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Improved method for synthesis of cysteine modified hyaluronic acid for *in situ* hydrogel formation†

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We developed a new strategy for the functionalization of hyaluronic acid by chemical modification of its C-6 hydroxyl groups through an ether bond to obtain a cysteine–hyaluronic acid conjugate. This conjugate is suitable to prepare injectable and *in situ* formed hydrogels cross-linked by native chemical ligation and Michael addition under mild conditions.

Hyaluronic acid (HA) is a non-sulfated anionic linear polysaccharide consisting of β-(1, 4) linked D-glucuronic acid-β-(1, 3)-*N*-acetyl-D-glucosamine disaccharide repeats. HA has a molecular weight ranging from 100 kDa to 8 MDa and is ubiquitous in the human body, especially in the synovia of joints, the corpus vitreum of the eyes and the dermis of the skin.[1] As an essential signalling biomacromolecule, HA is located in the extracellular matrix (ECM), at the cell surface and inside the cell, where it is involved in many cellular functions such as water homeostasis, cell migration, proliferation and adhesion.[2]

HA has a short half-life of only about 1 to 2 days in tissue, limiting its applications as a biocompatible, biodegradable and non-immunogenic polymer.[3] Chemical modification and cross-linking of HA has been used to achieve longer residence time *in vivo* while maintaining biocompatibility and viscoelastic properties of the naturally occurring biomolecule.[4] Modified HA has been used in various physical forms – viscoelastic solutions, soft or stiff hydrogels, macroporous and fibrillar sponges, nanoparticulate fluids and flexible sheets.[5] A wide range of biomedical applications utilizing HA derivatives have been developed, such as viscosupplementation,[6] cell differentiation,[7] tissue sealants,[8] tissue augmentation,[9] ophthalmic surgery,[10] engineering of bioactive surfaces,[11] drug delivery,[12] and minimally invasive *in situ* formation of hydrogels under physiological conditions.[13] Such applications are facilitated by modification strategies that permit chemical functionalization while preserving the biological activity of HA.[14]

Strategies for modification of HA often target the carboxylic acid of the glucuronic acid and primary hydroxyl groups of the biomacromolecule.[15] The carboxyl groups have been commonly modified by amidation and esterification using carbodiimide-mediated chemistry. However, derivatization of HA in this manner may form *N*-acylurea-type EDC adduct,[16] which causes the resulting HA conjugate to be immunogenic.[17] Furthermore, the biological functions of HA are considered to be mediated through specific ionic interactions between HA carboxylates and cationic amino acids of the cell surface receptor CD44, and five or more HA disaccharide units in length have been shown to be necessary for optimal CD44 binding.[18]

Alternative HA modification strategies to target the hydroxyl groups of HA have employed divinyl sulfone, diglycidyl ether, glutaraldehyde, and ester linked glycidyl methacrylate, or methacrylic anhydride.[4,5,15a,19] For the preparation of thiolated HA, a previous report described an HA derivatized with free thiol groups (HASH) through ether bond linkage by reaction of the hydroxyl groups of HA with ethylene sulphide.[20] Unfortunately, the reported HASH was unable to form cross-linked HA hydrogels *via* disulfide bonds, or through bivalent thiol-reactive cross-linkers, indicating that employing this strategy for HA gel formation was rather challenging.

In the interest of developing cysteine derivatized HA (Cys–HA) for chemo-selective gel formation under mild conditions,[21] here we report a new strategy for the preparation of Cys–HA useful for *in situ* formation of hydrogels by native chemical ligation. The method preserves the free carboxylic acids by conjugating the cysteine through a stable ether linkage *via* the HA hydroxyl groups (Scheme 1). Starting with commercially available L-cysteine hydrochloride **1**, compound **3** was prepared in two steps,[22] followed by activation of the carboxylic acid with *N*-hydroxysuccinimide (HOSu) to afford the active ester **4**. Cystamine is an unstable liquid and is generally handled as the dihydrochloride salt,

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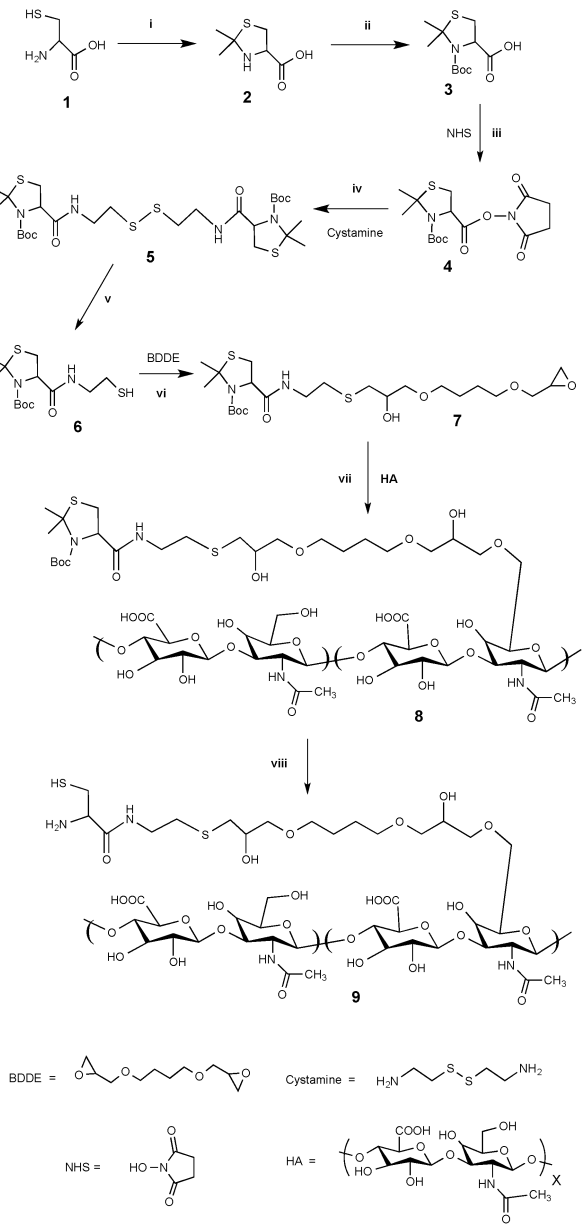


Scheme 1 Synthesis of Cys–HA conjugate **9**.

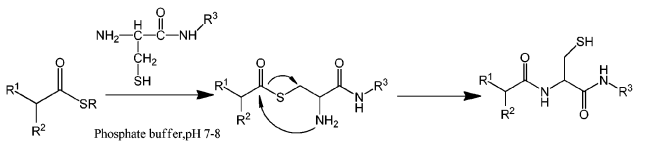
scarcely soluble in organic solvents. Therefore, compound **4** as an active ester can facilitate the reaction with cystamine dihydrochloride in aqueous solution to generate compound **5** without an extra step for the conversion of the salt to the free amine. Selective reduction of the disulfide of compound **5** with $NaBH_4$ yielded compound **6** with free thiol group,[23] a nucleophile that is highly reactive with epoxides under mild conditions. Addition of compound **6** to one epoxide group of 1,4-butanediol diglycidyl ether (BDDE) produced compound **7** containing an epoxide group, which was then used to modify HA at its alcohol groups through an ether bond linkage.

The hydroxyl groups in HA are nucleophiles, which can react with epoxides in a ring-opening process to form ether bonds at high pH conditions (usually above 11). However, we found that the reaction of the epoxide **7** with HA in alkaline solution (0.1 M NaOH solution) could not afford the expected modified HA, as indicated by the absence of the ^{1}HNMR signal of the Boc protecting group. A number of reaction conditions for this reaction, including mixtures of alkaline acetonitrile or THF as well as use of HA–TBA salt in DMSO after TBA salt conversion by ion exchange chromatography, were explored without success. Finally, we discovered that introduction of tetrabutylammonium hydroxide (TBAH) into the reaction mixture of compound **7** with HA enabled the synthesis of compound **8**, characterized by ^{1}HNMR data of the conjugate, and verified by Ellman test for detection of free thiol group[24] as well as ninhydrin test for free amino group after removal of protecting groups on the Cys moiety. Finally, Cys–HA conjugate **9** was obtained by cleavage of Boc protecting group of compound **8** with TFA, and followed by treatment with ethanol/H_2O (1 : 1) to free the Cys, monitored by ^{1}HNMR analysis and Ellman test.

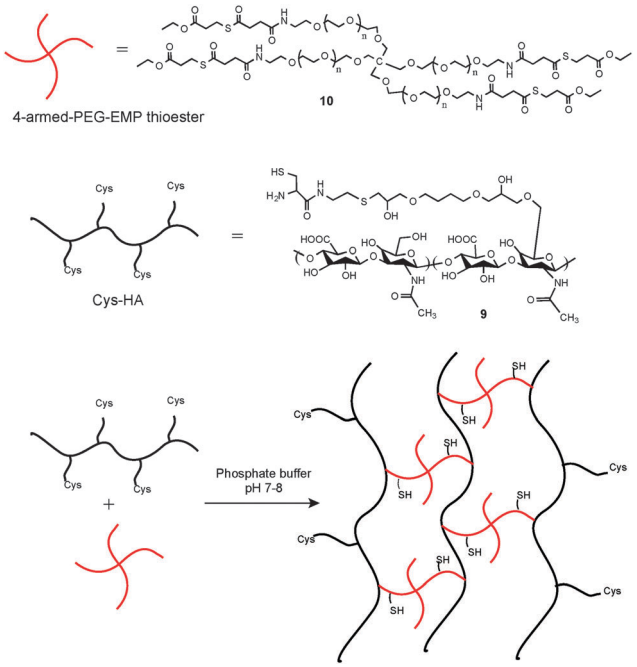
Measurement of the thiol content and the degree of modification (DS, HA disaccharide unit %), indicated that the DS was up to 3.3%. The modification sites on HA were proposed to be 6-position as illustrated, based on the hydroxyl group at 6-position being a primary alcohol and much less sterically and spatially hindered than the secondary alcohols at the 2- and 3- positions.[25] The molecular weight (M_w) of Cys–HA conjugate **9** was determined by gel permeation chromatography (GPC) to be 132 kDa (ESI†), indicating that the molecular weight was only minimally reduced compared to the HA starting material (143 kDa) during the chemical modifications shown in Scheme 1.

To confirm the functionality of modified Cys–HA conjugate **9** and its potential application for *in situ* hydrogel formation, we explored preparation of hydrogels of **9** cross-linked by native chemical ligation (NCL),[21a] and Michael addition[26] under mild conditions using this HA conjugate. In the case of NCL, a thioester can react with an N-terminal-Cys to form a new amide bond under mild conditions (Scheme 2).[27] Advantages of NCL include chemoselectivity (only an N-terminal-Cys is reactive), high efficiency, mild aqueous reaction conditions, and regeneration of free thiol side chain of the N-terminal Cys. NCL has been used to form covalently cross-linked hydrogels,[21a] allowing further biofunctionalization of the hydrogel network with bioactive compounds for tailoring the biological properties of *in situ* formed hydrogels.[21d]

We found that mixing aqueous solutions of Cys–HA conjugate **9** and poly(ethylene glycol) (PEG) thioester **10** resulted in hydrogel formation (Scheme 3). Gel formation time could be significantly affected by buffer pH, conjugate concentration, polymer architecture, reactant stoichiometry, and reaction temperature. Control experiments with 20 mM tricarboxyethylphosphine (TCEP) in water to reduce intermolecular disulfide bonds, and with 100 mM 2-mercaptoethanol (MCE) to cleave the disulfide bonds and thioester bond by thiol exchange in



Scheme 2 Chemistry of native chemical ligation (NCL).



Scheme 3 The hydrogel formed by Cys–HA conjugate **9** and 4-arm-PEG-EMP thioester **10** by native chemical ligation.

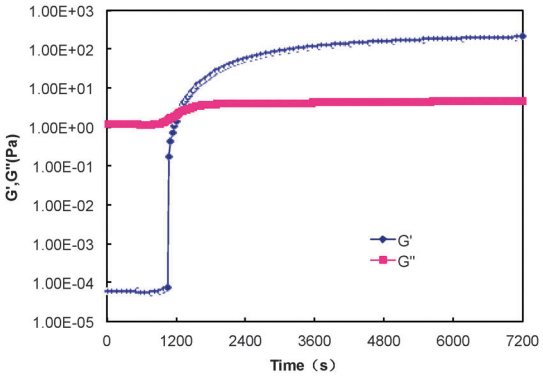


Fig. 1 Oscillatory rheology of the hydrogel formed by native chemical ligation of Cys–HA conjugate **9** (5% w/v) and 4-armed PEG-EMP thioester **10** (0.5% w/v) in phosphate buffer solution (pH 7.5) at 25 °C with the molar ratio of cysteine and thioester 2 : 1. Storage (G') and storage (G'') modulus *vs.* time are shown.

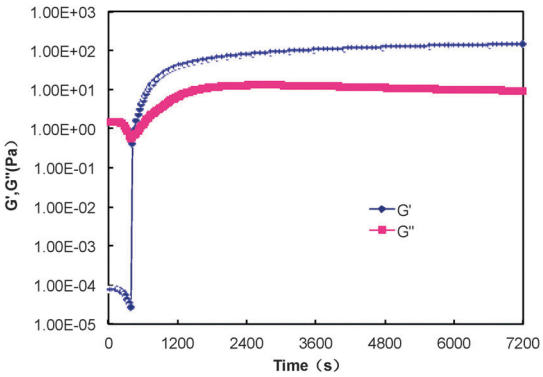


Fig. 2 Oscillatory rheology of the hydrogel formed by Michael addition of Cys–HA conjugate **9** (5% w/v) and 4-armed PEG-ACLT conjugate (0.5% w/v) in phosphate buffer solution (pH 7.5) at 25 °C with the molar ratio of cysteine and acrylate 2 : 1. Storage (G') and storage (G'') modulus *vs.* time are shown.

water, were performed to confirm the formation of amide cross-linked HA–PEG hydrogels. The persistence of hydrogels formed from Cys–HA conjugate **9** and PEG thioester **10** in the presence of TCEP and MCE confirmed that the gels formed mainly by cross-linking through NCL rather than intermolecular disulfide of Cys–HA conjugate, or thioester exchange.

Furthermore, the hydrogel formation process and viscoelastic behavior of the hydrogels formed by NCL of Cys–HA conjugate **9** and PEG thioester **10**, were investigated by oscillatory rheology using optimized conditions for measurement of a G'/G'' crossover point.[28] The time-dependent changes in storage modulus (G') and loss modulus (G'') for the hydrogel composition tested were characteristic of elastic hydrogel formation (Fig. 1), as indicated by a low initial G' ($G' < G''$), and a G'/G'' crossover point representing a theoretical gelling point, followed by rapid increase in G' to a plateau value. Subsequent frequency and strain sweep experiments conducted after G' reached a plateau indicated the storage modulus was frequency and strain independent as expected for a covalently cross-linked hydrogel.[29]

To illustrate the versatile nature of Cys–HA conjugate **9**, hydrogels cross-linked by Michael addition[26] were prepared under mild conditions by mixing Cys–HA conjugate **9** and 4-armed PEG–acrylate (ACLT) conjugate. It was found that hydrogels formed by Michael addition of Cys–HA conjugate **9** and 4-armed PEG–ACLT conjugate reached the gelling point more rapidly (Fig. 2) compared to those formed by NCL under the same conditions (8 min *vs.* 20 min).

In summary, we developed a new strategy for the functionalization of hyaluronic acid by chemical modification of alcohol groups through a stable ether bond to obtain a cysteine–hyaluronic acid conjugate. The method preserves the free carboxylic acid of the disaccharide repeat unit of HA, therefore facilitating the use of chemically modified HA in future biomedical applications where interactions between HA and biomolecules (*e.g.* CD44) may be important. We demonstrated that the Cys–HA conjugate is reactive with the polymers containing thiol-reactive groups and can be used to prepare hydrogels cross-linked by native chemical ligation and Michael addition under mild conditions, respectively. Injectable and *in situ* formed HA hydrogels could be useful in tissue engineering, tissue repair, and drug delivery in a minimally invasive way. The modified HA conjugate could be used for further bio-functionalization with bioactive molecules and engineering of bioactive surfaces through the thiol and amino groups. The method of HA modification reported here may also be applicable to the modification of other natural polysaccharides.

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