dichroism (CD) experiments after thiolation and conjugation steps. Oscillatory rheometry confirmed the formation of Dex-sHSA viscoelastic network and revealed that the mechanical properties can be tuned by varying the degrees of thiolation of sHSA and its concentration relative to Dex-Mal. *In vitro* drug release profiles were evaluated with ibuprofen, paclitaxel and dexamethasone demonstrating sustained release over one week.

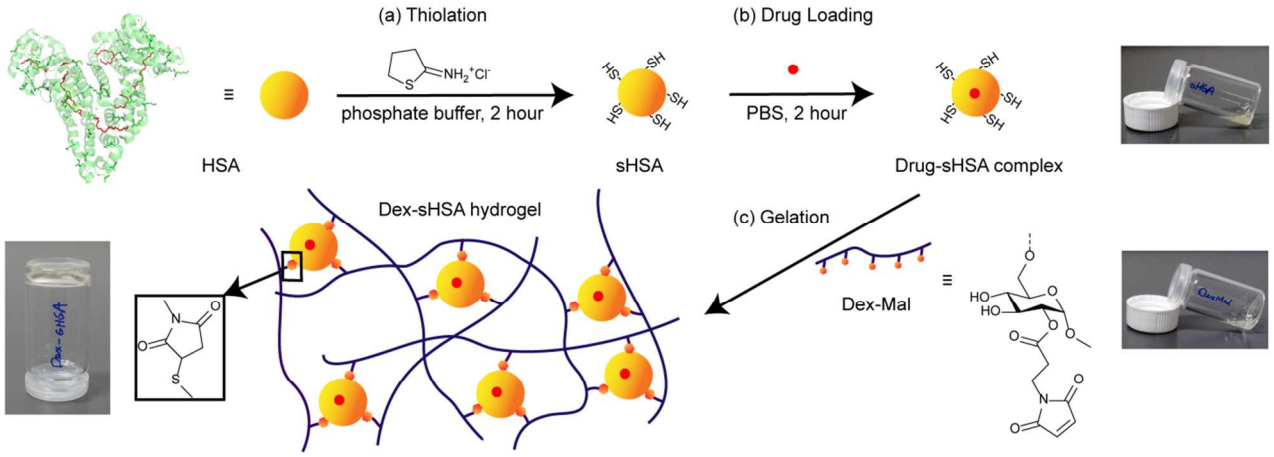

Fig. 1 Preparation of the Dex-sHSA macroscale delivery system. (a) HSA thiolation. (b) Drug loading. (c) Gelation *via* maleimide-thiol Michael addition.

Experimental

Materials

Dextran (70 kDa), human serum albumin (HSA), N,N'-diisopropylcarbodiimide (DIC), 2-iminothiolane (2-IT), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), ibuprofen (IBU), paclitaxel (PTX) and dexamethasone (DXM) were purchased from Sigma-Aldrich. Dextran was dried in a vacuum oven (30 °C) and DMSO was dried over 4Å molecular sieves before use. 3-maleimidopropionic acid and 4-(dimethylamino)pyridinium 4-toluenesulfonate (DPTS) were synthesized as previously reported.[42,43] Dialysis membranes (MWCO 3,500-5,000 Da) were obtained from Spectrum Laboratories, Inc.

Synthesis of Dex-Mal

Dextran (1 g, 6.17 mmol), 3-maleimidopropionic acid (417 mg, 2.47 mmol), and DPTS (115.7 mg, 0.247 mmol) were dissolved in DMSO (33 mL) followed by the addition of DIC (580 µL, 3.7 mmol). After stirring overnight at room temperature, N,N'-dialkylurea was removed by filtration and the crude product was obtained by precipitation in cold isopropanol. The precipitate was dialyzed against Milli-Q water using a 3500-5000 MWCO cut-off membrane and subsequently lyophilized. ^{1}H NMR (400 MHz, D_2O): δ 3.3-4.0 (m, dextran glucopyranosyl ring protons), 4.9 (s, dextran anomeric proton), 6.9 (s, maleimide).

The degree of substitution of the dextran polymer is defined as the number of maleimide groups per 100 glucopyranose residues. The DS is calculated using the ratio: $(100x)/(2y)$, in which x is the integral of the maleimide protons (δ 6.9) and y is the integral of the anomeric proton of dextran (δ 4.9) from the ^{1}H NMR spectra.[44] The DS_{MAL} of the dextran polymer (Dex-Mal) synthesized in this work was 4.6.

Synthesis of the thiolated human serum albumin (sHSA)

HSA was dissolved in 50 mM sodium phosphate buffer (pH 8, including 5 mM EDTA) and stirred gently at 0 °C on ice. Subsequently, an aqueous solution of 2-IT (20 mg mL^{-1}) was added drop-wise to the HSA solution. After 2 hours, the mixture was brought back to room temperature. The product was first dialyzed against 10 mM HEPES buffer (pH 7) three times over 24 hours and then, against Milli-Q water three times over 2 days at 4 °C. Finally, sHSA was lyophilized and stored at -20 °C.

The degrees of thiolation (DT) of sHSA were quantified by the Ellman's test. Briefly, 100 µL 2 mM DTNB, 200 µL 10 mg mL^{-1} sHSA and 700 µL 0.1 M sodium phosphate buffer (pH 8) were mixed and incubated for one hour at room temperature. During this reaction, the formation of the 2-nitro-5-thiobenzoate anion (TNB^{2-}) resulted in an intense yellow colour with an absorbance at 412 nm by UV-vis spectroscopy (Cary-300). The molar extinction coefficient of TNB^{2-} at 412 nm, ε = 14150 M^{-1} cm^{-1}, was used to calculated the concentration of free thiol groups on sHSA.

Circular Dichroism (CD)

Circular dichroism spectra were obtained on a Jasco J-815 spectrometer. All spectra were collected with a scan speed of 50 nm min^{-1} and a response time of 1 second at 20 °C. Each spectrum was averaged over 5 scans. Samples of native HSA, sHSA (DT=3.2), Dex-sHSA conjugate (molar ratio of thiol to maleimide 1:1) were prepared in sodium phosphate buffer (50 mM, pH 7). Native HSA and sHSA solutions were filtered through 0.2-µm syringe filters before CD measurements. For near-UV measurements (250-330 nm), a 1 cm path length quartz cuvette was used for 0.5 mg mL^{-1} protein samples. For far-UV measurements (200-250 nm), a 0.1 cm path length quartz cuvette was applied for 0.1 mg mL^{-1} protein samples.

CD data were presented in terms of ellipticity [θ] (degrees) and converted to mean residue ellipticity (MRE) in deg cm^2 dmol^{-1} by equation **1**, where n is the number of amino acid residues (585), l is the path length of the cuvette, and C_p is the mole fraction.

$$MRE = \frac{\theta(mdeg)}{C_p \times n \times l \times 10} \quad (\mathbf{1})$$

α-helix content was calculated from the MRE values at 222 nm[45] by equation **2**:

$$\alpha - helix\ \% = \left(\frac{MRE - 2430}{30300}\right) \times 100 \quad (\mathbf{2})$$

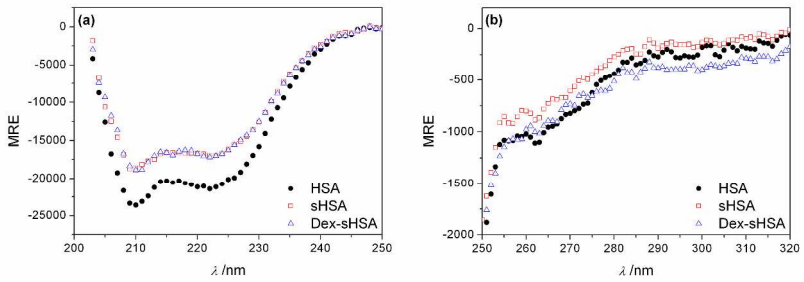

Fig. 2 Far-UV CD (a) and near-UV CD (b) spectra of HSA, sHSA and Dex-sHSA (1:1 maleimide:thiol) in sodium phosphate buffer (50 mM, pH 7).

Dex-sHSA hydrogels

Dex-Mal and sHSA were dissolved in phosphate buffered saline (PBS, 150 mM, pH 7.4) individually and then mixed together using a pipette to prepare the hydrogel samples (1:1 molar ratio of maleimide to thiol).

An AR-G2 rheometer (TA Instruments) was used to measure the mechanical properties of Dex-sHSA hydrogels in oscillatory mode at 37 °C using the parallel plate geometry (40 mm diameter). Solutions of Dex-Mal and sHSA were loaded at 300 µL of each component onto the bottom plate and the geometry was lowered to a gap of 0.40 mm for all experiments. Time sweep measurements were performed to follow gel curing at 1 rad s^{-1} with 5% strain. The linear viscoelastic regime was determined using an amplitude sweep measurement at 1 rad s^{-1} from 0.1% to 100% strain. Frequency sweep measurements were performed from 100 to 0.1 rad s^{-1} with 5% strain in the linear viscoelastic regime.

In vitro drug release

Lipophilic drugs (IBU, PTX or DXM) and sHSA were co-dissolved in PBS (150 mM, pH 7.4, 0.02% NaN_3) and incubated for 2 hours at 37 ºC prior to gelation. The molar ratio of sHSA and drug was kept at 1:1 in all samples. The drug-sHSA solution was subsequently cross-linked with the Dex-Mal polymer solution to form the drug-loaded hydrogels for *in vitro* drug release.

To examine the drug loading efficiency, 1 mL 0.25% trypsin solution (25 mM HEPES buffer, pH 7) was added on top of 200 µL drug loaded Dex-sHSA gel (4.5 wt%) and incubated at 37 ^{0}C. 2 mL acetonitrile was added to the mixture and the precipitates were removed by ultracentrifuge at 13 000 rpm for 10 minutes. The amount of drug was subsequently quantified by reversed-phase high performance liquid chromatography (RP-HPLC) analysis using two LC-8A pumps, SPD-10AVP UV-VIS and ELSD-LTII detectors from Shimadzu. The separation was performed on a Gemini C18 column at a flow rate of 1 mL min^{-1} with a linear gradient from 90% B to 10% B, where A was acetonitrile with 0.1 vol% TFA and B was H_2O with 0.1 vol% TFA. The eluate was monitored at both 220 and 254 nm. The drug loading efficiency was calculated from the amount of drug recovered from the gel divided by the amount of drug loaded expressed in percent.

For *in vitro* drug release study, the drug loaded Dex-sHSA gel was immersed in PBS at 37 °C. At predetermined time intervals, the supernatant buffer solution was collected and replaced with fresh PBS. The collected buffer solutions at each time point were analyzed by RP-HPLC. All drug release experiments were performed in triplicate.

Results and discussion

Synthesis of sHSA

The native HSA protein contains 35 cysteine residues, one free thiol (Cys 34) and 17 disulfide bridges. The disulfide double loops of HSA play an important role in the formation of the two main hydrophobic cavities within the protein, that can be used for ligand binding.[34] Since the goal was to use sHSA as a simultaneous cross-linker and drug carrier, a gentle thiolation agent that helps to retain the native conformation and binding capacity of albumin was required. To this end, Traut's reagent, 2-IT, was selected due to its inherent water-solubility and ability to react with primary amines quantitatively in the pH range (7-10) of biological solutions. In comparison to reducing reagents such as dithiothreitol (DTT), 2-mercaptoethanol and tris(2-carboxyethyl)phosphine (TCEP) 2-IT retains intramolecular disulfide bridges and lysine residues' charge upon thiolation, thus aiding protein's stability and solubility in water.[46, 47] Using Ellman's test prior to thiolation with native HSA, 0.24 free thiol groups per protein were detected. This value increased to 3.16 free thiol groups per protein when thiolated with 20-fold of 2-IT. As shown in **Fig. 3e** *insert*, the degree of thiolation can be easily varied by the molar ratio between HSA and 2-IT, thus providing a handle to control the mechanical properties of the final Dex-sHSA hydrogel (*vide infra*).

Conformation of HSA, sHSA, and Dex-sHSA

CD spectroscopy is a convenient technique to study the structure of proteins in aqueous solution.[48] More specifically, absorption signatures in the far-UV region (below 240 nm) provide insight into protein's secondary structure while absorption in the near-UV region (260-320 nm) is often correlated with protein's tertiary structure. Hence, to investigate the effects of the thiolation by 2-IT and the conjugation of the dextran polymer on the conformation of HSA, CD spectra of a series of samples containing native HSA were collected.

A comparison of CD spectra of HSA and sHSA suggests that albumin's secondary and tertiary structure is largely retained post-thiolation by 2-IT (**Fig. 2**). Generally, the CD spectrum of native HSA in the far-UV has two major minima of a similar ratio

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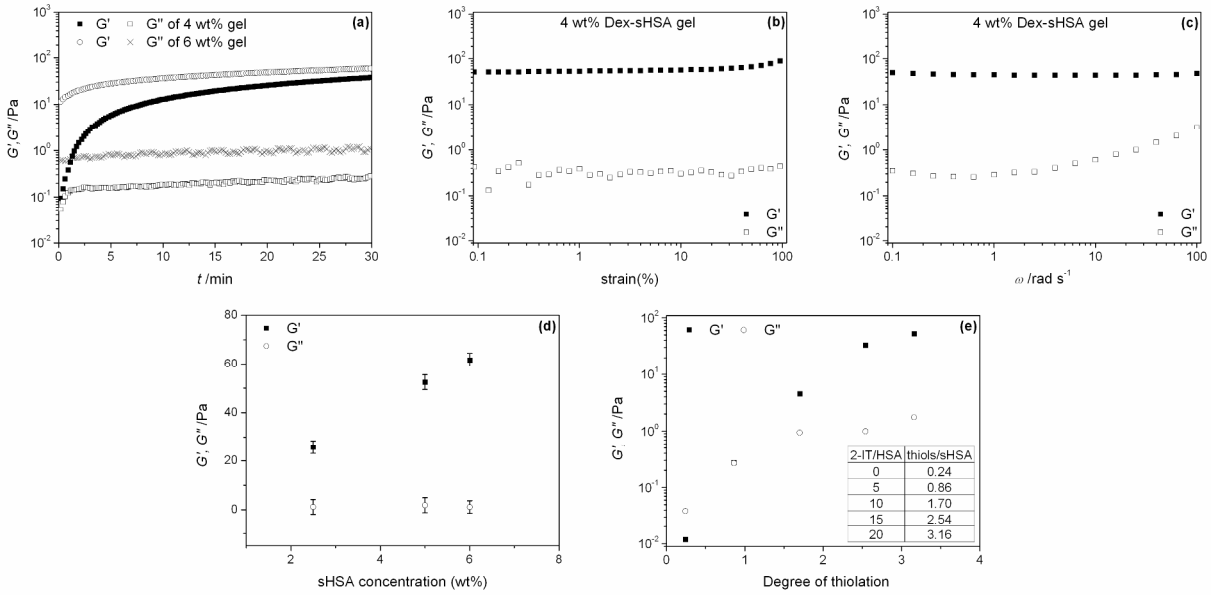

Fig. 3 Oscillatory rheometry of Dex-sHSA hydrogels at 37 °C. (a) Time sweep of 4 wt% and 6 wt% hydrogels at 1 rad s^{-1} and 5% strain. (b) Amplitude sweep from 0.5% to 100% at 1 rad s^{-1}. (c) Angular frequency sweep from 100 rad s^{-1} to 0.1 rad s^{-1} and 5% strain. Effect of (d) the relative concentration of sHSA (DT=3.16) to Dex-Mal, and (e) the degree of thiolation of sHSA (5 wt%) on the storage and loss modulus (G' and G") of Dex-sHSA hydrogels. (insert: sHSA with various degrees of thiolation synthesized by different molar ratio between 2-IT and HSA).

around 208 and 222 nm arising from the degree of protein α-helical character. The mean residue ellipticity (MRE) of thiolated sHSA at both 222 and 208 nm was found to decrease post-thiolation relative to the native form by 13%, thus indicating a slight loss of α-helicity. Additionally, the near-UV CD spectrum shows a subtle decrease in the MRE for the thiolated form. This decrease may be correlated with structural rearrangements occurring nearby HSA's disulfide bridges and tryptophan residues.[49, 50]

Subsequently, we examined the CD signature of the HSA protein upon reacting an equivalent molar ratio of maleimide functionalized dextran polymer and thiolated sHSA. To prevent gelation from occurring during conjugate formation in the CD cuvette, the concentration of sHSA was kept at 0.5 mg mL^{-1}. The CD spectrum of HSA recorded after formation of the Dex-sHSA conjugate is nearly identical to the far-UV CD curve of sHSA and is suggestive of α-helical character retention. More specifically, in the near-UV region, the MRE intensity increased slightly after conjugation, on par with the native protein. A similar result was previously reported by Antonov,[51] whom suggested that hydrogen bond formation between the hydroxyl groups of dextran with the tryptophan residues located on bovine serum albumin leads to the observed increase in CD signal. Gratifyingly, a negligible effect on HSA conformation is observed upon reaction with 2-IT and subsequently, with the dextran polymer by CD spectroscopy.

Formation of Dex-sHSA hydrogels

Dex-sHSA hydrogels were formed *via* maleimide-thiol Michael addition, a rapid, widely used click reaction in biological applications. Since this reaction does not require any initiator, catalyst or cross-linking agent, cell viability can be retained after *in situ* hydrogel formation.[2, 52]

The gelation process of Dex-sHSA hydrogels was monitored by an oscillatory time sweep. After loading and mixing of the Dex-Mal and sHSA solutions on the rheometer plate, the hydrogel was formed on the order of seconds (**Fig. 3a**). Values of storage modulus (G') were observed above the loss modulus (G") from the start of the experiment due to the rapid gelation of the sample. Hence, the cross-over point of G' and G" (generally considered as the gel point) could not be observed. In less than half an hour, G' reached a plateau indicating that the bulk of the gelation process occurred. Moreover, the gelation time was found to be dependent on the initial concentrations of Dex-Mal and sHSA. In comparison with the 4 wt% Dex-sHSA hydrogel, 6 wt% samples were found undergo a more rapid gelation process, resulting in a greater storage modulus on the initial reading.

The resulting hydrogels were further examined by amplitude and angular frequency sweep measurements. Amplitude sweep measurements (**Fig. 3b**) provided information regarding the linear viscoelastic region of the Dex-sHSA hydrogel, which was found to be in the range of 0.1% to 100%. Angular frequency sweep measurements (**Fig. 3c**) showed that G' was at least an order of magnitude greater than G" over the range of 0.1-100 rad s^{-1}; in agreement with the formation of a three-dimensional cross-linked

network.

Since sHSA acts as a cross-linker in the dextran polymeric network, both the relative concentrations and the degrees of thiolation (DT) of sHSA can influence the mechanical properties of Dex-sHSA hydrogels. For the former method, the concentration of Dex-Mal was held constant while the concentration of sHSA was varied corresponding to the molar ratio of between thiols and maleimide. As shown in **Fig. 3d**, the storage modulus (G') of Dex-sHSA gels increased from 25 Pa to 52 Pa when sHSA concentration doubled from 2.5 wt% to 5 wt%. By further increasing the concentration to 6 wt%, G' of the gels went up to 62 Pa. In the latter method, increasing degrees of thiolation of the sHSA protein also yielded a concomitant increase in the mechanical properties of the resulting Dex-sHSA conjugates. In **Fig. 3e**, at low DT, sHSA can be covalently ligated to Dex-Mal, but a limited number of thiol groups on the albumin surface prevents the formation of hydrogels. For these samples, G" was greater than G' in all oscillation measurements exhibiting predominant viscous-behavior. When the DT increased to 1.7 and over, G' was found to be greater than G" in angular frequency sweep measurements and a viscoelastic material was formed. Hence, both methods, either increasing protein concentration or DT of the protein, provide a facile handle to tune mechanical properties of the macroscale delivery scaffold.

In vitro drug release

Drug release kinetics from HSA binding pockets within the Dex-sHSA hydrogels were evaluated using ibuprofen (IBU), paclitaxel (PTX) and dexamethasone (DXM). IBU is a typical non-steroidal anti-inflammatory drug. PTX is an antimicrotubule chemotherapeutic agent currently used in the treatment of solid tumor malignancies. Both IBU and PTX have been shown to be highly bound to plasma proteins (above 95%).[53, 54] DXM is a synthetic corticosteroid widely used as anti-inflammatory and immunosuppressant in clinical treatments, which is moderately bound to plasma proteins (70-80% depending on the test procedure).[55] Despite the lipophilic and poor water-soluble character of the three drugs tested (See supporting information **Table S1**), the drugs were co-dissolved with the HSA drug carrier to form water-soluble drug-HSA complexes and reacted with the hydrogel material. High drug loading efficiencies of IBU, PTX and DXM within the Dex-sHSA material were obtained (91.3%, 87.7% and 92.1%, respectively). By this method, organic solvents such as DMSO become unnecessary for the dispersion of various therapeutics, and further ligation to the hydrogel scaffold enables their prolonged delivery.

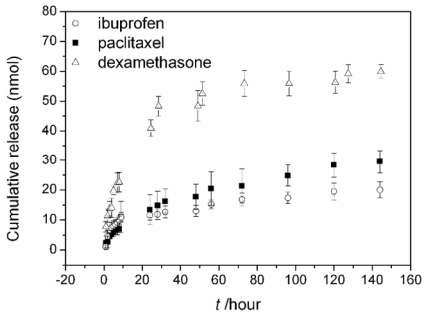

Fig. 4 *In vitro* drug release profiles of IBU, PTX and DXM from Dex-sHSA hydrogels (4.5 wt%, DT=3.16) in PBS (150 mM, pH 7.4) at 37 °C.

In vitro drug release of Dex-sHSA hydrogels were performed under physiological conditions (PBS, pH 7.4) at 37 °C. As shown in **Fig. 4**, the release of all three drugs from the hydrogels occurred in two stages: a fast release in the initial 10 hours, followed by a second stage of slower and sustained release up to 140 hours. In the first stage, hydrophobic drug release was driven by diffusion and swelling (See supporting information, **Fig. S1**) of the hydrogel matrix. In the second stage, the erosion of the hydrogel plays an important role in release kinetics. The release curve of DXM exhibited a faster release rate than IBU and PTX, which may arise from differences in hydrophobic character and binding interactions with HSA. However, it is anticipated that these release profiles will change when these networks are presented with proteases due to the enzymatically sensitive HSA cross-links. Overall, this assay shows that Dex-sHSA hydrogels can be easily loaded with hydrophobic therapeutics and show sustained release.

Conclusion

In this work, we have demonstrated a facile and benign method to simultaneously use HSA as a drug carrier and hydrogel cross-linker in a macroscale drug delivery system. Thiol groups were introduced to HSA by a mild thiolation agent 2-IT, which assisted in the preservation of the native protein conformation as observed by CD spectroscopy. Mixing of sHSA and Dex-Mal resulted in rapid *in situ* gelation with the potential to tune mechanical properties by modulating protein concentration and the degrees of thiolation of sHSA. Moreover, the incorporation of HSA into these networks provided access to sustained delivery of hydrophobic therapeutics on the order of one week. This hydrogel based macroscale delivery system combines the advantage of the versatile drug-binding capability of HSA and the biocompatible, highly hydrophilic nature of dextran polymers. Beyond therapeutic delivery applications in an *in vivo* context, such scaffolds can also be candidates for high throughput screening of therapeutics *in vitro* in a 3D cell culture setting.

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Notes and references

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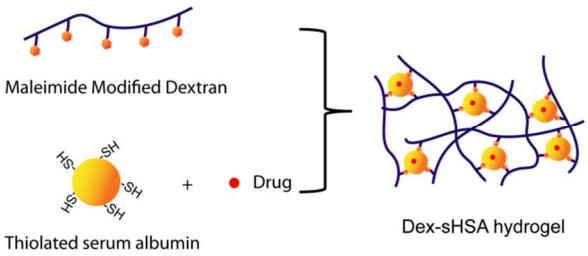
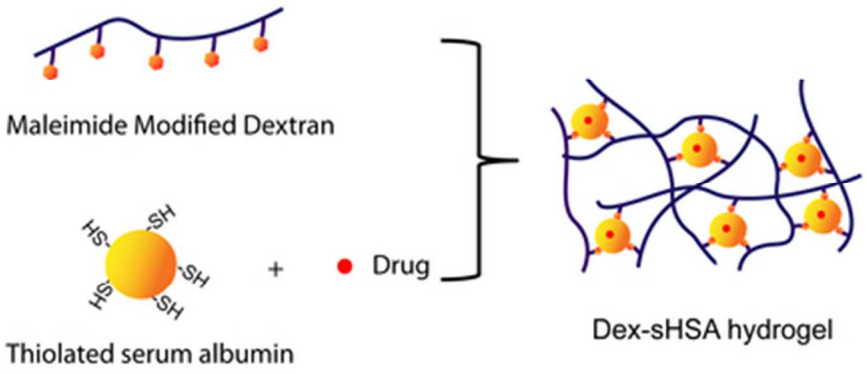


A biohybrid hydrogel system using human serum albumin as a simultaneous drug carrier and covalent cross-linker was established for macroscale drug delivery.



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