Hydrogel based drug carriers for controlled release of hydrophobic drugs and proteins

Ke, P.

Chapter 4

In situ Forming Covalently Crosslinked Albumin/Dextran Hydrogel as a Drug Carrier

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Abstract

An *in situ* forming, covalently crosslinked hydrogel system composed of human serum albumin and maleimide functionalized dextran has been prepared without any chemical modification on the protein. The obtained hydrogel was tested as a drug carrier, using diclofenac, ibuprofen and ketoprofen as model drugs.

Introduction

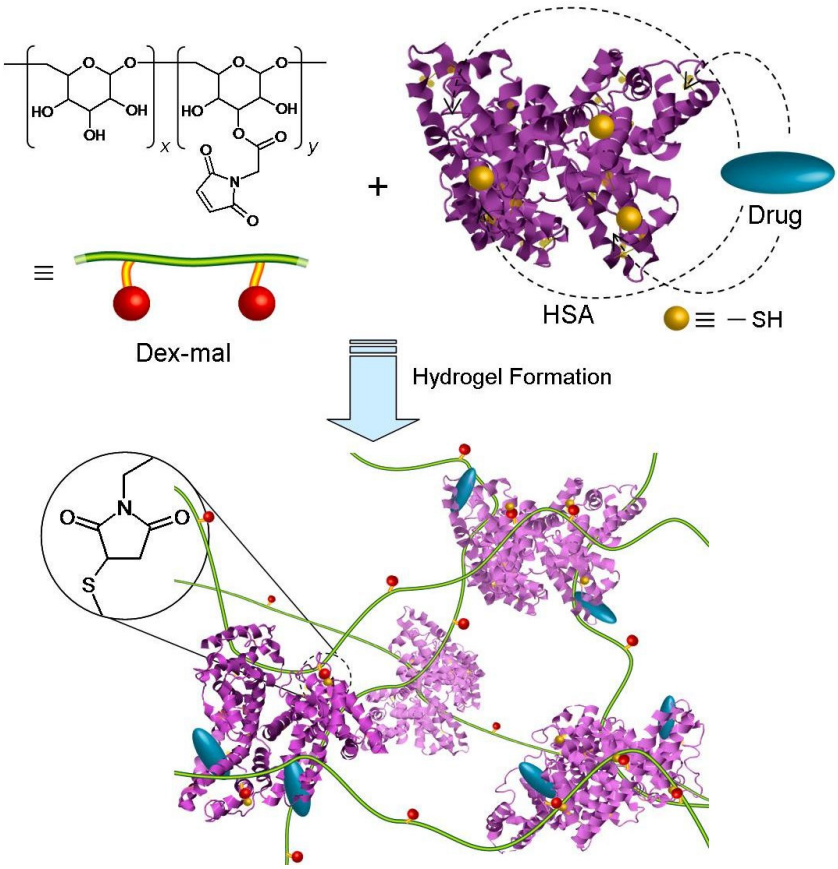
Hydrogels have drawn a great attention for biomedical applications because of their excellent biocompatibilities and the similarity between their highly hydrated three-dimensional polymeric networks and hydrated body tissues.[1] During the last decades, hydrogels have been functionalized with variety of molecules to provide additional values and to convert them to a "smart material".[2] For example, intelligent hydrogel based drug delivery systems with tunable permeability, stimuli responsiveness and biodegradability have been developed, which lead to the desired release.[3]

Recently we have developed *in situ* forming hydrogel systems composed of maleimide modified dextran (Dex-mal) and thiol functionalized β-cyclodextrins for the delivery of hydrophobic drugs.[4] The excellent biocompatibility of the obtained hydrogel was demonstrated *in vivo* using a zebrafish embryo assay.[5] It is important to note that the crosslinking reaction, a Michael addition between thiol and maleimide groups, can be performed under physiological conditions without the need of any additional reagents (e.g. radical initiators or catalysts, which are potentially toxic.) and no side-products are formed. Therefore, we believe that this strategy for fabrication of hydrogels is promising for the future biomedical applications.[6]

Protein functionalized hydrogels have been of great interest due to their high biocompatibility and inherited characteristic functions of proteins that can be preserved in the highly hydrated environment.[7] Human serum albumin (HSA) is one of the most abundant proteins in blood plasma. In the blood stream, it can act as a transporter for a wide variety of compounds including hormones, fatty acids, and drugs.[8] Since HSA has easy accessibility and high affinity for various (bio)molecules, HSA is emerging as an attractive component to construct biomaterials.[9] Thus a HSA functionalized hydrogel can be beneficial for efficient drug delivery systems, for example, it can improve the solubility of hydrophobic drugs, decrease the toxicity caused by aggregation and release the drugs in a controlled manner.[10]

In this contribution, we will present an *in situ* forming, covalently crosslinked hydrogel by using HSA as a crosslinker *via* the thiol-maleimide reaction. Hydrogel can be obtained by mixing solutions of HSA and maleimide modified dextran (Dex-mal) without any chemical modification on HSA, since HSA has several thiol groups on its surface.[11] The

resulted hydrogel was characterized with rheometry and scanning electron microscopy. Furthermore, the drug release property from the obtained HSA functionalized hydrogel was evaluated with model drugs diclofenac, ibuprofen and ketoprofen to show its potential as a drug carrier. (Scheme 1)



Scheme 1 Hydrogel formation of maleimide modified dextran (Dex-mal) and human serum albumin (HSA). Upon the mixing, maleimide and thiol groups undergo Michael addition to form crosslinking points. In the HSA structure, there are several drug binding sites,[12] thus the affinity of drugs to the resulting hydrogel becomes higher.

Results and discussion

Maleimide modified dextran (Dex-mal; 150 kDa, the number of maleimide group per 100 anhydroglucosidic rings was 7) was prepared by the same procedure as we reported previously.[4] Human serum albumin (HSA) was used without any pretreatment and the

amount of thiol was determined to be 10 per HSA molecule by Ellman test.‡ Furthermore, to test the reactivity of those free thiol groups on HSA to maleimide groups, consumption of maleimide groups by addition of HSA was confirmed by ^{1}H NMR. (Fig. 1)

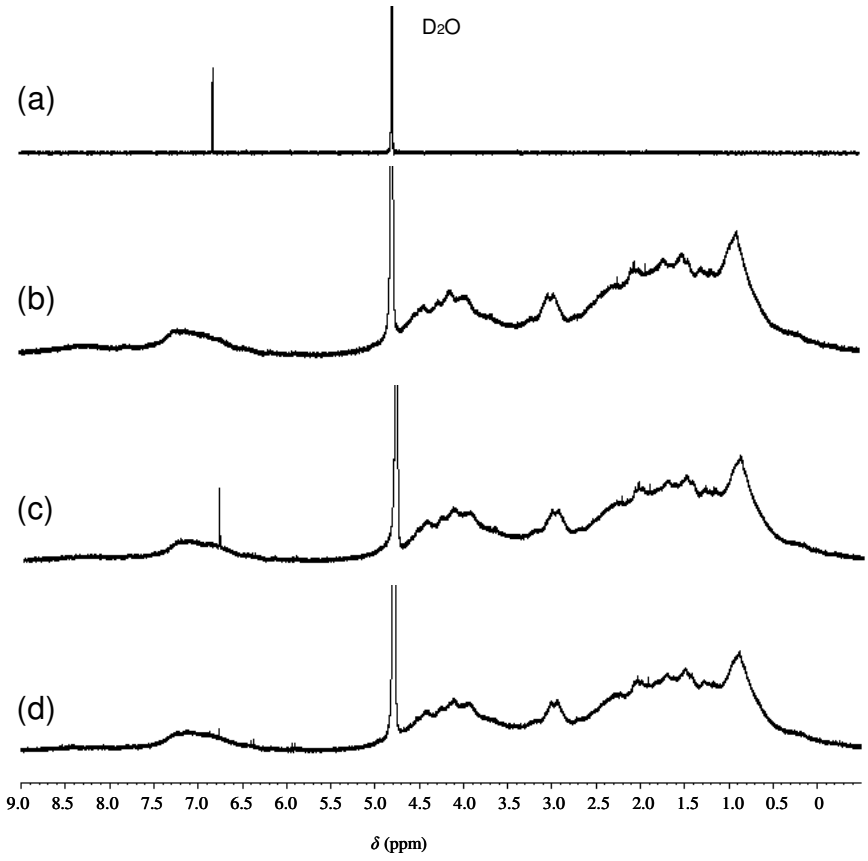


Fig. 1 ^{1}H NMR spectra of 105 μg/mL maleimide in D_2O (a), 29.55 mg/mL HSA in D_2O (b), 2 hours after mixing of maleimide (210 μg/mL, 400 μL) and HSA (59.1 mg/mL, 400 μL) in D_2O (c) and 12 hours after mixing of maleimide (210 μg/mL, 400 μL) and HSA (59.1 mg/mL, 400 μL) in D_2O (d).

As shown in Fig. 2, hydrogels were formed by mixing solutions of Dex-mal and HSA in phosphate buffered saline (PBS), in which the ratio between maleimide and thiol groups were kept at 1:1. Upon the addition of HSA solution (59.1 mg/150 μL), a solution of Dex-mal (22.5 mg/150 μL) turned into a gel within 30 min at 37 °C. The gelation time was shown to depend on the polymer concentrations.

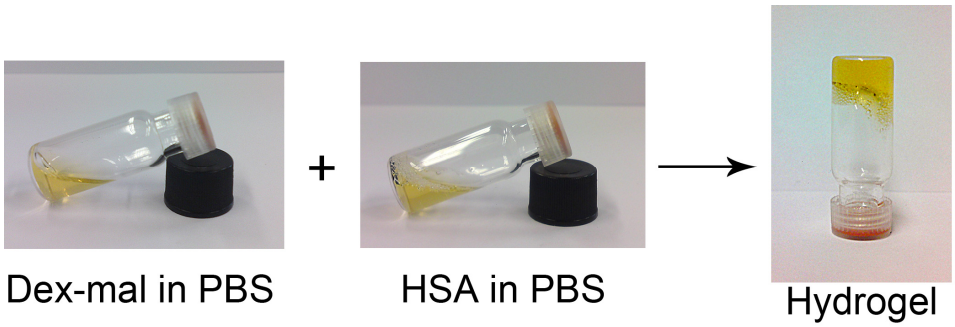


Fig. 2 Photographs of *in situ* hydrogel formation. Hydrogel was formed after mixing solutions of Dex-mal (22.5 mg/150 µL) and HSA (59.1 mg/150 µL) in PBS.

The obtained hydrogels were characterized with rheometry, dependences of storage (G') and loss (G'') moduli on angular frequency (ω) were measured. As shown in Fig 3a, after mixing Dex-mal and HSA, it showed elastic behavior within a few minutes although it

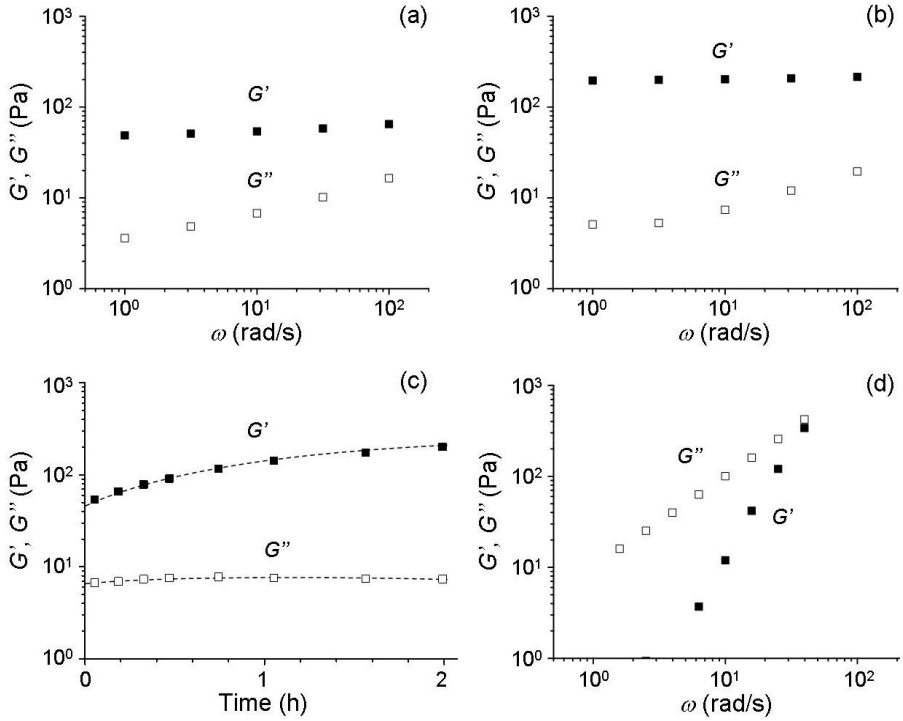


Fig. 3 Rheological properties of the hydrogel obtained by mixing of solutions of Dex-mal (22.5 mg/150 µL) and HSA (59.1 mg/150 µL) in PBS: just after mixing (a); and after 2 hours (b). Time developments of the G' and G'' at 10 rad/s were shown in panel (c). Control experiment was conducted with the mixture of unmodified dextran and HSA at the same concentrations as the hydrogel sample: even one day after mixing, it showed fluidic behavior (d).

showed a little flow in the vial tilting method until a half hour. 2 hours after mixing, the elastic behavior was pronounced as shown in Fig 3b. The time development of the G' and G'' measured at 10 rad/s was shown in Fig 3c. The values of G' increased 4 times in 2 hours, while G'' remained practically the same. It was confirmed that individual solutions of HSA and Dex-mal showed a dominant viscous property. Furthermore the mixture of unmodified dextran and HSA exhibited a characteristic behavior of a Newtonian liquid. (Fig 3d) This observation indicated that HSA crosslinked Dex-mal *via* a thiol-maleimide reaction resulting in a formation of hydrogel.

The structures of the obtained hydrogels were investigated with scanning electron microscopy (SEM) on freeze-dried hydrogel samples. As shown in Fig. 4, the hydrogel had a relatively regular porous structure, which indicated a highly hydrated structure and a homogeneous reaction during the gelation. The observed structure was not different from our previous dextran based hydrogels,[4] which implied the same mechanism of hydrogel formation.

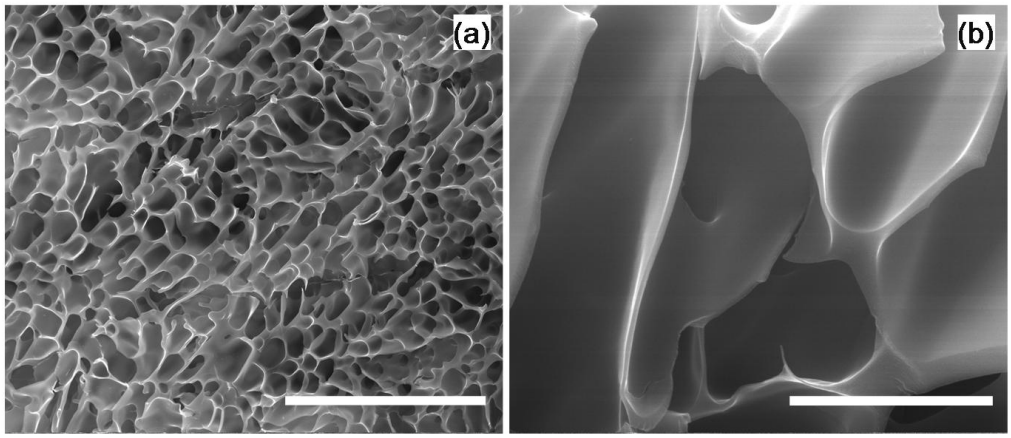


Fig. 4 SEM images of the freeze-dried hydrogel prepared from HSA and Dex-mal solutions in pure water; with lower and higher magnifications. Scale bars in the images (a) and (b) represent 50 and 5 μm, respectively.

To examine the release properties of the obtained HSA functionalized hydrogel, commonly used nonsteroidal antiinflammatory drugs, namely diclofenac, ibuprofen and ketoprofen were loaded to the gel. The drug loaded hydrogels were prepared with a fixed ratio of HSA to drug of 1:2. The drug carrying gels (300 μL) were put into a dialysis tube

with 2 mL of fresh PBS and placed in 25 mL of PBS which was refreshed every 24 hours. The amount of drug released from the hydrogel was determined by HPLC over a period of one week. It is noteworthy that addition of these drugs did not alter the gelation time and the solubility of the drugs were improved by HSA.

The release profiles of the three drugs were shown in Fig. 5. In all cases drugs were released in a linear manner, in which about 50 %, 50 % and 20 % of loaded drugs were released in 7 days, for diclofenac, ibuprofen and ketoprofen, respectively. The difference in the released amounts is presumably because of difference in binding constants to HSA.[14‡] It is important to note that the release profile did not show an initial rapid release phase (burst effect), which has been commonly observed in other hydrogel systems.[13] In this hydrogel, HSA acts not only as a crosslinker but also as a binding site for drugs, thus resulting in a constant release from the hydrogel without showing a burst effect. As HSA has several binding sites with different binding constants, a preferable release for each molecule can be achieved after careful optimizations (e.g. the density of HSA in the gel, the amounts of the drugs and existence of competitive binding molecules).

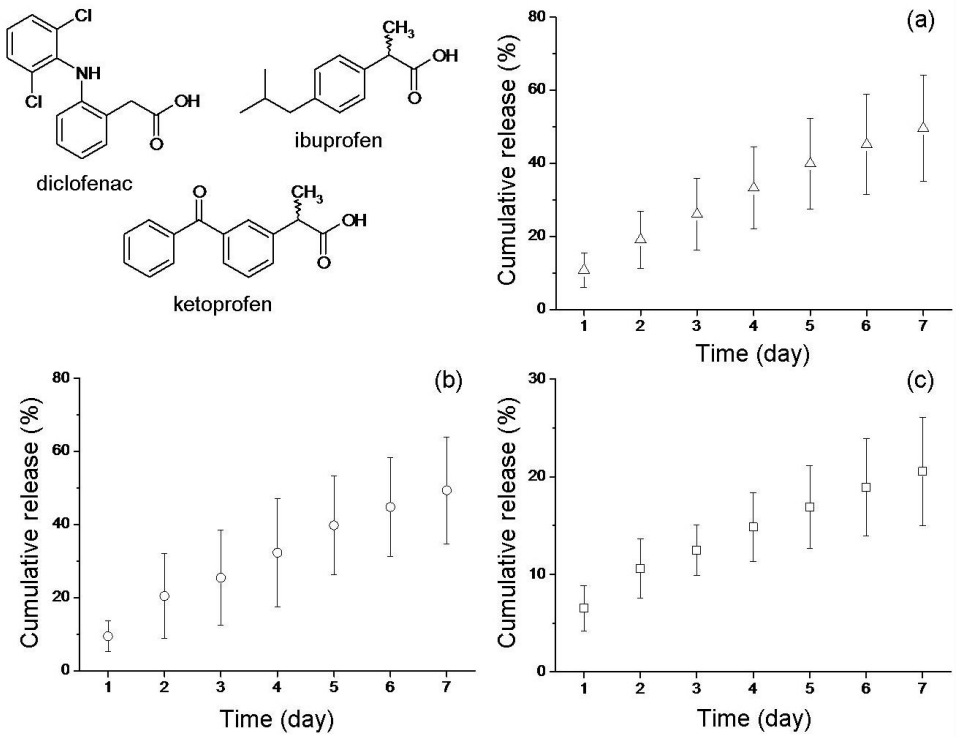


Fig. 5 *In vitro* release profile of diclofenac (a), ibuprofen (b) and ketoprofen (c) from the HSA functionalized hydrogel. In all cases, drugs were released linearly.

Conclusion

In conclusion, an *in situ* forming, covalently crosslinked hydrogel system composed of human serum albumin (HSA) and maleimide functionalized dextran (Dex-mal) has been prepared and its potential as a drug carrier was shown. By mixing aqueous solutions of HSA and Dex-mal, hydrogels were formed *via* a Michael addition. In this system, native HSA was directly used for hydrogel preparation without any chemical modifications. The *in vitro* release of three different model drugs diclofenac, ibuprofen and ketoprofen were investigated and the release of these model drugs did not show an initial burst effect making the current hydrogel an attractive drug carrier. In principle, this system can also be applied for many other drugs that can bind to HSA for an efficient delivery. Furthermore, using an enzymatic degradation of HSA (see Fig. 6), enzyme triggered drug release from the HSA functionalized hydrogel is currently under investigation.[15]

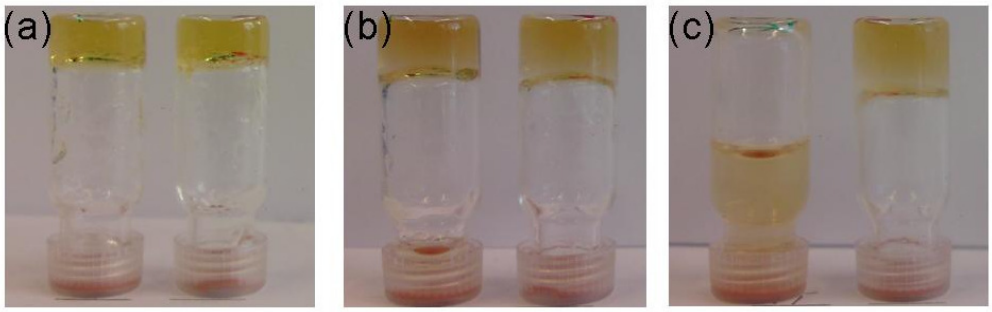


Fig. 6 Photographs of enzymaic degradation of HSA functionalized hydrogel. Freshly prepared hydrogel sample (a), 24 hours after adding 300 µL trypsin (0.25%) solution (left) and 300 µL PBS (right) (b), 48 hours after adding 300 µL trypsin (0.25%) solution (left) and 300 µL PBS (right) (c).

Experimental

Materials.

Human serum albumin (HSA), ketoprofen, ibuprofen, diclofenac sodium salt, 4-nitrophenyl disulfide and 4-dimethylaminopyridine were obtained from Aldrich. Maleic anhydride, *N,N′*-dicyclohexylcarbodiimide (DCC), *p*-toluene sulfonic acid monohydrate and maleimide was supplied by Fluka. Trypsin (2.5 %) was purchased from invitrogen. Dextran (Mw = 150, 000, Pharmacia Fine Chemicals, Sweden) was dried in the vacuum oven for several days before use. 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS) was synthesized from 4-(dimethylamino)-pyridine and *p*-toluenesulfonic acid

monohydrate and recrystallized from toluene. Water used in all experiments was purified through deionization and filtration with a Millipore purification apparatus.

Methods.

^{1}H NMR spectra were recorded on a Bruker AV-400 spectrometer operating at 400 MHz. The visco-elastic properties of the samples were measured by using a rheometer (TA Instruments AR-G2) with plate-plate geometry (40 mm parallel plate). Structure of the hydrogel was analyzed by a Nova NanoSEM (FEI) scanning electon microscope with an accelerating voltage of 15 kV and a spot size of 3.5. The amount of drug released from the hydrogel was detected by the HPLC (Shimadzu) with a C18 column (Gemini, Phenomenex; 50 x 4.6 mm; 3µm particle size).

Synthesis of maleimide functionalized dextran (Dex-mal).

Dex-mal was synthesized according to our previously reported procedure.[4] The degree of substitution (DS; defined as the number of substituents per 100 AHG units) was determined to be 7 from the ^{1}H NMR based on the glucosidic protons of dextran (δ 3.2 - 4.0) and the protons of the maleimides (δ 6.9). ^{1}H NMR (400 MHz, D_2O): δ 3.2-4.0 (m, dextran glucosidic protons), 4.4 (maleimide), 4.9 (dextran anomeric proton), 6.9 (maleimide).

Determination of free thiol groups of HSA.

The amount of free thiol groups of HSA were measured using Ellman test.[18] Typically, to a solution of 4 mL HSA (0.5 mg in 10 mL PBS) was added 1 mL PBS, 4 mL acetone and 1 mL 4-nitrophenyl disulfide solution (1 mM in acetone), the absorbance at 412 nm of the resulted mixture was measured. The amount of free thiol groups was calculated to be 10 per HSA molecule.

Michael addition between free thiol groups of HSA and maleimide.

The Michael addition between the free thiol groups of HSA and maleimide was investigated by ^{1}H NMR (Fig. 1). A singlet at δ 6.9 is attributed to maleimide (a) and HSA has a broad peak in the range δ 6.5 ~ 7.5 besides the other peaks between 0.5 ~ 4.5 (b). An obvious decrease of maleimide peak was observed 2 hours after mixing of maleimide and HSA at 37 °C with a thiol-maleimide ratio of 4:1 (c) and the maleimide peak was almost completely disappeared after 12 hours incubation at 37 °C (d), which indicate the Michael addition between free thiol groups of HSA and maleimide.

Hydrogel preparation.

Hydrogels were obtained by mixing solutions of Dex-mal and HSA at 37 °C. Typically, 150 μL Dex-mal (22.5 mg) and 150 μL HSA (59.1 mg) in phosphate buffered saline (PBS) solutions were mixed by vortexing. The obtained mixture was incubated at 37 °C and the gelation time was determined to be 30 min by the vial tilting method (it was regarded as a gel when there was no flow of the sample within 5 seconds).[19] The gelation time was shown to depend on the polymer concentrations: Dex-mal (30.0 mg/150 μL) and HSA (78.7/150 μL) gellated within 15 min; Dex-mal (15.0 mg/150 μL) and HSA (39.4 mg/150 μL) gellated within 100 min.

Characterizations of the hydrogel.

The visco-elastic properties of the gel were measured by using TA Instruments AR-G2 rheometer with plate-plate geometry (40 mm steel parallel plate), and equipped with the solvent trap. Hydrogel sample was prepared by mixing solutions of Dex-mal (22.5 mg) and HSA (59.1 mg) directly prior to the measurements. The sample was placed on the Peltier lower plate and squeezed into a 100 μm gap by the upper plate. Strain-sweep tests of the initial sample were performed to determine the limits of the visco-elastic regime. The gelation time and changes in the gel strength were measured with oscillatory time sweep tests (on frequencies 100, 10, and 1 rad/s), and to confirm the visco-elasticity of the sample during gelation, strain sweep tests were regularly performed. For a control experiment, strain-sweep and frequency sweep tests were performed on a mixture of 600 μL unmodified Dextran (M_w = 150, 000, 45.0 mg) with HSA (118.2 mg) with a 345 μm gap. All experiments were carried out at 37°C.

The structure of the hydrogel was analyzed by using scanning electron microscopy (SEM). 300 μL of freshly prepared hydrogel was freeze-dried and fractured pieces of the dried gel were placed on an aluminum stub and coated with carbon previous to the measurements.

Drug loading and release.

Diclofenac, ibuprofen and ketoprofen were taken as model to examine the release properties of the formed hydrogel. Typically, 520 μg of diclofenac was added to HSA (59.1 mg) in 150 μL PBS (the molar ratio between diclofenac and HSA was 2:1) and the resulted solution was vortexed and kept at 37°C for 30 minutes. After that, a solution of

Dex-mal (22.5mg) in 150 µL PBS was added and the resulted mixture was incubated at 37°C for 2 hours. The resulted diclofenac carrying hydrogel was then put into a dialysis tube (MWCO 3500) together with 2 mL of PBS. The dialysis tube was then placed in 25 mL of PBS. The surrounding PBS was stirred at 50 rpm at room temperature and refreshed every 24 hours. The released amount of diclofenac was determined by HPLC (Shimadzu, C18 column) using UV detection at 254 nm with isocratic flow of 1 mL/min with a mobile phase of methanol/water/acetic acid (400:600:5, v/v/v). For calibration standard diclofenac solutions of 5 – 10 µg/mL were used.

Drug loading and release processes for ibuprofen (360 µg) and ketoprofen (450 µg) were similar with diclofenac. The released amount of ibuprofen was detected using UV detection at 260 nm with a mobile phase of acetonitrile/NaH_2PO_4 (5 mM)/acetic acid (60:40:1, v/v/v). The signals were calibrated with standard solutions of 1 – 10 µg/mL. Ketoprofen was detected at 254 nm with an isocratic flow of acetonitrile/water/phosphate buffer (pH = 3.5) (40:58:2, v/v/v). The signals were calibrated with standard solutions of 0.1 – 1 µg/mL.

Enzymaic degradation of HSA functionalized hydrogel.

The HSA have amino acid sequences that are known as recognition sites for enzymes. For example, the protease trypsin is able to cleave the amide bond after the lysine residue within HSA. To test the enzymatic degradability, 300 µL trypsin (0.25%) and 300 µL PBS solution was added on top of the freshly prepared hydrogels respectively and refreshed every 24 hours. As shown in Fig. 6, there was not obvious difference between the hydrogels treated with trypsin and PBS solutions after 24 hours incubation at room temperature. After 48 hours, the tripsin treated hydrogel was completely degraded, in contrast with the hydrogel treated with PBS. This result indicates that the hydrogel degradation is in an enzymatically controlled manner.

Notes and references

‡ From the reported crystal structure of HSA,[16] the number of thiol groups that do not form disulfide bonds is 8.

‡ There was no apparent correlation between released amounts of drugs and physicochemical properties of them including pKa, solubility and log P.[17] (see Table 1)

Table 1. Physicochemical properties of the model drugs

Drugs	Mw	pKa	solubility (mM)	log P
Diclofenac	296	3.8	6	4.4
Ibuprofen	206	4.6	185	3.5
Ketoprofen	254	4.6	21	3.1

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