Chitosan-g-hematin: Enzyme-mimicking polymeric catalyst for adhesive hydrogels

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Abstract

Phenol derivative-containing adhesive hydrogels has been widely recognized as having potential for biomedical applications, but their conventional production methods, utilizing a moderate/strong base, alkaline buffers, the addition of oxidizing agents or the use of enzymes, require alternative approaches to improve their biocompatibility. In this study, we report a polymeric, enzyme-mimetic biocatalyst, hematin-grafted chitosan (chitosan-g-hem), which results in effective gelation without the use of alkaline buffers or enzymes. Furthermore, gelation occurs under mild physiological conditions. Chitosan-g-hem biocatalyst (0.01%, w/v) has excellent catalytic properties, forming chitosan–catechol hydrogels rapidly (within 5 min). In vivo adhesive force measurement demonstrated that the hydrogel formed by the chitosan-g-hem activity showed an increase in adhesion force (33.6 ± 5.9 kPa) compared with the same hydrogel formed by pH-induced catechol oxidation (20.6 ± 5.5 kPa) in mouse subcutaneous tissue. Using the chitosan-g-hem biocatalyst, other catechol-functionalized polymers (hyaluronic acid–catechol and poly(-vinyl alcohol)–catechol) also formed hydrogels, indicating that chitosan-g-hem can be used as a general polymeric catalyst for preparing catechol-containing hydrogels.

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requires the use of significant amounts of the enzymes. Therefore, time and labor for large-scale bacterial culture are required.

Another class of phenolic derivative used for crosslinking functional groups includes catechol-containing small molecules, such as dopamine and 3,4-dihydroxyhydrocinnamic acid. The pH-induced oxidation of catechol results in catecholquinone, which subsequently forms catechol–catechol adducts [24,25]. Other routes for catecholquinone-involved chemical crosslinkings involve the formation of catechol–amine and catechol–thiol adducts [26,27]. Catechol-containing hydrogels have recently been used for the preparation of adhesive hydrogels, generating interesting underwater adhesive properties from the conjugated catechol [28]. A variety of hydrogels formed by a catechol-mediated chemical crosslinking have been reported, including end-functionalized poly(ethylene glycol) (PEG)–catechol [29], catechol-grafted PEG [30], catechol-conjugated hyaluronic acid (HA-C) [31], HA-C/pluronic-thiol [32] and catechol-conjugated chitosan (CHI-C)/pluronic-thiol [33] hydrogels. Catechol-mediated hydrogels are formed by a non-enzymatic process, which is an obvious advantage compared with tyramine-containing hydrogels. However, the disadvantages of the catechol-mediated hydrogels include a gelation that is triggered by relatively high pH values (>8) or by the presence of highly concentrated oxidizing agents, such as periodate ions, often resulting in the cytotoxicity of the encapsulated cells (Fig. 1a). The development of a new approach that can effectively trigger gelation in the physiological pH range without using enzymes, such as HRP, is necessary for further improvement of the biocompatibility of catechol-containing hydrogels (Fig. 1b).

We hypothesize that the use of a water-soluble biocatalyst to initiate catechol oxidation and subsequent crosslinking could be a possible solution to overcome the aforementioned challenges in catechol hydrogel formation. In this study, a new polymer-based biocatalyst, hematin-grafted chitosan (chitosan-g-hem) (Fig. 2), was investigated. Hematin, an iron-containing heme group, has been studied as an oxidative catalyst for phenol compounds [34,35]. HRP and hematin commonly catalyze covalent crosslinks between phenolic compounds, consuming hydrogen peroxide via radical transfer reactions occurring in the heme group [34–38]. One major problem encountered with hematin is its poor solubility in water, because the porphyrin structure in hematin is a water-insoluble functional group [35,39]. Because of this poor solubility, the effective use of hematin is significantly hindered. Previously, hematin has been dissolved in alkaline buffer, with the pH of the solution being subsequently reduced to 7.4 for further use [35]. This method is useful for the simplicity of the hematin dissolutions, but its solubility is nevertheless limited, resulting in crude aggregates, and the time needed to dissolve the hematin is significantly long. As a potential solution to the poor solubility, we have investigated conjugating hematin to chitosan. Chitosan-g-hem is highly water soluble, but retains the HRP-mimicking catalytic activity of hematin to facilitate the rapid gelation of catechol-containing polysaccharides. With this conjugate, the resulting hydrogels exhibited tissue adhesion properties, demonstrating increased adhesiveness compared with the same hydrogels formed by conventional pH-induction processes.

2. Experimental

2.1. Materials

Chitooligosaccharide (MW 3–5 kDa, 95.2% deacetylated) was purchased from Kitto Life Co., Ltd. (Gyeonggi-do, Republic of Korea). Chitosan 100 (Mv = 1.31 MDa [40], 80% deacetylated) was purchased from Wako Pure Chemical Co. (Osaka, Japan). Hyaluronic acid was obtained from Amore Pacific (Seoul, Republic of Korea).

Chitosan-g-hem was synthesized via a two-step reaction: a standard EDC coupling of chitosan oligosaccharide with 3,3-dithiodipropionic acid and standard click chemistry of thiolated chitosan oligosaccharide with hematin. Briefly, chitosan oligosaccharide (500 mg, 3.2 mmol for the monomer) was dissolved in Poly(vinyl alcohol) (MW 85–123 kDa), Hematin, hydrocaffeic acid (3,4-dihydroxy hydrocinnamic acid), 1-ethyl-3-(3-dimethylaminopropyl)-carbohdiimide hydrochloride (EDC), 3,3-dithiodipropionic acid, and DL-dithiothreitol (DTT), 2,2-azobis(2-methylpropionitrile) (AIBN), HRP (150 units mg⁻¹) were purchased from Sigma–Aldrich. Ethanol was purchased from Merck Chemicals (Darmstadt, Germany). Dimethylsulfoxide (DMSO) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Synthesis of chitosan-g-hem

Fig. 1. (A) Graphical illustrations of the crosslinking methods for polymer–tyr (left) and polymer–catechol (right). Generally, tyramine-containing hydrogels were crosslinked by the enzyme HRP in a neutral pH solution. In contrast, enzymes are not necessary for catechol-containing hydrogels, but the addition of sodium periodate as a strong oxidant or an increase in the pH of the solution is required for hydrogel formation. Thus, developing a new strategy for hydrogel formation that occurs at physiological pH and avoids enzyme usage is desirable. (B) The alternative, biocompatible strategy for adhesive hydrogel formation.
deionized and distilled water (DDW, 50 ml), followed by the slow addition of 3,3-dithiodipropionic acid (341.3 mg, 1.6 mmol) and EDC (622.4 mg, 3.2 mmol) in 50 ml of DDW to the chitosan oligosaccharide solution. The reaction time was 12 h and the solution pH was maintained at 5.5. After completion of this reaction, DTT (1252 mg, 8.1 mmol) was added to the reaction solution and further reacted for 3 h. The product was dialyzed using a membrane with a molecular weight cut-off (MWCO) of 1000 (SpectraPor, USA) for 3 days, and subsequently lyophilized. Thiolated chitosan (50 mg) was further reacted with hematin (23.4 mg, 37 μmol) using AIBN (24.3 mg, 147 μmol) in 50 ml of DMSO at 60 °C for 24 h. The final product was dialyzed and precipitated by the addition of 5 N HCl. Thiolated chitosan was confirmed by using either 1H-nuclear magnetic resonance (NMR) (Bruker Avance, 400 MHz) or Fourier transform infrared (FTIR) spectroscopy (Thermo Nicolet Instrument Co., Madison, WI). Chitosan-g-hem was confirmed by using either FTIR or an ultraviolet–visible (UV–vis) spectrophotometer (UV-1601, Shimadzu, Japan). The degree of thiolation on the chitosan oligosaccharide backbone was 11.4%, as measured using Ellman’s assay. Ellman’s reagent solution was prepared by dissolving 20 mg of 5,5'-dithio-bis-(2-nitrobenzoic acid) in 5 ml of sodium phosphate-buffered solution (pH 8). The thiolated chitosan (0.1 wt.%) was dissolved in pH 8 buffered solutions, then Ellman’s reagent was added into polymer solutions. After mixing and incubation at room temperature for 15 min, the absorbance change at 412 nm was measured using the UV–vis spectrophotometer. The standard curves for quantitating sulfhydryl groups were generated by 3-thiopropionic acid standard solutions as a function of concentration. UV–vis spectroscopy was used to estimate the degree of hematin substitution in the chitosan backbone using its dominant absorbance peak at 395 nm in pH 12 phosphate-buffered saline (PBS) solutions and dimethyl formamide (DMF). Standard hematin concentration curves (0.0001–0.05 mg ml−1) were generated using a standard hematin solution in pH 12 PBS solutions and DMF, then the UV–vis absorption of the conjugated sample was measured for the quantification of hematin tethering. The estimated degree of hematin substitution of chitosan-g-hem was 8.2 mol.% in the PBS solution (pH 12) and 7.8 mol.% in DMF, as determined using the UV–vis spectrophotometer.

2.3. Synthesis of CHI-C

CHI-C was synthesized by conjugating a carboxylic acid group of hydrocaffeic acid to the amino groups of chitosan using carboximide chemistry. Briefly, chitosan oligosaccharide (1 g, 6.5 mmol of the monomer) was dissolved in 100 ml of DDW and the pH was adjusted to 5.5. Hydrocaffeic acid (1181.2 mg, 6.5 mmol) and EDC (1866.2 mg, 9.7 mmol) in 50 ml with equal portions (by vol.) of DDW and ethanol were slowly added to the chitosan solution and reacted for 12 h. The pH of the reaction solution was maintained at 5.5. The product was purified by dialysis (MWCO: 12,000–14,000; SpectraPor, USA) against an HCl solution (pH 5.0) for 2 days, followed by DDW for 4 h. The final product was lyophilized and held in a moisture-free desiccator until further use. The chemical structure of CHI-C was examined by 1H NMR (Bruker Avance, 400 MHz). The degree of substitution was estimated to...
be ca. 14.5 mol.% by comparing the relative peak area of three protons on the catechol (δ = 6.5–6.9 ppm) and the acetyl (–COCH₃, δ = 1.95 ppm) groups of the monosaccharide residue in the chitosan.

2.4. Characterization of chitosan-g-hem

The solubility and turbidity of chitosan-g-hem was studied using a UV–vis spectrophotometer (Hewlett-Packard 8453, Groton, CT). Briefly, a 10 mM buffer solution exhibiting a wide pH range buffering capacity (from pH 2 to 12) was prepared by the mixture of tris-(hydroxymethyl)aminomethane, sodium acetate and disodium hydrogen. For the solubility test, chitosan-g-hem (0.001–0.6 mg ml⁻¹) was dissolved in the buffer solution with various pH values. The absorbance spectra of hematin and chitosan-g-hem were measured using the UV–vis spectrometer. To observe the turbidity, hematin and chitosan-g-hem were dissolved first in disodium phosphate solution titrated at pH 12. UV–vis absorbance values at 800 nm were monitored as a function of pH values (12, 10, 8, 7, 6, 4 and 2) by the addition of HCl. Dynamic light scattering (DLS) equipment (Zeta-Plus, Brookhaven, NY) was used to measure nanoaggregates of hematin and chitosan-g-hem. To obtain details of the structures of the particles and their chemical compositions, transmission electron microscopy (TEM) using a microscope (JEOL-3011 (HR), Japan) equipped for energy-dispersive X-ray spectroscopy (EDX) was used. The final concentration of chitosan-g-hem was fixed to 0.1 mg ml⁻¹ to form the assembled nanoaggregates.

2.5. Cell viability test using chitosan-g-hem

The cytototoxicity of chitosan-g-hem was evaluated using a CCK assay (cell counting kit-8, Dojindo Laboratories, Japan). Polymer solutions were prepared in serum-supplemented medium with various final concentrations, ranging from 0.5 to 0.0001 mg ml⁻¹. NIH3T3 cells were seeded onto 96-well plates (5000 cells well⁻¹). Twenty-four hours after seeding the cells, the above-mentioned polymer solutions (200 μl) were added to the 96-well plates. After standing for 24 h, the CCK solutions were added to the well plates, and the plates were incubated for 4 h at 37 °C. The absorbance of the well plates at 450 nm was measured by a microplate reader to quantify the viability of the cells (BioRad, Model 550, Hercules, CA). All experiments were performed in triplicate.

2.6. Catalytic activities of chitosan-g-hem

To evaluate the catalytic profile of chitosan-g-hem at pH 7, the UV–vis spectra using pyrogallol as a substrate were monitored. A mixture containing 5.0 mM pyrogallol, 0.01 mg ml⁻¹ chitosan-g-hem and H₂O₂ in a total volume of 0.75 ml was poured into a quartz cell. The increase in absorbance at 420 nm was recorded after the addition of H₂O₂. The concentration was varied from 10 to 100 mM H₂O₂, HRP (0.01 unit ml⁻¹) was used as a control. The amount of hematin in solution was determined for the A₃₉₅ values before the addition of H₂O₂ and pyrogallol. The changes in the A₄₂₀ values were monitored to quantifying the activities through the equation: (change in A₄₂₀)/(A₃₉₅ of hematin or chitosan-g-hem). The total activity was calculated by the change in A₄₂₀ from t = 0 s to t = 20 s, where time t = 0 s was defined as the time point of the addition of H₂O₂. The relative catalytic activities using various H₂O₂ concentrations were calculated by normalizing the increase in the absorbance at 420 nm at each H₂O₂ concentration with the maximum values detected at both 100 and 10 mM H₂O₂ for hematin and HRP, respectively. The data for the catalytic profiles were expressed as the average of three measurements.

2.7. Preparation of adhesive CHI-C hydrogels

The hydrogels were prepared by dissolving CHI-C in pH 7 solutions to various concentrations (4, 5 and 6 wt.%). Hydrogen peroxide was dissolved in a separate pH 7 solution. The two solutions were homogeneously mixed with various molar concentrations of H₂O₂ (10, 40, 70 and 100 mM) and then 0.01 wt.% chitosan-g-hem was added to the CHI-C solutions. The final concentration of the hydrogels (4 wt.% CHI-C, 70 mM H₂O₂ and 0.01 wt.% chitosan-g-hem) was determined using 1.25 × concentrated CHI-C solutions, 10× molar concentrated H₂O₂ and 10× concentrated chitosan-g-hem. Using a volume ratio of 8:1:1 (v/v/v), the three solutions were mixed to produce the final concentration. For the preparation of the pH-induced hydrogels, CHI-C was dissolved in a pH 7 solution (4 wt.%), followed by the addition of 5 M NaOH at a volume ratio of 9 (CHI-C):1 (5 M NaOH).

2.8. Rheological analysis of chitosan-g-hem-catalyzed CHI-C hydrogels

The rheological properties were evaluated using a rotating rheometer (Bohlin Advanced Rheometer, Malvern Instruments, UK). Parallel plate geometry was used to monitor the elastic modulus values (solid-like property, G') and the viscous modulus values (liquid-like property, G''). For the frequency sweep measurements, the sweep of the frequency varied from 0.1 to 10 rad s⁻¹, and each frequency sweep took 21 points to be completed. For the time sweep measurements, 150 μl of the polymer solution with the chitosan-g-hem was placed on the rheometer plate, followed by an addition of the hydrogen peroxide at predetermined concentrations. The frequency was fixed at 1 Hz, and the time t = 0 s was defined as the time point of the hydrogen peroxide addition. The dynamic viscoelastic functions were measured as a function of time. The crossover point of G' and G'' was considered for the gelation time. A constant stress of 100 Pa was used for all measurements. All samples were measured in triplicate.

2.9. In vitro biocompatibility of extracts of chitosan-g-hem catalyzed CHI-C hydrogels

In order to evaluate the cytotoxicity of extracts of polymer hydrogels, the cell viability test was performed using the eluting test methods. Briefly, the extracts (10 ml) were obtained by incubation of hydrogels (1 g) after pre-curing for 30 min in separate media at 37 °C in a humidified 5% CO₂ atmosphere for 24 h, after which the hydrogels were removed by centrifugation (500 rpm, 5 min). NIH 3T3 cells were seeded onto 96-well plates (5000 cells well⁻¹) and the cells were incubated for 24 h. The extracts (200 μl) were then added to the 96-well plates. After 24 h of standing in the extract medium, CCK solutions were added to the well plates, and the plates were incubated for 4 h at 37 °C. The absorbance of the well plates at 450 nm was measured by a microplate reader to quantify the viability of the cells (BioRad, Model 550, Hercules, CA, USA). All experiments were performed in triplicate.

2.10. Tissue adhesion test using a universal testing machine (UTM)

Tissue adhesion was examined using a UTM (Instron 5583, Instron, USA) with a 150 N load cell, equipped as previously described [33]. Briefly, mouse subcutaneous tissue samples with cylindrical geometry were used to measure the tensile strength of the hydrogels between the tissues. The cylindrical tissue samples were placed between the adjustable crosshead and the UTM plate, which was fixed with a clamp. For each test, the polymer solution (135 μl) was injected between both tissues and then hydrogen peroxide was injected into the solution. The force was
pushed into the tissue for 5, 15 and 30 min with a normal force of 0.1 mN. Tensile strength values were collected by pulling the probe with a crosshead speed of 1 mm min⁻¹. The data collection rate of the tensile strength was fixed at 5 pts s⁻¹. All measurements were performed in triplicate.

3. Results and discussion

3.1. Synthesis of chitosan-g-hem

We hypothesized that a water-soluble polymer might increase the poor water solubility of hematin. We chose water-soluble, low-molecular weight chitosan (MW 3–5 kDa) as the polymeric backbone. The chitosan was reacted with 3,3-dithiodipropionic acid, resulting in the formation of chitosan-S-S-chitosan conjugates. To reduce the disulfide bonds, DTT was added to the solution, generating thiolated chitosan (chitosan-SH). The chitosan-SH was further reacted with hematin via radical coupling using AIBN to form chitosan-g-hematin (chitosan-g-hem) conjugates (Fig. 2). One might speculate that EDC-involved bioconjugation, which directly couples the carboxylic acid in the hematin with the amine on the chitosan, might be an easy route to prepare chitosan-g-hem. However, the resulting product following this procedure turned out to be water-insoluble, and the reaction yield was too low (data not shown). The poor solubility of chitosan-g-hem prepared using this method may have resulted from the elimination of the hydrophilic carboxylic acid present in hematin. For this reason, an alternative bioconjugation strategy was developed, as mentioned in Fig. 2, which preserves the carboxylic acids of the hematin. This chitosan-g-hem conjugate showed excellent water solubility compared with the unconjugated hematin. As shown in Fig. 3A, hematin did not dissolve in buffer solutions with pH values of 6, 7 and 8 (top). In contrast, the chitosan-g-hem developed a brownish-yellow color in the same buffer solutions, demonstrating immediate water solubility (bottom). To demonstrate the solubility of chitosan-g-hem, we measured the absorbance spectra of hematin and chitosan-g-hem. The solubilized chitosan-g-hem showed strong UV–vis absorption, with a maximum absorbance at 395 nm (Fig. 3B) [39,41]. With the precipitation resulting from the water insolubility of the unconjugated hematin, no peaks were observed in the UV–vis spectroscopy experiments. Thus, we used a previously used hematin dissolution protocol in which hematin was dissolved in pH 12 PBS solution and then the pH was adjusted to pH 7.4 by adding pH 4.5 monosodium phosphate solution [35]. Two solutions containing equivalent amounts of hematin were prepared: 8.2 μg ml⁻¹ for hematin and 100 μg ml⁻¹ for chitosan-g-hem. In low concentrated solutions (8.2 μg ml⁻¹ hematin basis), the hematin solution was slightly turbid, exhibiting light scattering, as detected by an upshift from 700 to 1000 nm (black). However, chitosan-g-hem showed near-100% dissolution, exhibiting no background scattering from the same range of wavelength (red). Furthermore, we increased the concentration of hematin to 50 μg ml⁻¹, the equivalent concentration for chitosan-g-hem being 609 μg ml⁻¹. The chitosan-g-hem exhibited excellent enhancement of water solubility, showing no light scattering caused by aggregates (i.e. background level adsorption from 700 to 1000 nm, red dots). UV–vis spectral measurements were also used to characterize the further water solubility of chitosan-g-hem, as shown in Fig. 3C. Chitosan-g-hem was soluble in the pH values ranging between 6 and 12. Two wavelengths were chosen: A₉₅₀ and A₈₀₀–A₉₅₀ is the wavelength at which hematin is absorbed, so that the absorption was abruptly increased around pH 6 (square dots, black line). In contrast, the turbidity at A₈₀₀ was decreased suddenly from pH 4 to pH 6 as the conjugate became soluble (circle dots, red line). The chitosan-g-hem solution was transparent in the pH values ranging between 6 and 12. The observed water insolubility of chitosan-g-hem in acidic solutions may have resulted from the electrostatic neutralizations with the interactions between the –NH₃⁺ groups in the chitosan and the –COO⁻ groups in the hematin. The πKₐ value of the amino groups in chitosan is below 6.5 [42,43]. The zeta-potential values of chitosan-g-hem demonstrated charge inversion (− to + mV) as a function of pH (−17.3 ± 1.8 mV at neutral pH and 7.8 ± 7.4 mV at pH 5). This large deviation in zeta-potential values indicated that the charge of the polymers might be near neutral values, possibly explaining the water insolubility of chitosan-g-hem in acidic solutions. Zwitter-ionic chitosans have exhibited similar water-insoluble behavior in acidic solutions [44,45].

3.2. Characterization of chitosan-g-hem

It was found that the chitosan-g-hem behaves similar to amphi-philic molecules in aqueous buffers depending on its concentra-
tion. The conjugate was rapidly dissolved in the buffer (pH 7.4 PBS), but there is no indication that it formed nanoparticles or aggregates (Fig. S1A, left). When the concentration was increased to 0.1 mg ml\(^{-1}\), we found nanoparticles (Fig. S1A, right), but the DLS signal became weak and inconsistent when the solution was diluted twofold to a concentration of 0.05 mg ml\(^{-1}\) (middle panel). Thus, the conjugate is soluble in aqueous solution at low concentrations (<0.05 mg ml\(^{-1}\)) and subsequently forms nanoparticles stably dispersed in the solution above the concentration of 0.05 mg ml\(^{-1}\). Fig. 3D shows the hematin and chitosan-g-hem solutions. Chitosan-g-hem instantaneously dissolved within 1 min, exhibiting a transparent and brown-colored solution. The upper left (0.1 mg ml\(^{-1}\) hematin basis) and lower left photographs (0.01 mg ml\(^{-1}\) hematin basis) showed no aggregation after 24 h dissolution, which remained stable even after 2 months (middle). In contrast, the hematin that was dissolved in the disodium phosphate buffer first and then neutralized exhibited a certain degree of particulate aggregation observed after 24 h of dissolution (upper and lower right panels). DLS measurements of a chitosan-g-hem solution showed two distinct populations of particles, 9–120 nm and 230–300 nm (Fig. S1A). The DLS data were consistent with the TEM image (Fig. S1D). The amphiphilicity of chitosan-g-hem can result in nanocolloidal assemblies. The self-assembly of hematin in aqueous solutions is thermodynamically favorable due to the hydrophobic nature of hematin [46]. Thus, multiple chains of chitosan-g-hem conjugates may have been inter-molecularly associated by the hematin-driven hydrophobic force. We also confirmed that Fe signals were detected by EDX from the catalytic chitosan-g-hem nanoparticles (Fig. S1E). In addition, we further examined the toxicity of chitosan-g-hem to verify the optimal dose of the biocatalyst. The chitosan-g-hem conjugates were non-cytotoxic to mammalian cells at concentrations up to 0.1 mg ml\(^{-1}\) (~97.3% cell viability). Approximately 50% of the cells remained alive at a chitosan-g-hem concentration of 0.5 mg ml\(^{-1}\) (Fig. 4). Considering the negligible cytotoxicity of chitosan alone at 0.5 mg ml\(^{-1}\) concentration (Fig. S2), the cytotoxicity apparent with 0.5 mg ml\(^{-1}\) chitosan-g-hem might be caused by the chemically tethered hematin. For further experiments, a chitosan-g-hem concentration of 0.1 mg ml\(^{-1}\) was used for the adhesive hydrogel preparations.

3.3. Catalytic activity of chitosan-g-hem

The catalytic activity of chitosan-g-hem was evaluated using pyrogallol as a standard substrate [35,41]. Oxidative crosslinking with the substrate pyrogallol was initiated by the catalytic activity of chitosan-g-hem. HRP and hematin were also used for comparison. The activity was calculated by the change in \(A_{420}\) for 0–120 s, the time \(t = 0\) s being defined as the time point of the addition of H\(_2\)O\(_2\). The poor solubility of unconjugated hematin limited the measurement of the maximum catalytic activity in aqueous conditions. In contrast, the total observed activity of chitosan-g-hem was largely increased because of the enhanced solubility in water (Fig. S3). It should be noted that the intrinsic catalytic activity of hematin remained unchanged. Utilizing the very small amount of dissolved hematin and the same amount of hematin conjugated to the chitosan, the specific activities could be measured and were found to be similar to the specific activities of chitosan-g-hem and hematin (Fig. S3). The catalytic activities of chitosan-g-hem as a function of H\(_2\)O\(_2\) concentration were also measured. The normalized catalytic activity of chitosan-g-hem was increased from 0.04 in 1 mM H\(_2\)O\(_2\) to 0.83 in 10 mM H\(_2\)O\(_2\). Further increasing the concentrations up to 100 mM H\(_2\)O\(_2\) caused a slight catalytic enhancement of approximately 17% (Fig. 5B). This data indicated that chitosan-g-hem was stable in high H\(_2\)O\(_2\) concentration conditions, and the hematin groups catalyzed oxidative polymerization of the catechol groups consuming H\(_2\)O\(_2\). For the enzyme HRP, the optimal concentration of H\(_2\)O\(_2\) was measured to be approximately 10 mM, with the catalytic activity decreasing with

![Fig. 4](image-url)  
**Fig. 4.** Cell viability as a function of chitosan-g-hem concentration.

![Fig. 5](image-url)  
**Fig. 5.** (A) Absorbance (420 nm) value difference between 0 and 120 s for hematin and chitosan-g-hem. Relative activities of (B) chitosan-g-hem and (C) HRP as a function of H\(_2\)O\(_2\) concentration.
increasing concentration due to the non-specific oxidative degradation of HRP (Fig. 5C).

3.4. Hydrogel formations

Utilizing chitosan-g-hem catalytic reactions, we demonstrated for the first time that catechol-containing hydrogels can effectively be prepared at physiological pH without using HRP. A CHI-C conjugate was prepared as a model catechol-containing polymer with a degree of catechol modifications of approximately 14.5% [33]. After gelation, the elastic modulus of the CHI-C hydrogel was approximately $10^4$ Pa (Fig. 6A). The proposed mechanism of hematin-catalyzed catechol oxidation is based on radical formation, with a subsequent transfer in the process of the chelated Fe reduction (III → IV). The recovery from Fe(IV) to Fe(III) in hematin results from the consumption of H$_2$O$_2$ [34]. Additionally, the gelation time was shown to be controllable by the concentration of H$_2$O$_2$. As shown in Fig. 6B, the gelation time was 130 s for 100 mM H$_2$O$_2$ and 206 s for 70 mM H$_2$O$_2$. Low concentrations, such as 40 and 10 mM H$_2$O$_2$, exhibited prolonged CHI-C gelation times, of 5 and 18 min, respectively (Fig. 6C). Similar to the HRP activity characteristics, the polymeric biocatalyst chitosan-g-hem required hydrogen peroxide for effective hydrogel formation. As noted in the introduction, gelation under physiological conditions without the use of expensive enzymes would be beneficial for the production of catechol-containing hydrogels. In the control experiments, CHI-C alone did not form a hydrogel under physiological conditions (pH 7.4) (Fig. 6D, first black bar), chitosan-g-hem without H$_2$O$_2$ did not initiate CHI-C hydrogel formation (second red bar) and H$_2$O$_2$ itself did not form a hydrogel (third green bar). Chitosan-g-hem in the presence of H$_2$O$_2$ produced a CHI-C hydrogel under physiological conditions (fourth yellow bar) (4 wt.% CHI-C, 0.1 mg ml$^{-1}$ of chitosan-g-hem and 70 mM H$_2$O$_2$ for 1 h). Concerns about cytotoxicity related to the presence of H$_2$O$_2$ can be raised in this formulation, but our cytotoxicity evaluation showed >95% cell viability (Fig. 6E). This is due to the consumption of H$_2$O$_2$ by the catalytic

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**Fig. 6.** (A) Frequency sweep measurements of chitosan-g-hem-catalyzed CHI-C hydrogels. (B) Gelation kinetics of chitosan-g-hem-catalyzed CHI-C hydrogels at various H$_2$O$_2$ concentrations. Viscous modulus (black), elastic modulus (red). (C) Gelation time of the CHI-C hydrogels as a function of H$_2$O$_2$ concentration. (D) Elastic (G') modulus of the CHI-C solution (black), 0.01 wt.% chitosan-g-hem added to CHI-C (red), 70 mM H$_2$O$_2$ added (green) and chitosan-g-hem with 70 mM H$_2$O$_2$ added (yellow). (E) Cell viability of the extracts of CHI-C hydrogels after 24 h of incubation. Medium (first bar), the extract from the hydrogel prepared by 100 mM H$_2$O$_2$ and chitosan-g-hem (second bar) and the extract from the hydrogel by 0.5 N NaOH (third bar).
activity of hematin in chitosan-g-hem. In contrast, CHI-C hydrogels formed by the conventional pH-induction method exhibited significant cytotoxicity (~20% cell viability) because of basicity of the extracts (Fig. 6E, third bar). Thus, chitosan-g-hem is a promising polymeric catalyst for the fabrication of biocompatible, catechol-containing hydrogels.

3.5. In vitro tissue adhesion

Interestingly, the adhesive properties of the hydrogels formed by chitosan-g-hem were enhanced compared with the same catechol-containing hydrogels prepared by the pH triggering process. Adhesion forces were measured by a UTM to evaluate the adhesion properties of the catechol-containing hydrogels. In addition to CHI-C, two additional catechol-conjugated polymers, HA-C and PVA-C, were prepared. The degree of catechol conjugation was 24% for HA-C and 2% for PVA-C. Fig. 7A shows the standard stress–distance curves, demonstrating the superior adhesion force in the CHI-C hydrogel compared to the same polymer hydrogel formed by pH induction. Increased detachment stress (20.6 ± 5.5–33.6 ± 5.9 kPa, Fig. 7A, left bars) with improved ductile properties (top red vs. black curves, Fig. 7A) were observed in the CHI-C hydrogels prepared by chitosan-g-hem. Other catechol-containing hydrogels fabricated by the catalytic activity of chitosan-g-hem showed similar results, with enhanced adhesion and ductile properties. As shown in Fig. 7B (middle bars), the detachment stress of HA-C was 26.0 ± 17.5 kPa for preparations using catechol-g-hem, but was 1.1 ± 0.6 kPa for preparations initiated by a change in pH. The stress–distance curve indicated a significantly enhanced work of adhesion (i.e. ductile property; Fig. 7A, middle red vs. black curves). Hyaluronic acid is non-sticky, and is used in anti-adhesive materials for biomedical applications [47–49]. Chitosan-g-hem-catalyzed HA-C hydrogels showed a greater than eightfold increase in these properties compared with the untreated HA. This same enhancement in both properties was observed for preparations of PVA-C hydrogels (39.6 ± 5.1 kPa for biocatalyst-induced gelation, 35.7 ± 3.8 kPa for pH-induced gelation). The increased instantaneous work of adhesion suggested that these hydrogels may be suitable for in vivo injection. With large deformation characteristics, the injected hydrogels are expected to remain attached within the tissues, improving the adhesion properties [50–52]. A molecular-level explanation of the adhesive properties of the hydrogels prepared by chitosan-g-hem is challenging. Based on our previous experiences with hydrogel preparation utilizing catechol-containing polymers, such as PEG–catechol, hyaluronic acid–catechol [31] and CHI–catechol/pluronic-SH [33], kinetic factors might be important in the resulting adhesion properties. To evaluate the crosslinking kinetics of chitosan-g-hem catalyzed CHI-C hydrogels, we monitored UV–vis spectral changes of CHI-C solutions after adding chitosan-g-hem and H2O2 (Fig. S4A) or NaOH (Fig. S4B). Compared to the predominant existence of a quinone peak appearing at 346 nm when treated with NaOH, the oxidation by chitosan-g-hem resulted in a significant degree of crosslinking, exhibiting an overall upshift of the UV–vis spectra (Fig. S4B). This result was displayed by the kinetics of spectral change at 600 nm (Fig. S4C). Thus, we can conclude that the chitosan-g-hem resulted in rapid inter-chain crosslinking, which might contribute to the enhancement of tissue adhesion. In addition, optimal windows for gelation time and polymer concentration appear to exist, suggesting that unreacted catechol residues can maximally contribute to the interfacial adhesion. Nevertheless, chitosan-g-hem can be a very effective biocatalyst in the fabrication of in situ gelling adhesive catechol-functionalized polymer hydrogels for biomedical applications (Fig. 8).

4. Conclusions

In this study, a novel enzyme-mimicking chitosan-g-hem biocatalyst was developed to fabricate adhesive catechol-containing hydrogels prepared by chitosan-g-hem.
hydrogels. Using chitosan-g-hem, the gelation effectively occurred at physiological conditions even without the use of the enzyme HRP. Chitosan-g-hem biocatalysts showed enhanced solubility and activity compared with the unmodified hematin. The catechol-containing hydrogels prepared by chitosan-g-hem exhibited enhanced tissue adhesion compared with the hydrogels formed by the conventional pH-initiated methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.09.014.

Appendix B. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–8, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.actbio.2013.09.014.

References


