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Modulating thiol pK_a promotes disulfide formation at physiological pH: An elegant strategy to design disulfide crosslinked hyaluronic acid hydrogels

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Abstract

The disulfide bond plays a crucial role in protein biology and has been exploited by scientists to develop antibody-drug conjugates, sensors and for the immobilization other biomolecules to materials surfaces. In spite of its versatile use, the disulfide chemistry suffers from some inevitable limitations such as the need for basic conditions (pH > 8.5), strong oxidants and long reaction times. We demonstrate here that thiol-substrates containing electron-withdrawing groups at the β-position influence the deprotonation of the thiol group, which is the key reaction intermediate in the formation of disulfide bonds. Evaluation of reaction kinetics using small molecule substrate such as L-cysteine indicated disulfide formation at a 2.8-fold higher (k_1 = 5.04 x 10^{-4} min^{-1}) reaction rate as compared to the conventional thiol substrate, namely 3-mercaptopropionic acid (k_1 = 1.80 x 10^{-4} min^{-1}) at physiological pH (pH 7.4). Interestingly, the same effect could not be observed when N-acetyl-L-cysteine substrate (k_1 = 0.51 x 10^{-4} min^{-1}) was used. We further grafted such thiol-containing molecules (cysteine, N-acetyl-cysteine, and 3-mercaptopropionic acid) to a biopolymer namely hyaluronic acid (HA) and determined the pK_a value of different thiol groups by spectrophotometric analysis. The electron-withdrawing group at the β-position reduced the pKa of the thiol group to 7.0 for HA-cysteine (HA-Cys); 7.4 for N-acetyl cysteine (HA-ActCys) and 8.1 for HA-thiol (HA-SH) derivatives respectively. These experiments further confirmed that the concentration of thiolate ($R\text{-}S^-$) ions could be increased with the presence of electron-withdrawing groups, which could facilitate disulfide cross-linked hydrogel formation at physiological pH. Indeed, HA grafted with cysteine or N-acetyl groups formed

hydrogels within 3.5 minutes or 10 hours, respectively at pH 7.4. After completion of crosslinking reaction both gels demonstrated a storage modulus G' ≈3300–3500 Pa, indicating comparable levels of crosslinking. The HA-SH gel, on the other hand, did not form any gel at pH 7.4 even after 24 h. Finally, we demonstrated that the newly prepared hydrogels exhibited excellent hydrolytic stability but can be degraded by cell-directed processes (enzymatic and reductive degradation). We believe our study provides a valuable insight on the factors governing the disulfide formation and our results are useful to develop strategies that would facilitate generation of stable thiol functionalized biomolecules or promote fast thiol oxidation according to the biomedical needs.

Introduction

Disulfide formation is crucial in many biological processes, such as protein folding,[1, 2] redox signaling,[3] and the regulation of protein function.[4] Disulfide linkages are of particular interest for bioconjugation, controlled drug delivery, and cell encapsulation, because of the ability of cells to cleave disulfide bonds by secreting natural reductants such as glutathione.[5-7] The strategy most commonly used in organic chemistry to form disulfide bonds is the oxidation of thiol compounds in presence of oxygen (**Equation 1**). Thiol-oxidation, in general is a very slow process under physiological conditions (pH 7.4) because it involves the deprotonation of the thiol groups to form thiolate ions ($R\text{-}S^-$) that react with the molecular oxygen to generate the reactive radical species. Since thiols have a pK_a of ≈8-10, it requires basic conditions (pH > 8.5) to drive the forward reaction.[8] Other strategies involve the use of radical initiators such as peroxides that yield disulfides following a complex multistep reaction intermediates.[9] Therefore, the rate of disulfide formation through thiol oxidation can be improved by increasing the reaction pH[9, 10] or by the addition of oxidants such as hydrogen peroxide or iodine.[9, 11]

$$R\text{-}SH + H_2O \rightleftharpoons R\text{-}S^- + H_3O^+$$

$$4R\text{-}S^- + O_2 + 4H_3O^+ \rightleftharpoons 4R\text{-}S^{\cdot} + 6H_2O$$

$$2R\text{-}S^{\cdot} \rightarrow R\text{-}S\text{-}S\text{-}R \qquad (1)$$

Another strategy to form disulfide bonds is the disulfide exchange reaction, which also involves the deprotonation of the thiol group, followed by nucleophilic substitution reaction

with other disulfides having labile thiol groups (**Equation 2**). The use of reactive pyridyl disulfide compounds highly improves the disulfide exchange reaction rate at neutral conditions and is extensively used for conjugation reactions.[7, 12]

$$R\text{-}SH + H_2O \rightleftharpoons R\text{-}S^- + H_3O^+$$

$$R\text{-}S^- + R'\text{-}SS\text{-}R' \rightleftharpoons R\text{-}SS\text{-}R' + R'\text{-}S^- \qquad (2)$$

Even though disulfide chemistry is slow at physiological pH, it is intriguing to note that most of the proteins form three-dimensional (3D) structure employing cysteine dimerization with high fidelity. It was also reported previously by Lutolf et al. that cysteine pK_a could be increased or decreased by changing the neighboring amino acid in a peptide sequence that regulate the rate of Michael addition reactions.[13] This prompted us to investigate the role of thiol-pK_a in driving disulfide formation at physiological pH using different thiol derivatives. We subsequently validated our finding by developing hyaluronic acid (HA) based injectable hydrogels under physiological conditions.

Hydrogels are water containing 3D polymeric networks with highly tunable physical and chemical properties[14] and have been extensively used for a range of biomedical applications, including tissue engineering,[15-17] and drug delivery[18-20] owing to their ability to mimic the native extracellular environment. Hydrogels based on natural polymers such as HA, that is present in the extracellular matrix (ECM), have defined interactions with cells that support cellular activities, has inherent biocompatibility and biodegradability and can preserve cell-secreted sensitive proteins.[21] In addition, cells cultured on HA-hydrogels secrete factors that regulate cell response and function.[22] HA is composed of β-1,4-D-glucuronic acid-β-1,3-D-*N*-acetylglucosamine disaccharide repeat units and represents the only non-sulfated glycosaminoglycan present in the ECM. It is known to play a diverse biological role because of its ability to hold large amounts of water, lack of immunogenicity and degradability by ubiquitous enzymes.[23] Therefore, HA-based hydrogels have been applied as tissue fillers,[24] drug delivery systems,[25, 26] and is intensively studied for tissue regeneration.[17, 27] We have previously developed hydrazone crosslinked HA-hydrogel to deliver clinically relevant protein (recombinant human bone morphogenetic protein-2 or rhBMP-2) to induce bone formation in vivo.[28, 29]

Disulfide cross-linked HA hydrogels have been also developed by several groups but it requires either a high degree of chemical modifications (≈50%), basic pH or oxidants.[30-32] The use of pyridyl disulfide exchange reaction, though significantly improves reaction rates, it liberates 2-mercaptopyridine as a side-product, which may not be suitable for in vivo applications.[33, 34] Recently, we have shown the 1,2-aminothiols such as cysteine could form stable thiazolidine linkages at pH 7.4 when mixed with reactive aldehydes.[35] Unlike other chemistries, disulfide cross-linking reaction has an added advantage for cell-based therapies because the hydrogels could be cleaved enzymatically or chemically by cell-secreted reductants such as glutathione.[5, 34, 36] We, therefore, envisioned developing disulfide crosslinked HA hydrogels by tuning the pK_a of the thiol group. We anticipated that by reducing the thiol pK_a, we could promote thiolate anion formation at physiological pH, which could favor oxygen catalyzed disulfide formation. For this purpose, we explored the influence of electron withdrawing groups at the β-position of thiol (cysteine and N-acetyl-L-cysteine) to drive the disulfide bond formation and compared the reaction with conventional thiol derivative without any electron-withdrawing groups having a similar degree of substitution on HA. The hydrogels were developed by performing reaction at basic and physiological pH and were characterized by evaluating their rheological properties, hydrolytic stability, and dual degradability using enzymatic and non-enzymatic conditions.

Experimental section

General

Hyaluronic acid (HA) sodium salt (200 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). All reagents were of the highest purity available from Sigma-Aldrich (Steinheim, Germany). All the organic solvents were purchased from Fisher Scientific (Loughborough, UK). Dialysis membranes Spectra/Por® 6 (3500 g/mol cut-off) were from VWR International (Compton, CA, USA). All NMR-spectra were recorded on a JEOL JNM-ECP Series FT-NMR spectrometer at magnetic field strength 9.4 T, operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. The residual solvent peak was used as the internal reference when possible. Chemical shifts were expressed in ppm. The pH measurements were carried out on Mettler Toledo pH meter. The dihydrazide reagent for the synthesis of thiol-modified HA (HA-SH) derivative (3,3'-dithiodi(propanehydrazide) (**6**)[37] and the consequent HA-SH derivative[30] were synthesized according to previously reported procedures.

NMR-spectroscopy kinetic study of disulfide formation

L-Cysteine (**1**), N-acetyl-L-cysteine (**2**) or 3-mercaptopropionic acid (**3**) were dissolved in deuterated phosphate buffer saline (dPBS) at 20 mM concentration. The resulting solutions pD (pH + 0.4) was adjusted to 7.4 or 9 by adding NaOD or HCl 0.1 M in D_2O. The oxygen concentration was kept constant by bubbling air in the reaction mixture. Disulfide formation was followed by periodically recording ^{1}H-NMR spectra of the reaction mixture during 24 hours. Resonances of the newly formed disulfides were compared with the resonances from the original thiol compounds and expressed as % of disulfide formation. Pseudo-first-order reaction constant (k_1) was defined as the slope of the line obtained by representing the ln(disulfide concentration/thiol initial concentration) as a function of reaction time.

Synthesis of the dihydrazide used for the modification of HA with cysteine groups

The reagent used in the synthesis of HA-Cys (3,3'-dithiodi(2-aminopropanehydrazide)) (**4**) was synthesized following a three-step synthetic procedure (**Scheme 1**).

Dimethyl 3,3'-dithiodi(2-(tert-butoxycarbonyl)aminopropanoate) (8). N-[(tert-butoxy)carbonyl]-L-cysteine methyl ester (**7**) (2.66 g, 11.31 mmol) was dissolved in EtOAc (32 mL). Solid sodium iodine (14.7 mg, 0.098 mmol) was added followed by hydrogen peroxide 30% (1.6 mL, 12.44 mmol). The reaction mixture was stirred at room temperature. The reaction was followed by TLC on silica gel plate revealed by ninhydrin. When the reaction was completed, a saturated aqueous solution of sodium sulfite (40 mL) was added to quench the reaction. The product was extracted with EtOAc (3 x 50 mL) and the combined organic phases were washed with brine and dried over $MgSO_4$. The solvent was evaporated and the product was dried under vacuum. Yield: 2.57 g (97%). ^{1}H-NMR (CD_3OD, 400 MHz) δ (ppm): 4.46 (dd, J = 8.3, 4.9 Hz, -*CH*-*NHCO*-, 2H), 3.75 (s, *CH*$_3$*O*-, 6H), 3.19 (dd, J = 14.0, 4.8 Hz, -*CH*$_2$-*S*-, 2H), 2.96 (dd, J = 13.9, 8.8 Hz, -*CH*$_2$-*S*-, 2H), 1.45 (s, (*CH*$_3$-)$_3$,18H). ^{13}C-NMR (CD_3OD, 100 MHz) δ (ppm): 173.1 (-*CO*-*OCH*$_3$), 157.8 (-*CO*-*OC*(*CH*$_3$)$_3$), 80.9 (-*C*-(*CH*$_3$)$_3$), 54.3 (-*CH*-*NHCO*-), 52.9 (*CH*$_3$*O*-), 41.1 (-CH_2-S-), 28.7 ((*CH*$_3$)$_3$-).

3,3'-dithiodi(2-(tert-butoxycarbonyl)aminopropanehydrazide) (9). Hydrazine hydrate 80% (5.6 mL, 76.73 mol) was added to a stirred solution of Compound **8** (2.57 g, 5.48 mmol) in ethanol (55 mL). The reaction mixture was stirred for one hour at room temperature. The obtained white precipitate was filtered, washed with cold ethanol and dried under vacuum. Yield: 1.49 g (58%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 4.42 – 4.35 (m, -*CH*-*NHCO*-, 2H), 3.12 (dd, J = 13.8, 5.5 Hz, -*CH*$_2$-*S*-, 2H), 2.91 (dd, J = 13.6, 8.6 Hz, -*CH*$_2$-*S*-, 2H), 1.45

(s, (*CH₃-)₃*, 18H). ^{13}C-NMR (100 MHz, CD_3OD) δ (ppm): 172.2 (-*CO-NHNH₂*), 157.6 (-*CO-OC(CH₃)₃*), 80.9 (-*C-(CH₃)₃*), 54.1 (-*CH-NHCO-*), 42.0 (-*CH₂-S-*), 28.7 (*(CH₃)₃-*).

3,3'-dithiodi(2-aminopropanehydrazide) (4). Hydrochloric acid 37% (13 mL) was added to a stirred clear solution of Compound **9** (767.2 mg, 1.64 mmol) in 1,4-dioxane (25 mL). The reaction mixture was stirred for one hour at room temperature. The obtained white precipitate was filtered, washed with cold ethanol and dried under vacuum. Yield: 458.8 mg (76%). ^{1}H-NMR (400 MHz, D_2O) δ (ppm): 4.46 (dd, J = 8.1, 5.4 Hz, -*CH-NHCO-*, 2H), 3.41 (dd, J = 15.1, 5.4 Hz, -*CH₂-S-*, 2H), 3.21 (dd, J = 15.1, 8.2 Hz, -*CH₂-S-*, 2H). ^{13}C-NMR (100 MHz, D_2O) δ (ppm): 167.2 (-*CO-NHNH₂*), 51.3 (*CH-NH₂*), 37.4 (-*CH₂-S-*).

Synthesis of the dihydrazide used for the modification of HA with N-acetyl cysteine groups

The reagent used in the synthesis of HA-ActCys (3,3'-dithiodi(2-acetamidopropanehydrazide) (**5**) was synthesized following a three-step synthetic procedure (**Scheme 1**).

Methyl 3-sulfanyl-2-acetamidopropanoate (11). N-acetyl-L-cysteine (**2**) (10.67 g, 65.37 mmol) was dissolved in methanol (130 mL). Sulfuric acid was added to the solution (30 drops) and the reaction mixture was refluxed overnight. The solvent was evaporated and the transparent oil obtained was dissolved in ethyl acetate (150 mL). The organic phase was washed with water (3 x 20 mL) and dried over $MgSO_4$. The solvent was evaporated and the product was dried under vacuum. Yield: 7.11g (61%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 4.63 (dd, J = 6.6, 5.0 Hz, -*CH-NHCO-*, 2H), 3.75 (s, *CH₃O-*, 6H), 2.91 (qd, J = 14.0, 5.8 Hz, -*CH₂-SH*, 4H), 2.02 (s, *CH₃-*, 6H). ^{13}C-NMR (100 MHz, CD_3OD) δ (ppm): 173.3 (-*CO-CH₃*), 172.1 (-*CO-OCH₃*), 56.2 (-*CH-NHCO-*), 52.9 (*CH₃O-*), 26.6 (-*CH₂-SH*), 22.3 (*CH₃-*).

Dimethyl 3,3'-dithidi(2-acetamidopropanoate) (12). Compound **11** (7.1 g, 40.07 mmol) was dissolved in EtOAc (125 mL). Sodium iodide (66.3 mg, 0.44 mmol) was added followed by hydrogen peroxide 30% (5 mL, 44.08 mmol). The reaction mixture was stirred at room temperature. The reaction was followed by TLC on silica gel plate revealed by ninhydrin. When the reaction was completed, a saturated aqueous solution of sodium sulfite (40 mL) was added to quench the reaction. The product was extracted with EtOAc (3 x 20 mL) and the combined organic phases were dried over $MgSO_4$. The solvent was evaporated and the obtained white product was dried under vacuum. Yield: 5.16 g (73%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 4.74 (dd, J = 8.6, 5.0 Hz, -*CH-NHCO-*, 2H), 3.75 (s, *CH₃O-*, 6H), 3.23 (dd, J = 14.5, 5.3 Hz, -*CH₂-S-*, 2H), 2.97 (dd, J = 13.8, 8.6 Hz, -*CH₂-SH*, 2H), 2.00 (s, *CH₃-*, 6H).

^{13}C-NMR (100 MHz, CD_3OD) δ (ppm): 173.4 (-*CO*-*CH*$_3$), 172.4 (-*CO*-*OCH*$_3$), 53.0 (-*CH*-*NHCO*-), 49.0 (*CH*$_3$*O*-), 40.5 (-*CH*$_2$-*S*-), 22.3 (*CH*$_3$-).

3,3'-dithiodi(2-acetamidopropanehydrazide) (5). Hydrazine hydrate 80% (15 mL, 204.6 mmol) was slowly added to a stirred solution of compound **12** (5.15 g, 14.61 mmol) in ethanol (120 mL). The white precipitate obtained was filtered, washed with cold ethanol and dried under vacuum. Yield: 4.68 g (91%). ^{1}H-NMR (D_2O, 400 MHz) δ (ppm): 4.60 (dd, *J* = 8.5, 5.6 Hz, -*CH*-*NHCO*-, 2H), 3.16 (dd, *J* = 14.3, 5.6 Hz, -*CH*$_2$-*S*-, 2H), 2.93 (dd, *J* = 14.2, 8.5 Hz, -*CH*$_2$-*S*-, 2H), 2.03 (s, *CH*$_3$-, 6H). ^{13}C-NMR (100 MHz, CD_3OD) δ (ppm): 174.3 (-*CO*-*CH*$_3$), 170.8 (-*CO*-*NHNH*$_2$), 51.6 (-*CH*-*NHCO*-), 38.6 (-*CH*$_2$-*S*-), 21.8 (*CH*$_3$-).

HA modification with different thiol groups (HA-Cys, HA-ActCys, HA-SH)

Sodium hyaluronate was dissolved in deionized water at a concentration of 9 mg/mL. The correspondent hydrazide reagent (4, 5 or 6) was added to the clear solution at the molar ratio specified in **Table 1**. The pH of the solution was adjusted to 4.5 by adding NaOH (1 M). The coupling reaction was initiated by the addition of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (see **Table 1** for EDC/HA disaccharide ration). EDC was added portion-wise during 1 hour while maintaining the pH at 4.5 by adding HCl (2M). The reaction mixture was stirred overnight at room temperature. DTT (see **Table 1**) was then added and the pH was adjusted to 8.5 by adding NaOH (5M). The reaction mixture was stirred overnight at room temperature. The solution was diluted to 5 mg/mL, acidified to pH 3 by adding HCl (2M) and transferred to a dialysis membrane. After exhaustive dialysis against dilute HCl (pH 3) containing NaCl (0.1M) for 24 h (3 x dialyzing solvent change) and dilute HCl (pH 3) for 24 h (3 x dialyzing solvent change). The solution was lyophilized to give the corresponding thiol-modified HA derivatives (HA-Cys, HA-ActCys, HA-SH). The incorporation of different thiol functionality was verified by ^{1}H-NMR. Specifically, the signals corresponding to the methine (-*CHNH*$_2$, 4.36 ppm) and methylene (-*CH*$_2$*SH*, 2.88 ppm) protons of HA-Cys, the methine (-*CHNHCO*-, 4.59 ppm), methylene (-*CH*$_2$*SH*, 2.97 ppm) and methyl (*CH*$_3$*CONH*-, 2.07 ppm) protons of HA-ActCys and the methylene (-*CH*$_2$*CH*$_2$*SH*, 2.87 ppm and 2.72 ppm) protons of HA-SH. The degree of substitution (DS) was determined by the Ellman's test[38] (see **Table 1**).

pK_a Determination

The determination of the pK_a values for the thiol groups in HA-Cys, HA-ActCys, and HA-SH was conducted spectrophotometrically based on previously reported procedures.[30] Briefly, thiol-modified HA derivatives were dissolved in HCl (1 mM) containing NaCl (0.1M) (to

provide stable ionic strength) at a concentration of 0.5 mg/L. Different amounts of NaOH (0.01 M) were added to the freshly prepared solutions and the absorbances (242 nm) were immediately measured on Perkin Elmer Lambda 35 UV/VIS spectrometer. The pH of the resulting solutions was determined after the absorbance measurements. The pK_a value was obtained by representing the -log[(A_{max} – A_i)/A_i] as a function of the pH. The pK_a values are displayed where the linear fit crosses the abscissa.

Hydrogels formation and characterization

HA derivatives were dissolved in acidified (pH 5) and degassed PBS (2% w/v) to avoid disulfide formation during solubilization. The hydrogel formation was initiated by raising the pH of the solutions to 7.4 or 9 with NaOH (1 M). The solutions (200 µL) were transferred to a cylindrical mold, sealed with parafilm and allowed to cross-link overnight. The viscoelastic properties of the hydrogels were evaluated on a Discovery Hybrid Rheometer 2 (DHR2) from TA Instruments. The cylindrical hydrogels were carefully transferred from the molds to the rheometer plate and the gap was adjusted to reach a normal force of 30 mN. The rheological properties were determined with frequency 0.1-20 Hz and 1% strain at 25°C using 8 mm parallel plate geometry. The gelation time was determined using the same rheometer. All the gelation kinetics measurements were performed inside a humidity chamber to avoid hydrogel desiccation. The solutions of the HA derivatives (350 µL) were immediately transferred to the rheometer after pH adjustment and the gap was lowered to a size of 100 µm. The storage (G') and loss modulus (G") were recorded over time using 20 mm parallel plate geometry (strain: 1%, frequency: 0.5 Hz). The time at which G' cross G" was defined as the gelation time.

Hydrogel hydrolytic stability and degradation

Newly formed hydrogels were removed from the mold, weighed (m_0) and immersed in 5 mL Eppendorf tube containing 2 mL of degradation media. At specific time intervals, the remaining hydrogels were weighed (m_t) after removing the excess solution. After measuring their mass, the hydrogels were immersed in fresh degradation media. All experiments were performed in triplicates. The hydrolytic stability was studied in PBS (pH 7.4) as the degradation media. The swelling ratio was calculated from the equation [(m_t/m_0)x100]-100. The enzymatic and reductive degradation was studied using PBS containing hyaluronidase from bovine testes (Type IV-S, 0.3 mg/mL, 210 U/mL) or DTT (1 mM) as the degradation media, respectively. The degradation ratio was express as (m_t/m_0)x100.

Results and discussion

Kinetics of disulfide formation by ^{1}H NMR spectroscopy

In order to determine the effect of an electron-withdrawing substituent at the β-position of the thiol residue on disulfide formation, we first determined the reaction kinetics by ^{1}H-NMR analysis using a small molecule model (**Figure 1**). We performed the reaction by dissolving L-cysteine (**1**), N-acetyl-L-cysteine (**2**) and 3-mercaptopropionic acid (**3**) in deuterated phosphate buffer at 20 mM concentrations. The reaction was performed at physiological (pD 7.4) and basic (pD 9) conditions. Since molecular oxygen concentration is a key factor for such reactions, we constantly bubbled air in the reaction mixture.

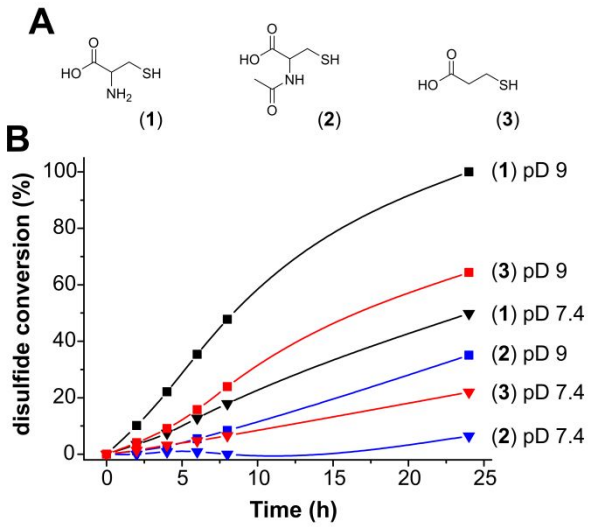


Figure 1. a) Chemical structure of L-cysteine (**1**), N-acetyl-L-cysteine (**2**) and 3-mercaptopropionic acid (**3**). b) Disulfide formation of the above molecules followed by ^{1}H-NMR at pD 9 and pD 7.4.

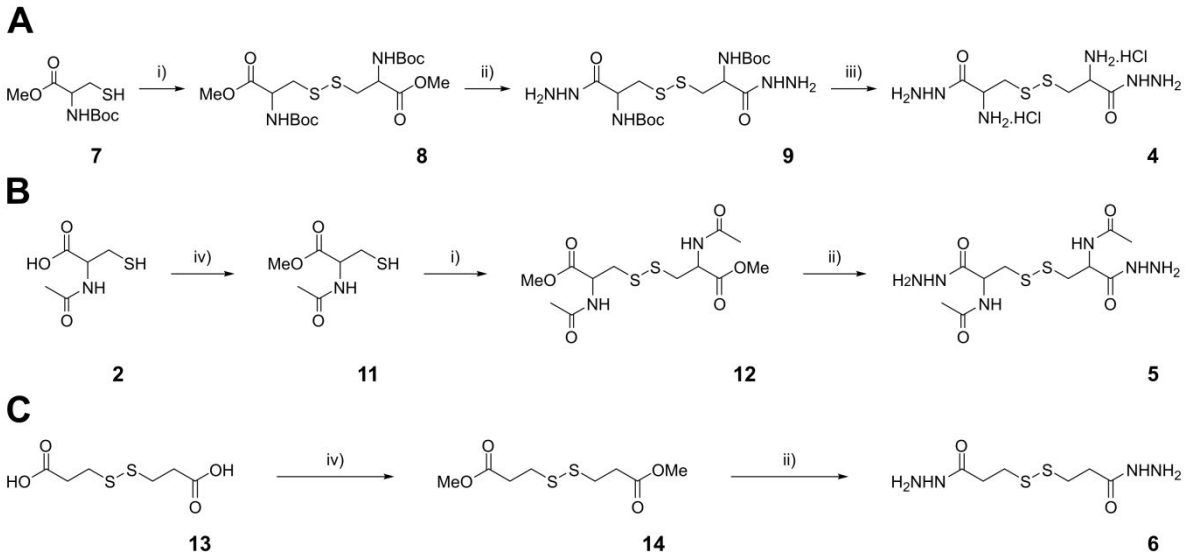
Monitoring the reaction by ^{1}H-NMR showed complete conversion of L-cysteine to disulfide at pD 9 within 24 hours, as the cysteine resonances were completely disappeared (**Figure S1, in the Supporting Information**). At pD 7.4, disulfide formation considerably slowed down and only 49.8% conversion was observed after 24 hours. The rates of reaction were calculated using pseudo-first-order conditions, which indicated that the rate of reaction at pD 9 (k_1 = 15.1 x 10^{-4} min^{-1}) was 3-fold higher than at pD 7.4 (k_1 = 5.04 x 10^{-4} min^{-1}; **Figure S2 and Figure S3, in the Supporting Information**). The observed increase in reaction rate at higher pH corroborates with the hypothesis that the rate of disulfide formation is directed by the thiolate anion concentration. Since the electron-withdrawing group at the β-position (protonated amine) of L-cysteine favors the deprotonation of the thiol group, we anticipated

an increase in reaction rate as compared to the unmodified thiol substrate (3-mercaptopropionic acid). ^{1}H-NMR analysis of the disulfide formation with 3-mercaptopropionic acid (**Figure S4, in the Supporting information**) revealed that the reaction rate of such a substrate indeed was ≈2-fold (k_1 = 7.68 x 10^{-4} min^{-1}) lower at pH 9 and ≈2.8-fold (k_1 = 1.80 x 10^{-4} min^{-1}) lower at pD 7.4, as compared to the L-cysteine substrate (**Figure S5 and Figure S6, in the Supporting Information**). Since the N-acetylation of cysteine eliminates the protonation of nitrogen atom we anticipated a reduction in the reaction rate. ^{1}H-NMR based study of the reaction kinetics with N-acetyl-L-cysteine group indeed demonstrated reduction of the reaction rate by ≈5–10 folds at pH 9 (k_1 = 3.30 x 10^{-4} min^{-1}) and pH 7.4 (0.51 x 10^{-4} min^{-1}) respectively, as compared to the L-cysteine substrate (**Figure S7-S9, in the Supporting Information**). Such a significant drop in reaction rate could be attributed to the drop in nitrogen pK_a and also due to the steric hindrance of the N-acetyl group that slows down the reaction. Taken together, the observed reduction of the reaction rates corroborates with our hypothesis that electron-withdrawing substituent favors the oxidation of thiols to disulfides by increasing the acidity of the thiol group, though steric factors could also affect the reaction rate. These experiments clearly demonstrated the pH dependence on the reaction and the influence of the thiol electronic environment on the reaction kinetics.

HA modification with different thiol groups (HA-Cys, HA-ActCys, HA-SH)

Encouraged by the results obtained with small molecule analogs, we prepared HA derivatives modified with cysteine (HA-Cys), N-acetyl-cysteine (HA-ActCys) and mercaptopropionic acid moieties (HA-SH). For this purpose, we first synthesized the dihydrazide derivatives of the corresponding carboxylic acids molecules with a dimeric structure (**Scheme 1**). Such a modification enabled incorporation of the pendant functional groups using carbodiimide-mediated hydrazide coupling chemistry, as optimized previously in our group.[29]

Scheme 1. Schematic representation of the synthesis of disulfide-containing dihydrazide compounds. a) 3,3'-dithiodi(2-aminopropanehydrazide) (**4**) used in the preparation of HA-Cys. b) 3,3'-dithiodi(2-acetamidopropanehydrazide) (**5**) used in the preparation of HA-ActCys. c) 3,3'-dithiodi(propanehydrazide) (**6**) used in the preparation of HA-SH.



Reaction conditions: i) cat. NaI, H_2O_2, EtOAc; ii) $NH_2NH_2 \cdot H_2O$, EtOH; iii) HCl, 1,4-dioxane; iv) cat. H_2SO_4, MeOH.

It is important to note that the amine functionality of the dihydrazide **4** was not protected during the EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) mediated coupling reaction as such a reaction was performed at pH 4.5 that prevents the participation of free amines that remain primarily as protonated species. The progress of the reaction was evident by a visible increase in the viscosity of the reaction mixture, indicating that the dihydrazide molecules acted as cross-linkers between HA macromolecules (**Figure 2a**). However, the subsequent reduction of the central disulfide bonds with 1,4-dithiothreitol (DTT) liberated the desired free thiol groups yielding the soluble form of thiolated HA derivatives. The excess of reagent and DTT was removed by dialyzing against acidified water (pH 3) that prevented the formation of new disulfide bridges during the purification process. The dialyzed product was lyophilized to obtain the thiolated HA derivatives as a white fluffy material.

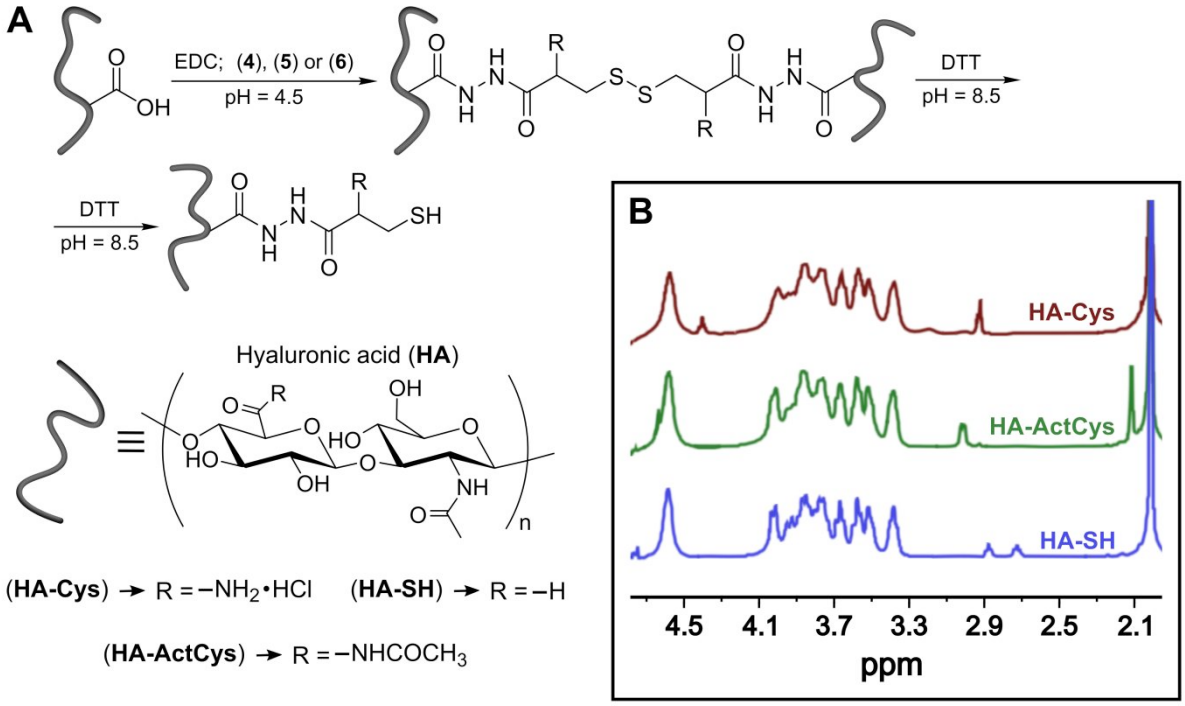


Figure 2. a) Synthetic scheme and chemical structure of thiolated hyaluronic acid (HA) derivatives (HA-Cys, HA-ActCys, and HA-SH). b) ^{1}H-NMR spectra of thiolated HA derivatives.

The chemical characterization of the obtained HA derivatives was carried out using ^{1}H-NMR analysis (**Figure 2b and Figure S10-S13, in the Supporting Information**). Specifically, new resonances appeared at 4.36 ppm (-*CHNH*$_2$) and 2.88 ppm (-*CH*$_2$*SH*) for HA-Cys derivative, whereas a downfield shift at 4.59 ppm (-*CHNHCO*-), 2.97 ppm (-*CH*$_2$*SH*) and 2.07 ppm (*CH*$_3$*CONH*-) was observed for the acetylated product (HA-ActCys). For the mercaptopropionic acid derivative we observed a chemical shift at 2.87 ppm and 2.72 ppm for the -*CH*$_2$*CH*$_2$*SH* signals. The degree of substitution (DS) could not be determined by integrating the proton signals due to overlapped with the HA signals. The DS was therefore determined by the modified Ellman's method, which is a colorimetric assay for the determination of free thiol groups.[38] The colorimetric analysis indicated that the DS of different thiolated HA derivatives were 11% for HA-Cys, 11% for HA-ActCys and 9% for HA-SH. Additionally, HA-SH derivative with higher DS (19%) was also prepared. The DS was controlled by regulating the molar ratios between HA and the coupling reagent EDC (**Table 1**).

Table 1. The molar ratio of the reagents used for the synthesis of thiol-modified HA derivatives and the obtained degree of substitution (DS). The values were expressed based on HA disaccharide repeating units.

HA sample	Molar ratio			DS
	EDC	dihydrazide	DTT	
HA-Cys	0.3	0.9[a]	4.5	11%
HA-ActCys	0.175	0.525[b]	2.625	11%
HA-SH	0.15	0.45[c]	2.25	9%
HA-SH	0.3	0.9[c]	4.5	19%

[a] Compound **4** was the hydrazide reagent used for the synthesis of HA-Cys. [b] Compound **5** was the hydrazide reagent used for the synthesis of HA-ActCys. [c] Compound **6** was the hydrazide reagent used in the synthesis of HA-SH.

Determination of the thiol pK_a in the HA derivatives

Since the concentration of thiolate anion ($R\text{-}S^-$) has been found to be the key intermediate for disulfide formation, we determined the pK_a of different thiolated derivatives using UV spectrophotometric method. This was performed by dissolving the HA derivatives in an acidified (1 mM of HCl) aqueous media with a stable ionic strength (NaCl 0.1M). The absorbance at 242 nm was recorded after pH adjustment, following a previously reported procedure.[30] The absorbance increased with the pH indicating the formation of thiolate ions. **Figure 3a** shows the sigmoidal curves of the absorbance versus pH. The absorbance of the HA-Cys solution showed an abrupt increase within a very short range of pH, while the absorbance of HA-ActCys and HA-SH solution increased in a larger range of pH. This difference may be explained by the chemical microenvironment near the thiol group that strongly influences the formation of thiolate ions. The graphical representation of $-\log[(A_{max} - A_i)/A_i]$ versus pH (**Figure 3b**) displays the pK_a values of the modified HA where the linear fit crosses the abscissa. Interestingly, the pK_a of the HA-Cys was found to be 7.0, which is significantly lower than what is known in cysteine-containing peptides (≈8.3). Such a decrease in pK_a is presumably due to salt formation between the amino residue of cysteine and carboxylate backbone of HA. We recently demonstrated that electrostatic interactions between HA and a biologically active protein (recombinant human bone morphogenetic protein-2) result in a retained molecular interaction between the two biopolymers even after shifting to physiological pH.[28] The N-acetyl derivative (HA-ActCys) on the other hand, indicated a pK_a value of 7.4, which is anticipated as the N-acetyl protection of primary amine will limit the amino protonation and as a result will reduce the electron-withdrawing effect as

compared to the parent molecule. The pK_a value of the HA-SH was found to be 8.1, which is closer to the reported pK_a of HA-thiol.[30] Of note, the reported pK_a of mercaptopropionic acid is ≈10.8[39] while the HA derivative has a significantly lower pK_a indicating the role of the molecular structure of HA in increasing the acidity of the thiol group.

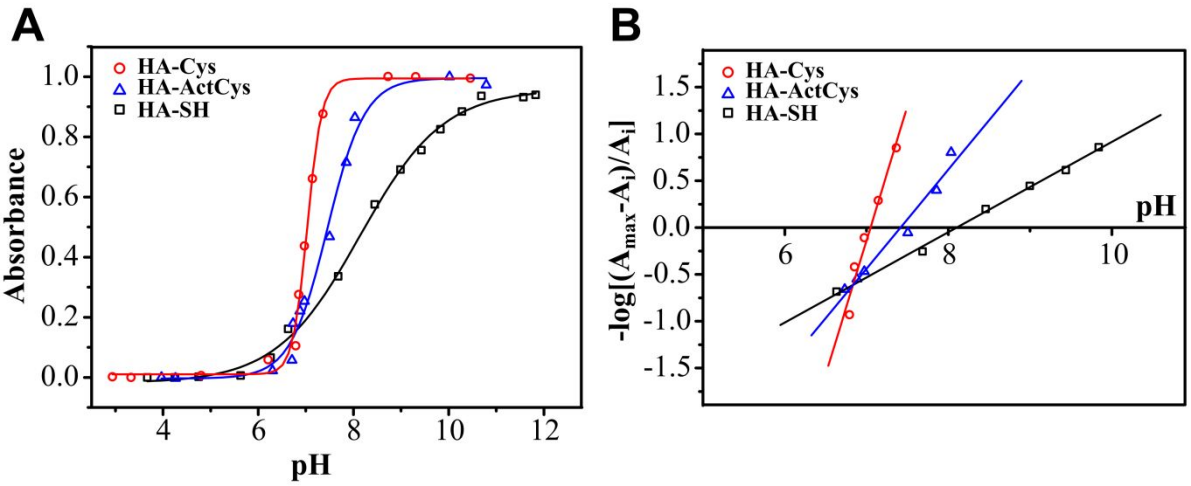


Figure 3. a) Absorbance of the thiol-modified HA derivatives (HA-Cys, HA-ActCys, and HA-SH) as a function of pH. b) Logarithmic representation of the normalized absorbance (-log[(A_{max}-A_i)/A_i]) as a function of the pH. The pK_a values correspond to the interception with the abscissa.

Hydrogel preparation and characterization

The observed decrease in thiol pK_a encouraged us to test disulfide crosslinked HA-hydrogel formation at physiological pH and at basic pH without any addition of oxidants. In earlier works, the degree of thiol modification is kept very high (for oxidant-free hydrogel preparation),[30, 31] due to the low reactivity of thiol groups but we aimed to keep the modification degree to ≈10%. The higher degree of chemical modification is expected to influence the HA interaction with cells as it is known that a minimum of ten saccharide units is needed for HA-CD44 interaction.[40] To prepare HA-hydrogels with 2% solid content, the three thiol-modified HA derivatives (HA-Cys, HA-ActCys, and HA-SH) were first dissolved in an acidified (pH 5) phosphate buffer and then the pH was carefully adjusted to 7.4 or 9.0. The gelation kinetics was determined using rheological measurements and the gel point was determined by measuring the crossover point between the storage modulus (G') and the loss modulus (G'') (**Table 2 and Figure S14, in the Supporting information**).

Table 1. The gelation time of all thiol-modified HA derivative (HA-Cys, HA-ActCys, and HA-SH). The gelation was performed at pH 7.4 and pH 9.0.

DS	Sample	pH 7.4	pH 9.0
11%	HA-Cys	3,5 min	3,5 min
11%	HA-ActCys	10 h	6,5 h
9%	HA-SH	-	-
19%	HA-SH	-	5 h

We first observed the hydrogel formation with HA-SH derivative having 9% or 19% of thiol groups at physiological pH (7.4) and surprisingly we did not observe any hydrogel formation within the 24 h experiment. The absence of gelation at pH 7.4 was presumably due to the low concentration of thiolate anions (16.6% according to the Henderson-Hasselbalch equation) in the reaction mixture (pK_a = 8.1). For this reason, most of the reported procedures employ a very high degree of modification (≈50%) or a supplement to the reaction with additional oxidants to obtain hydrogels within a reasonable time.[30, 31] By increasing the pH of the reaction to 9.0, the concentration of thiolate anions increased (88.8%) which resulted in hydrogel formation with HA-SH derivative having 19% modification, however, no gelation was observed with 9% modified HA-SH derivative. We anticipated that HA-Cys with a pK_a of 7.0 should form hydrogels at physiological pH because of the higher abundance of the reactive thiolate anions. Indeed, HA-Cys formed hydrogels within ≈3.5 minutes at pH 7.4. Surprisingly, the gelation time, in this case, was not significantly affected by increasing the pH to 9.0, presumably due to the pK_a of HA-Cys is lower than the experimental conditions (pH 7.4 and pH 9.0). This will allow more than 70% free thiolate anions (71.5% of R-S$^-$ at pH 7.4 and 99% of R-S$^-$ at pH 9) at both the pH's, resulting in high reaction rate. The most interesting case of pH dependence on disulfide formation was seen for HA-ActCys having a pK_a of 7.4 (50% of R-S$^-$ at pH 7.4 and 97.6% of R-S$^-$ at pH 9). The sol to gel transition took over 10 h for HA-ActCys at pH 7.4 but the gel time could be reduced to ≈6.5 h when the pH was raised to 9.0. In spite of the close pK_a values between HA-Cys and HA-ActCys, the observed large differences in their gelation time could be attributed to steric factors with the acetyl group being in close proximity to reactive thiol moiety. Nevertheless, the short gel time of HA-Cys at both pHs demonstrates the significance of introducing electron-withdrawing groups near the thiol residue that decrease the pK_a of the thiol groups and promote disulfide formation at physiological pH.

Table 3. Mechanical properties of disulfide cross-linked HA hydrogels.

DS	Sample	pH 7.4 G' (Pa)	pH 9.0 G' (Pa)
11%	HA-Cys	3312.3	2261.2
11%	HA-ActCys	3523.5	1734.4
19%	HA-SH	-	1910.6

We further evaluated the rheological properties of the HA-Cys and HA-ActCys hydrogels and compared it with HA-SH hydrogels (**Table 3**). The average storage modulus (G') of HA-Cys and HA-ActCys hydrogels prepared at pH 7.4 gave comparable results (≈3300 Pa) demonstrating that in spite of the different reaction rates, the hydrogels reached a similar degree of cross-linking upon completion. The G' was considerably reduced for hydrogels prepared at pH 9.0, suggesting that the high concentration of thiolate ions favored the formation of intramolecular loops reducing the effective intermolecular cross-links. Additionally, HA-SH hydrogels containing 19% modification showed nearly similar G' at pH 9.0 as obtained from HA-Cys and HA-ActCys with approximately half a degree of functionalization (11%), demonstrating that the introduction of electron-withdrawing groups not only improved the reaction kinetics but also improved the efficiency of the cross-linking reaction. Such an observation could be due to the dynamic disulfide exchange reaction as a result of a higher degree of thiol substitution.

Hydrogel hydrolytic stability and degradation

Finally, we evaluated the hydrolytic stability of the hydrogels at physiological pH (7.4) by quantifying the change in hydrogel mass after incubation in phosphate buffer saline (PBS). The most interesting aspect of our swelling studies was that both HA-Cys and HA-ActCys hydrogels showed excellent stability and exhibited only limited swelling (**Figure 4a**). In fact, HA-Cys hydrogels showed moderate shrinking during the 8 days of study. This atypical shrinking behavior is attributed to the presence of free thiolate ions that spontaneous oxidation reaction to form new cross-linkages. The HA-ActCys hydrogels demonstrated a moderate swelling (≈15%) within the first 24 h followed by moderate shrinking of the gels indicating the formation of new cross-links after incubation in PBS. The initial increase of mass could be explained by the stretching of the polymeric network that allowed an increasing influx of water (hydrogel swelling).[29] It should be noted that even though there was shrinking of the

gels, the hydrogels were intact even after 30 days incubation in the swelling medium. We did not perform swelling studies with the HA-SH hydrogels as such gels could not be formed at pH 7.4 but required pH over 9.0 to form hydrogels.

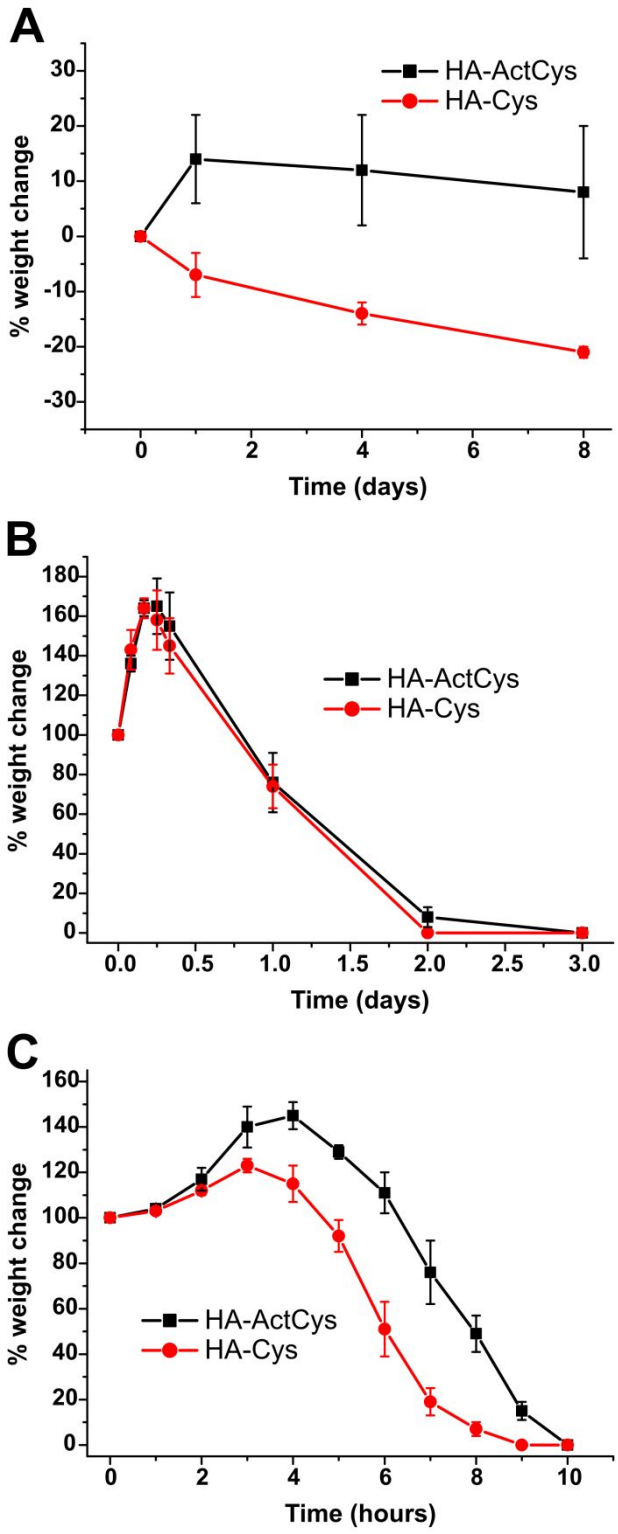


Figure 4. a) Gravimetric swelling study of HA disulfide hydrogels at physiological conditions (pH 7.4). b) Enzymatic degradation of HA hydrogels studied using hyaluronidase (0.3 mg/mL) solution. c) Reductive degradation of HA hydrogels studied using DTT (1 mM) solution.

Since hydrogel swelling characteristics and degradation are closely related we evaluated the enzymatic and non-enzymatic degradation properties of the HA hydrogels. For enzymatic degradation, we incubated different hydrogels with hyaluronidase (0.3 mg/mL), a ubiquitous enzyme known to cleave glycosidic linkages of the HA backbone, and measured the weight loss with time (**Figure 4b**). These experiments indicated that both the hydrogels showed a similar degradation profile, which is expected for hydrogels with similar cross-linking density. We further performed degradation experiments with DTT, a well-known reductant known to cleave disulfide bonds. Interestingly, these experiments revealed that HA-Cys and HA-ActCys hydrogels showed different reductive degradation profiles (**Figure 5c**). The sterically hindered acetyl group of HA-ActCys hydrogel retarded the DTT-mediated disulfide cleavage reaction slowing down the hydrogel degradation. The HA-Cys hydrogel, on the other hand, responded to the added reductant resulting in hydrogel degradation at a higher rate. Since cell-produced reductant such as glutathione is known to degrade disulfide bonds, we believe the two types of hydrogels will behave differently in presence of cells. However, the quick gelation time of HA-Cys hydrogels in less than 5-minutes will favor this gel in vivo applications over the HA-ActCys hydrogel or HA-SH hydrogel.

Conclusions

Oxidation of thiols requires thiolate anion and molecular oxygen to form disulfide bonds exploiting a radical chemistry. We demonstrate here that incorporation of an electron-withdrawing group at the β-position as in cysteine results in significant increase in reaction rate for disulfide formation at physiological pH. We exploited this observation to develop the first disulfide-linked HA-based hydrogel system that forms crosslink at physiological pH in just 3.5 minutes. To tailor such highly reactive thiol containing biopolymer, we designed the HA-thiol derivative having cysteine units with free thiols. Incorporation of the cysteine moiety onto HA reduced the pK_a of thiol group from ≈8.3 (as in peptides) to 7.0, which promoted thiolate anion formation at physiological pH. Acetylation of the amino residue of cysteine reduced the protonation ability of the amino group that resulted in an increase in thiol pK_a to 7.4. To determine the effect of electron-withdrawing group at the β-position, we

utilized the HA-thiol derivative that does not contain any substituent at the β-position using hydrazide modified 3-mercaptopropionic acid unit. Such a thiol moiety displayed a pK_a of 8.1. Such differences in pK_a of different thiols were reflected in disulfide-crosslinked hydrogel formation at pH 7.4 without any addition of external oxidants. The HA-Cys derivative formed hydrogel is just 3.5 minutes, however, the HA-ActCys derivative required over 10 h to form crosslinks. The HA-SH derivative, on the other hand, did not form the hydrogel network at pH 7.4 even after increasing the degree of chemical modification to 19% with respect to the disaccharide units. The swelling studies of the HA-Cys and HA-ActCys hydrogels indicated shrinking of the hydrogel without any visible change in gel structure. The hyaluronidase mediated enzymatic degradation of the HA-Cys and HA-ActCys hydrogels were identical however hydrogel degradation by the cleavage of disulfide bonds with DTT indicated a clear difference between the two materials. The presence of the acetyl group near the disulfide linkage hindered the cleavage of the disulfide resulting in slower degradation of HA-ActCys hydrogels. We believe that the understanding of the factors that regulate disulfide formation will provide new insight to design materials with stable thiol groups or fast disulfide formation for different applications. Moreover, the thiol-modified HA developed in this study undergo fast gelation under physiological pH without any need for catalysts. Such materials will allow developments of clinically translatable hydrogels for cellular delivery or for delivering therapeutic drugs.

Associated content

Supporting information

^{1}H-NMR spectra showing the disulfide conversion of small molecule substrates, representative plots of time-dependent disulfide conversion, representative linear least-squares fits for disulfide formation, ^{1}H-NMR spectra of thiolated HA derivatives, and rheological evaluation of the gelation kinetics of the different thiolated HA derivatives.

Acknowledgment

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