



In situ Crosslinking System of Gelatin with Acrylated β -cyclodextrin Towards the Fabrication of Hydrogels for Sustained Drug Release

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Abstract: Cyclodextrins (CDs) are of interest in the fabrication of various polymeric platforms, especially the crosslinked networks that possess unique advantages in biomedical applications. When one benefits from the abilities of CDs to form inclusion complexes with hydrophobic drug molecules, hydrogel-based supported drug platforms employ CDs as nano-buckets in the network structure. In the current study, we report on the fabrication of gelatin-based hydrogels chemically crosslinked with acrylated β -cyclodextrin (β -CD) as sustained drug release platforms. A practical and straightforward synthesis of crosslinked networks was achieved by Michael's addition reaction of gelatin amino functionalities onto acryloyl groups of modified β -CD. Tunable synthesis of hydrogels incorporating a different amount of β -CD molecules was maintained by simple adjustment of the reaction stoichiometry between amine and acrylate groups. The resulting hydrogels were characterized by their surface morphology, equilibrium swelling ratios, and rheological properties. Thus obtained hydrogels were evaluated in terms of their drug loading capacities and sustained release efficiencies. The results demonstrated that the amount of drug loading and prolonged release is dependent on the amount of β -CD in the gel network. Thanks to their in situ gel-forming abilities, these hydrogels can be used as injectable formulations for various biomedical applications.

Keywords: Drug releasing hydrogels, β -cyclodextrin, aza-Michael addition, injectable hydrogels.

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INTRODUCTION

Hydrogels purvey some unique and advantageous properties that make them ideal candidates in several biomedical applications. (1–3) Significant research has been devoted to engineered hydrogels in practical clinical utilization, such as in controlled/sustained drug release platforms or tissue construction scaffolds. In tissue engineering, hydrogels are employed as environments for adhesion, encapsulation, growth, or directed spread of cells. (4–6) In therapeutic applications, hydrogels are exploited as slow drug releasing platforms in which the rate of release can be controlled via response toward external stimuli such as temperature or pH of the local environment. (7–9) While the eximious features of hydrogels are advantageous, the challenges associated with the

practical introduction of the hydrogels into the body might limit their routine clinical implementation. Surgical implantation of the pre-formed hydrogels to defect sites is often associated with patient compliance and discomfort. In this context, non-invasive techniques such as employment of injectable hydrogel formulations to administrate regenerative factors or therapeutics to the body have been addressed. (10–12) In situ cross-linkable hydrogels that can form the network structure in vivo through physical or chemical interaction of gel, precursors could allow straightforward inclusion of drugs, (13) growth factors (14) or genes (15) to the target body tissues.

Gelatin is a biopolymer of animal-origin, derived from collagen by partial hydrolysis and denaturation. Due to essential characteristics such

as excellent biocompatibility, nontoxicity, high resemblance to extracellular matrix in a gel state, and low immunogenicity make gelatin widely used in the biomedical fields. (16–18) Gelatin contains peptide sequences that can bind to cell surface integrins, which is notable for cellular recognition. (19) Improved cell attachment and proliferation make gelatin-based scaffolds ideal for tissue regeneration, cell encapsulation, and 3D cell culture applications. (20–22) The utility of gelatin-based controlled-release systems covers a wide variety of drug and gene delivery applications. (23) Although gelatin can form temperature-induced physical gelation, a much-sought strategy is the fabrication of chemically crosslinked hydrogels to increase the stability of the gels. Toward this, several injectable gel-forming systems such as dextran-gelatin hydrogels via oxidized dextran and gelatin, (24) gelatin polymers with pendant norbornene (GelN) or tetrazine (GelT) groups, (25) and UV-curable acrylated gelatin hydrogels (26) have been reported.

Cyclodextrins (CDs) are torus-shaped molecules that are derived mainly degradation of starch. The structures of CDs possess an interesting molecular property with a hydrophobic inner cavity and hydrophilic outer structure. CDs can form inclusion complexes through the hydrophobic inner cavities with molecules that are physically compatible with the cavity size. The dynamic inclusion complexation abilities of CDs with low polarity organic compounds make them ideal materials in the fabrication of biomaterials augmenting the properties in various

applications. (27,28) To date, CDs have been employed in the fabrication of diverse hydrogel-based polymeric drug delivery systems, especially to modify the release kinetics. (29–32) While the overall hydrophilic nature of CDs increases the swelling state of the network structure, the hydrophobic inner core enables enhanced loading and prolonged/sustained release of hydrophobic therapeutic compounds.

In this contribution, we report on the synthesis of gelatin-based hydrogels incorporating β -cyclodextrin residues towards sustained drug release applications. Briefly, hydrogels were prepared via reacting gelatin amino groups with acrylate modified β -CD in aqueous buffer solutions (Figure 1). The method demonstrated a facile and efficient crosslinking process via aza-Michael addition of complementary functional precursors, which can be employed as an injectable gel-forming system. A series of hydrogels displaying tunable physical properties were obtained by changing the stoichiometric balance between gelatin amino groups and acrylate modified β -CD crosslinker. Drug loading and sustained release studies of fabricated hydrogels were investigated by using poorly water-soluble anti-inflammatory drug diclofenac sodium. The results indicated that the amount of drug loading and release profiles could be controlled by changing the β -CD crosslinker ratio. It is believed that the benign gelation methodology outlined here can be utilized in the design and synthesis of bulk and injectable hydrogels for tissue engineering, drug delivery, and various other biomaterial applications.

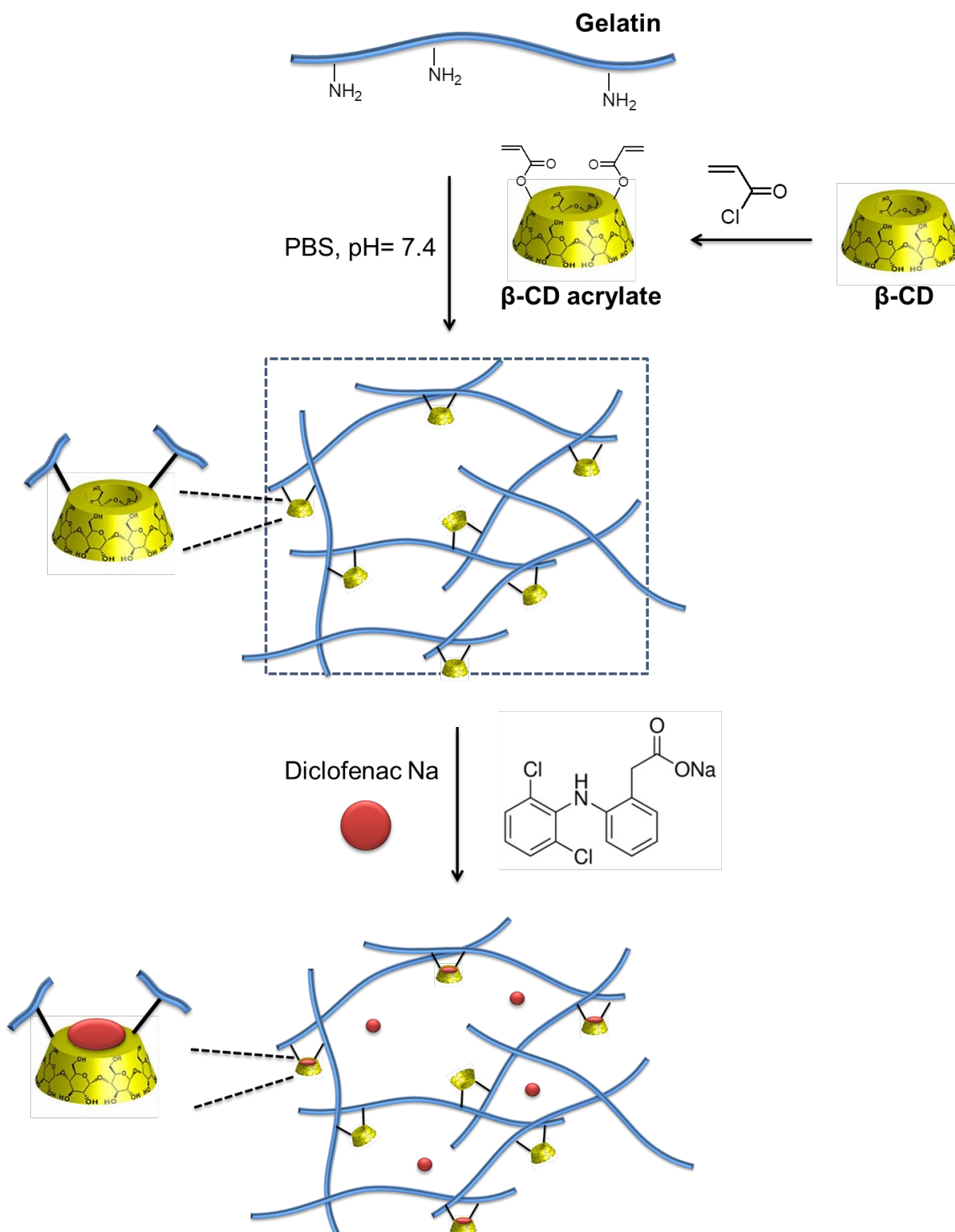


Figure 1. Schematic representation of the gelatin-based hydrogel synthesis with a β -CD-based crosslinker and the diclofenac Na loading into the hydrogel augmented by the inclusion complex formation.

EXPERIMENTAL SECTION

Materials and characterization

β -cyclodextrin, acryloyl chloride, diclofenac sodium salt, 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU), gelatin from porcine skin (type A, 300 g bloom,

average M_n : 50.000-100.000 g/mol) were obtained from Aldrich Chemical Co. All solvents and inorganic materials were obtained from Merck Co. and used as received unless otherwise noted. Synthesis of acryloyl-modified β -cyclodextrin (β -CD-acrylate, the average degree of acetylation ~ 5.9

per molecule. Detailed synthesis procedure is given in Supporting Information) was carried out based on the reported procedure. (33) Characterization of β -CD acrylate was performed using ^1H NMR spectroscopy (Varian 400 MHz). Fourier-transform infrared spectroscopy analyses were conducted on a Nicolet 380 spectrometer. Scanning electron microscopy analysis was carried out using an ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument with 10 kV accelerating voltage. The rheological measurements were performed using an Anton Paar MCR 302 rheometer between 0.05-100 rad/s at 37 °C. UV-vis studies were carried out using a Varian Cary 50 Scan UV/Vis spectrophotometer.

Methods

Synthesis of gelatin-based β -CD incorporated hydrogels

In a vial, gelatin (100 mg) was placed and dissolved in 1 mL PBS buffer (phosphate-buffered saline, pH of 7.4) at 37 °C. In a separate vial, a pre-determined amount of β -CD-acrylate and DBU (0.1 molar equivalents to acrylate groups) were placed and dissolved in 200 μL of PBS solution. The two-ingredient solutions were thoroughly mixed and sonicated briefly. The mixture solution was heated at 37 °C for 6 h for a crosslinking reaction. After that, the resultant hydrogel was washed with distilled water several times to remove unreacted species. Dried hydrogels were obtained after the freeze-drying of water-swollen samples.

Equilibrium swelling ratios (ESRs)

Equilibrium swelling of hydrogels in distilled water was determined at 25 °C. 20 mg hydrogel was conditioned in distilled water, and the mass increase of sample as a function of time was recorded until a constant weight was attained. The percent swelling was determined using empirical equation 1:

$$\text{ESR (\%)} = (W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}} \times 100 \quad (\text{Eq. 1})$$

The swelling tests were performed in triplicate for each sample, and average data with standard deviation was used for obtaining swelling curves.

Rheological characterization

The rheological properties of hydrogels were studied by dynamic oscillatory experiments at a constant 1

% strain. Loss (G'') and storage (G') moduli of disk-shaped water-equilibrated hydrogels were measured at 37 °C between 0.05 and 100 rad/s angular frequency. 8 mm diameter parallel plate was used for measurements and the plate distance was set to 2.0 mm.

Drug loading and release studies

Diclofenac-Na was loaded into the hydrogels by employing solution absorption. Briefly, 50 mg water-swollen gel sample was soaked in 0.5 wt.% drug solution in PBS (pH: 7.4). The hydrogel was incubated at 37 °C for 24 h, protected from light. Total drug loading was determined from the concentration difference between initial and final soaking solutions using a UV-Vis spectrophotometer (276 nm absorption maxima of diclofenac-Na in PBS solution).

Drug release profiles of hydrogels were investigated by immersing gel samples in 3 mL of PBS solution at 37 °C. Aliquots of the release medium (1 mL) were taken out and replaced with the new solution at pre-determined intervals. The total drug content in the collected medium was determined spectrophotometrically at 276 nm. The experiments were performed triplicate, and the release profiles were expressed in terms of cumulative release.

RESULTS AND DISCUSSION

Synthesis and characterization of gelatin-based β -CD containing hydrogels

Hydrogels composed of a gelatin matrix and distinct β -CD units were fabricated via amine-acrylate Michael addition of gelatin amino functionalities onto acryloyl groups of modified β -CD (Figure 2). Though it may vary depending on the source, the amino acid composition of gelatin mostly consists of glycine, proline, and hydroxyproline. Amine-containing lysine residues also constitute the structure of both bovine and porcine skin gelatin. (34) In chemical derivatization and modification of gelatin, amino groups of lysine have been exploited in reactions with isocyanates, (35) aldehydes, (36) vinyl sulfones(37), and genipin. (38) Towards the synthesis of gelatin-based hydrogels, reactive amino groups of amino acid chain strands were utilized in crosslinking reaction with activated carbonyls (39) as well as a modification with photo-curable acrylate functionalities. (26)

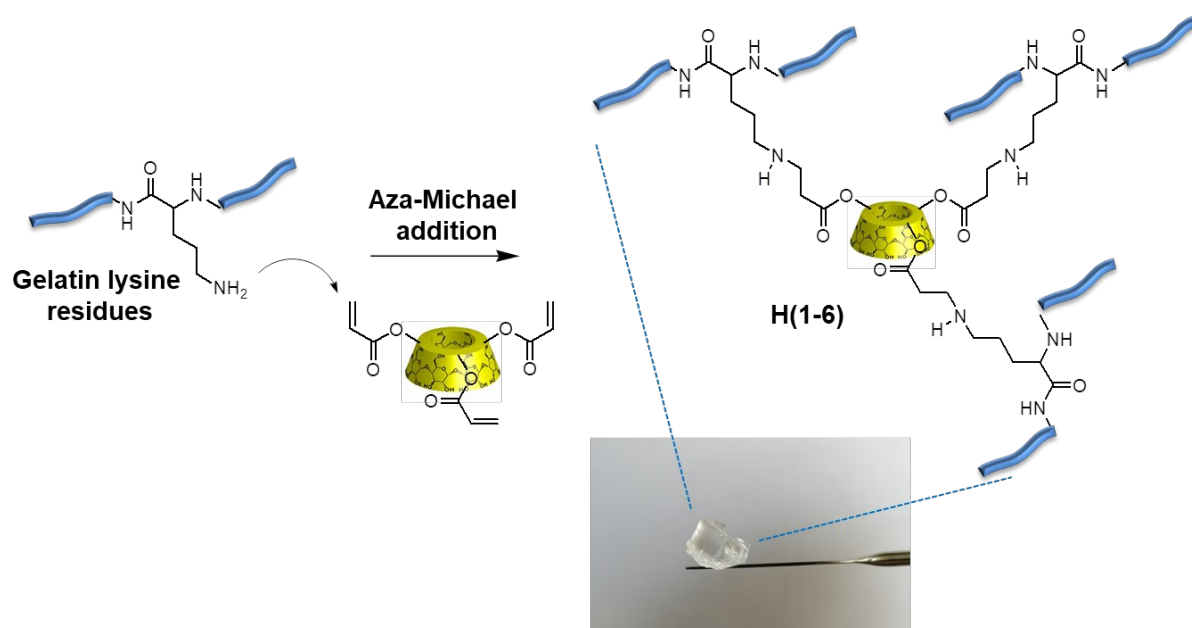


Figure 2. Crosslinking mechanism via multi-addition of gelatin amino groups onto acrylate functionalities of β -CD-acrylate. Representative picture of transparent hydrogel H-4.

A series of hydrogels composed of different β -CD-acrylate crosslinker ratio were synthesized in aqueous PBS buffer environment at 37 °C reaction temperature. Due to the multiple additions of amine groups onto acryloyl functionalities, relatively fast network formation was established within minutes. To ensure maximum available crosslinking, gelation reactions were continued for 6 h. Although the efficiency of the crosslinking process was prominent in uncatalyzed conditions (Table 1, Entry 1 and 2), gel formation was further promoted by using organo-catalysts DBU (Table 1, Entry 3-6). In aqueous conditions, β -CD is an efficient catalyst for aza-Michael addition of various amines onto electron-deficient carbonyls. (40) On the other hand, DBU possesses superior promotion of nitrogen

nucleophile reactivity towards α,β -unsaturated carbonyl compounds. (41)

A strong hydrogenation reaction was confirmed by FT-IR characterization of reaction ingredients and resulting hydrogels (Figure 3). The spectrum of β -CD-acrylate shows characteristic vibrations at 1720 cm^{-1} and 1664 cm^{-1} due to the stretchings of C=O and C=C, respectively. In the FT-IR spectrum of gelatin, typical amide peaks are resolved at 1639 cm^{-1} and 1535 cm^{-1} . The spectrum of hydrogel H-4 is characterized by the presence of the typical vibration peaks of both gelatin and β -CD-acrylate, demonstrating the structural integration of the hydrogelation components into the matrix network.

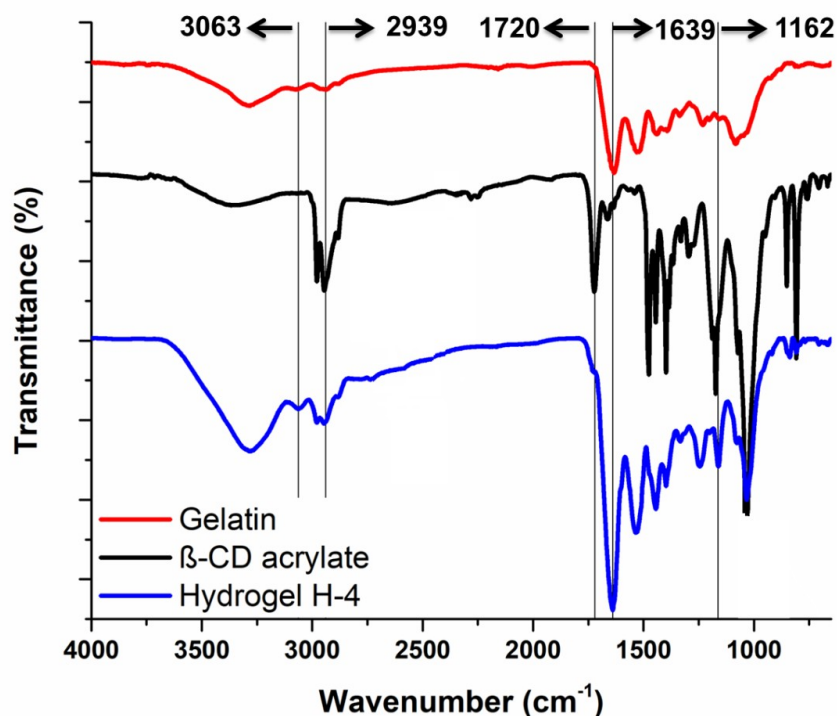


Figure 3. FT-IR structural analysis of gelatin, β -CD-acrylate, and hydrogel H-4.

The properties of fabricated hydrogels are summarized in Table 1. As expected, increased β -CD-acrylate feed in gel formulation resulted in higher gel conversions. The catalytic introduction of DBU in the gelation process has led to enhanced crosslinking efficiencies in different β -CD-acrylate

feeds. Obtained hydrogels are clear transparent samples in the water-swollen state (Figure 2). The morphological characterization of hydrogels using SEM analysis on freeze-dried samples revealed interconnected porous structures with varying pore sizes (Figure 4).

Table 1. Properties of hydrogels with varying gelatin / β -CD-Ac ratio.

Entry	Hydrogel ^a	Feed	Gel Conv. (%)	ESR ($\times 100\%$)	Drug Load (mg/g dry gel)
		Gelatin (mg) : β -CD-acrylate (mg)			
1	H-1	100.0 : 5.0	67	-	-
2	H-2	100.0 : 10.0	71	-	-
3	H-3	100.0 : 5.0	73	9.4 (± 2.7)	11.9 (± 4.4)
4	H-4	100.0 : 10.0	77	6.7 (± 2.1)	18.7 (± 5.8)
5	H-5	100.0 : 15.0	82	5.8 (± 1.8)	27.1 (± 5.3)
6	H-6	100.0 : 20.0	87	3.9 (± 1.3)	31.7 (± 7.4)

^a H-1 and H-2: No organo-catalyst; H (3-6): DBU catalystr.

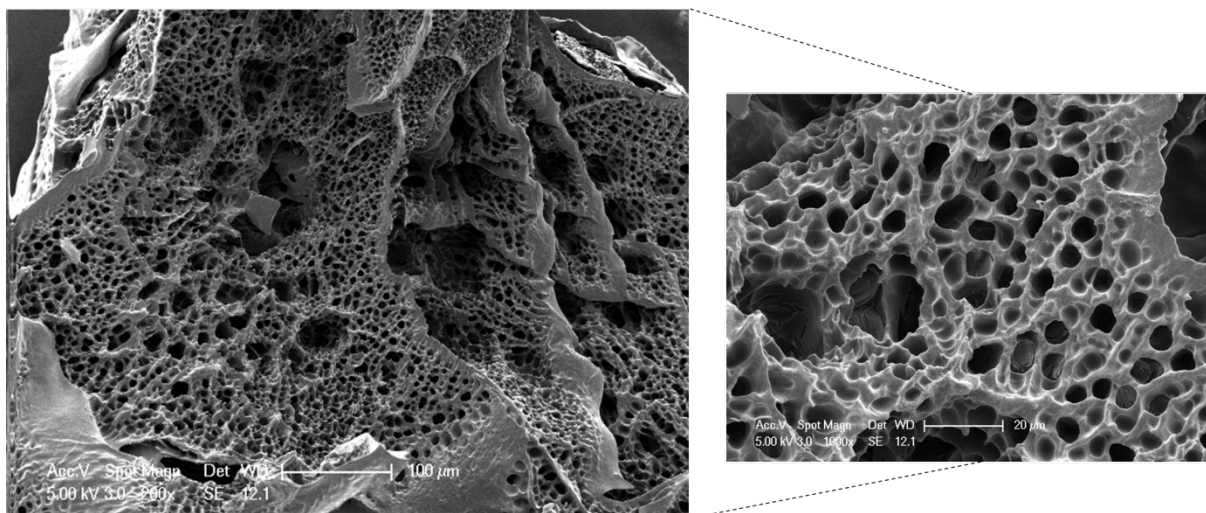


Figure 4. Representative SEM images of freeze-dried hydrogel H-4 with two different magnification (Scale bars: 100 μm for larger image and 20 μm for smaller image).

Equilibrium water uptake ratio of a hydrogel is an essential physical property in addressing gel mechanical properties, surface and network properties, and diffusion of solutes. (1) Though various parameters (i.e., pH, temperature) can contribute to ascertain the water uptake, maximum swelling is mostly related to the nature of polymer matrix and crosslinking degree. The swelling properties of the fabricated hydrogels were investigated in aqueous PBS conditions (pH: 7.4, 25 $^{\circ}\text{C}$). All the hydrogels displayed pronounced swelling degrees (\sim 940-390 % to the mass of gel sample)

because of the hydrophilic groups present on the gelatin backbone and cyclodextrin crosslinker (Figure 5). Equilibrium water uptakes were reached in relatively short periods in applied experimental conditions. It was observed that hydrogels exhibit maximum water uptake dependency on the feed ratio of the gelatin matrix and β -CD-acrylate crosslinker. Relatively higher maximum swelling degrees were observed in case of a lower crosslinker ratio in the feed. This can be attributed to higher free gel volumes and voids of network possessed by lower crosslinking degree.

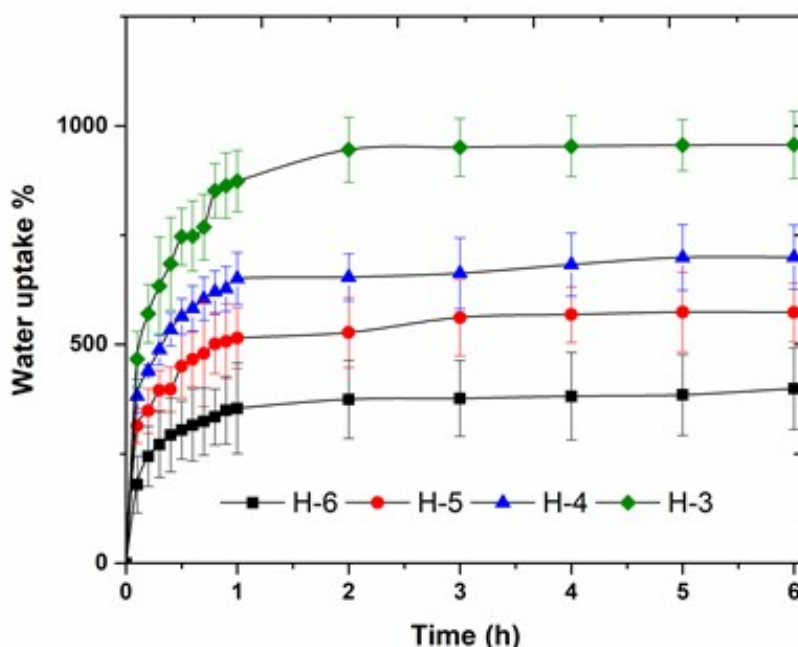


Figure 5. Equilibrium swelling profiles of gelatin-based β -CD containing hydrogels in PBS buffer.

Hydrogel viscoelastic properties were examined via dynamic frequency scan analysis of prepared hydrogels at 37 $^{\circ}\text{C}$. The tests of hydrogels having different crosslinker ratios were performed at a

constant 1.0 % strain between 0.05-100 rad/s. As it can be seen in Figure 6, all samples exhibited permanent elastic character in the test frequency interval where storage moduli (G') were higher than

loss moduli (G''). According to the results, G' and G'' values of the samples show relatively low oscillation frequency dependency maintaining highly homogenous crosslinking of gel networks. (42) An increase in the ratio of β CD-based crosslinker has

resulted in higher moduli, which could be due to the increased network structure. The damping factors ($\tan \delta$, G''/G') of hydrogels were less than 1 in all cases representing the higher elastic character of networks over viscous behavior. (43)

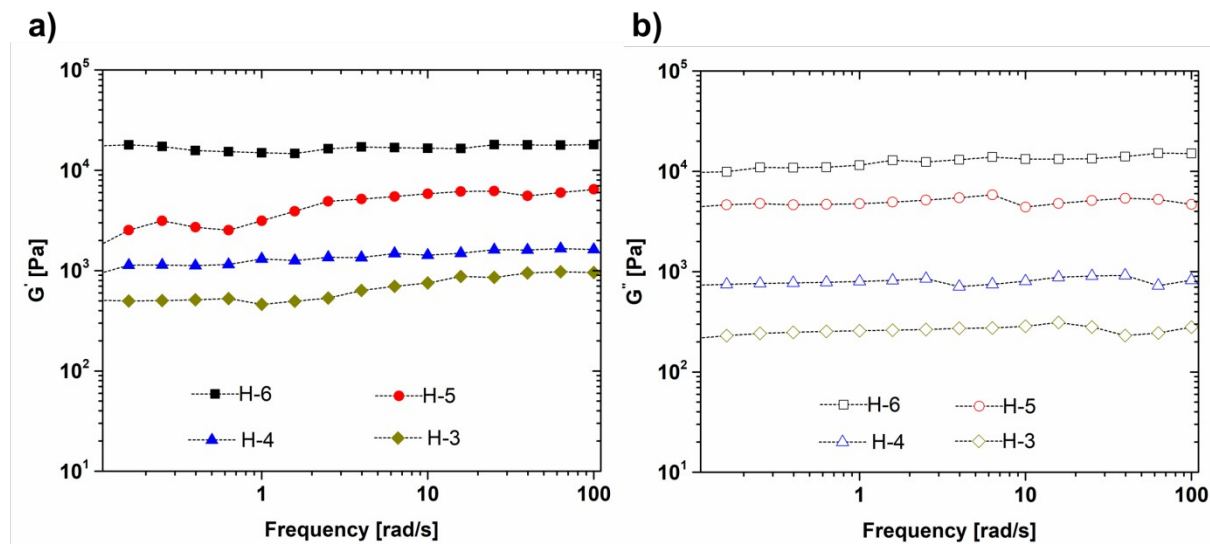


Figure 6. Dynamic frequency scan analysis of hydrogels: a) Storage and b) loss moduli.

Drug loading and release studies

Loading and sustained/controlled release of drugs, biomolecules, and other therapeutically relevant materials using hydrogels procure great potentials in realizing effective administration systems. The typical strategy for loading and release of drugs is based on the diffusion phenomena governed by weak interactions of the hydrogel matrix with subject molecules. In hydrogel networks that accommodate cyclodextrins as molecular buckets, inclusion complexation between hydrophobic molecules and cyclodextrins ensures another means of drug loading. Diclofenac-Na, widely used as a non-steroidal anti-inflammatory drug for various complaints and diseases, was employed as a model hydrophobic molecule to study the drug loading and release studies of fabricated hydrogels. It has been established that diclofenac-Na can form a molecular inclusion complex with β -cyclodextrin. (44) Hydrogels prepared as disk-shaped samples were loaded with the drug by solution absorption at 37 °C. 0.5 wt.% soaking solution was used for 24 h incubation, which is the required time to reach equilibrium as determined by UV-spectrophotometry. The drug loading efficiencies of hydrogels determined from the difference between initial and final soaking concentrations were given in Table 1. According to the results, the loaded drug amount increased from 11.9 (mg/g) to 31.7 (mg/g) for different hydrogels. A direct relation between crosslinker amount and drug levels was accounted for, where increased β -CD-acrylate crosslinker in the hydrogel resulted in considerably higher drug loading. The enhanced drug loading could be due to the higher available binding sites of cyclodextrin

cavities to complex with diclofenac-Na molecules. The inclusion complexation might have increased the solubility of slightly hydrophobic drug molecules within the hydrophilic gel network.

In vitro drug release profiles of the hydrogels containing different β -CD-acrylate crosslinker ratios were investigated in PBS buffer (pH: 7.4) at 37 °C. Initially, drug-loaded hydrogels taken from soaking solutions were gently washed with PBS buffer and then placed in the release medium. Aliquots of the release medium were taken out and replaced with the fresh solution at pre-determined intervals to resemble the physiological conditions. Time-dependent release profiles determined via UV-spectrophotometry were given in Figure 7. Overall, hydrogel H-3 having the lowest β -CD-acrylate, released its content faster than other hydrogels. An initial accelerated release was observed for all four hydrogels, which could be attributed to the burst removal of drug molecules present in the aqueous phase and adsorbed on the network matrix. (45) As expected, H-6 with the highest crosslinker content showed the lowest initial burst release and followed by H-4 and H-5. Incorporation of higher β -CD-acrylate caused a higher crosslinking degree of the network as well as enhanced drug/cyclodextrin complexation interactions, yielding slow diffusion of diclofenac-Na from the hydrogels. After the initial burst releases, all gelatin-based hydrogels exhibited sustained release profiles over a couple of days. In a specific time, relatively slower and lower total drug release from higher cyclodextrin content hydrogel could be ascribed to enhanced molecular inclusion interactions.

In an overall structure-property relationship of fabricated hydrogels, it was observed that the amount of β -CD content in the hydrogels maintained enhanced drug/matrix interactions. Hydrogel physical properties can also be manipulated by adjusting the crosslinking degrees, which of them are simply controlled by the stoichiometric ratios of

gelatin and β -CD-acrylate in gel formulations. The efficient covalent crosslinking reaction allows in situ gel formation that might improve the versatility of material to injectable drug release systems. One can suggest that the demonstrated approach could be utilized in the design and synthesis of hydrogel-based controlled/sustained drug delivery systems.

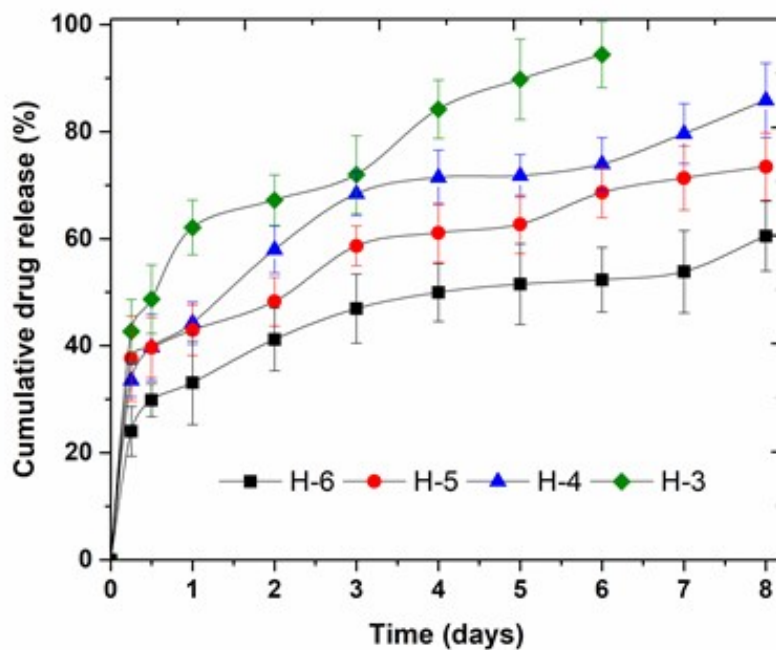


Figure 7. Cumulative release profiles of diclofenac-Na from gelatin-based β -CD containing hydrogels at 37 °C in PBS buffer (pH 7.4). Data with mean \pm SD (n=3).

CONCLUSION

A series of gelatin-based hydrogels incorporating β -CD units were fabricated under benign reaction conditions. Chemical crosslinking was established by utilizing unmodified pristine porcine gelatin via azo-Michael addition reaction. Practical and straightforward synthesis methodology allowed tunable control of the β -CD-based crosslinker amount in gel networks. It was shown that hydrogel physical properties could be tuned by adjusting the stoichiometry between amine and acrylate groups of precursor materials. The potential sustained drug release application of hydrogels was investigated by employing diclofenac-Na as a model hydrophobic molecule capable of inclusion complex formation with β -CD. The results suggested that the amount of β -CD content in the hydrogels maintained enhanced drug/matrix interactions leading to higher drug loading into the gel network. Lower initial burst releases and more sustained drug release for days was also attained in case of higher β -CD incorporation. It is believed that the efficient covalent crosslinking reaction demonstrated here could be utilized in the fabrication of biologically relevant gelatin and cyclodextrin based biomaterials.

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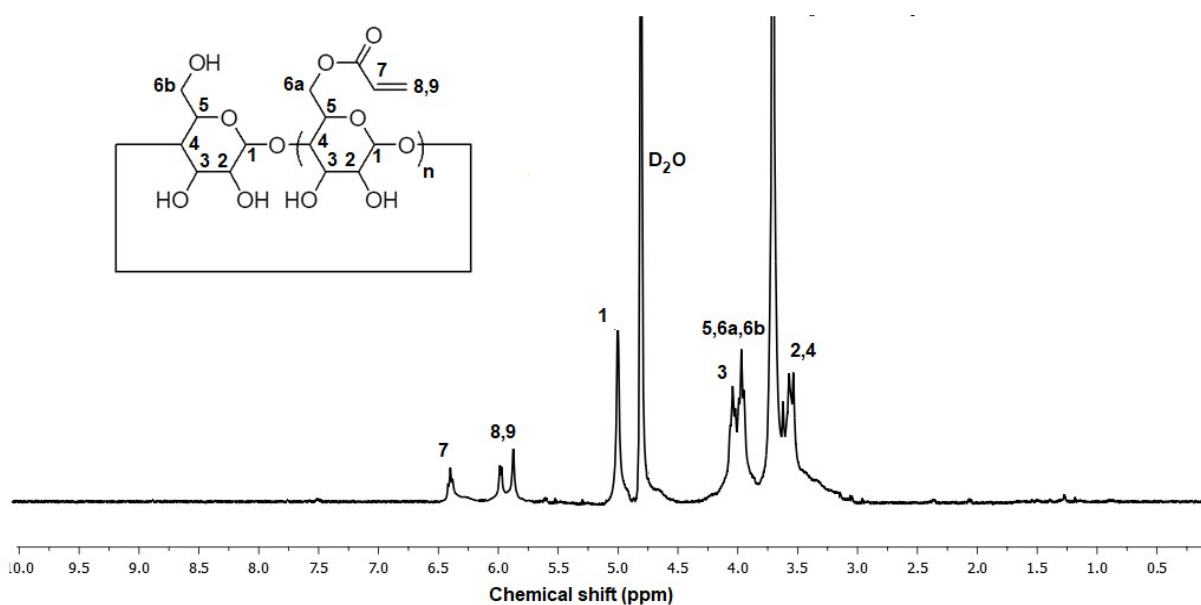
SUPPORTING INFORMATION

In situ Crosslinking System of Gelatin with Acrylated β -cyclodextrin Towards the Fabrication of Hydrogels for Sustained Drug Release

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Synthesis of Acryloyl-modified β -cyclodextrin (β -CD-acrylate): Synthesis of the β -CD-acrylate was conducted by following a previously reported procedure. (1) β -CD (1.0 g, 0.88 mmol) previously dried by lyophilization, and triethylamine (0.5 mL, 6.16 mmol) were dissolved in 10 mL dry dimethylformamide. The mixture was cooled to 0 °C in an ice bath and acryloyl chloride (0.85 mL, 6.16 mmol) was added dropwise. The reaction was continued for 16 h at room temperature. After the reaction was complete, a white precipitate was removed by filtration. The purified product was obtained by precipitation of the mixture into cold acetone and filtration (Yield: 41%). The structure of the compound is consistent with the reported literature data (Figure S1). Degree of acetylation based on peak integrals of H1 protons at 5.03 ppm and vinyl protons at 5.82 - 6.40 ppm revealed average degree of acetylation \sim 5.9 per molecule.



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