The self-crosslinking smart hyaluronic acid hydrogels as injectable three-dimensional scaffolds for cells culture

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Abstract

Although the disulfide bond crosslinked hyaluronic acid hydrogels have been reported by many research groups, the major researches were focused on effectively forming hydrogels. However, few researchers paid attention to the potential significance of controlling the hydrogel formation and degradation, improving biocompatibility, reducing the toxicity of exogenous and providing convenience to the clinical operations later on. In this research, the novel controllable self-crosslinking smart hydrogels with in-situ gelation property was prepared by a single component, the thiolated hyaluronic acid derivative (HA-SH), and applied as a three-dimensional scaffold to mimic native extracellular matrix (ECM) for the culture of fibroblasts cells (L929) and chondrocytes. A series of HA-SH hydrogels were prepared depending on different degrees of thiol substitution (ranging from 10% to 60%) and molecule weights of HA (0.1M Da, 0.3M Da and 1.0M Da). The gelation time, swelling property and smart degradation behavior of HA-SH hydrogel were evaluated. The results showed that the gelation and degradation time of hydrogels could be controlled by adjusting the component of HA-SH polymers. The storage modulus of HA-SH hydrogels obtained by dynamic modulus analysis (DMA) could be up to 44.6 kPa. In addition, HA-SH hydrogels were investigated as a three-dimensional scaffold for the culture of fibroblasts cells (L929) and chondrocytes cells *in vitro* and as an injectable hydrogel for delivering chondrocytes cells *in vivo*. These results illustrated that HA-SH hydrogels with controllable gelation process, intelligent degradation behavior, excellent biocompatibility and convenient operational characteristics supplied potential clinical application capacity for tissue engineering and regenerative medicine.

Keywords: hydrogel, controllable, self-crosslinking, thiolated hyaluronic acid, three-dimensional scaffold

1. Introduction

Hyaluronic acid (HA), or hyaluronan, is a kind of glycosaminoglycan (GAG) with repeating disaccharide units (α-1, 4-D-glucuronic acid and *β*-1, 3-*N*-acetyl-D-glucosamine) [1-4]. HA plays an essential role in the composition and structure of the extracellular matrix (ECM), which is mainly composed of GAGs and collagens through covalent and non-covalent interactions [5-7], could regulate cells adhesion, migration and morphogenesis, adjust cells proliferation and differentiation [8, 9] and has important effects on the development, organization or remodeling of tissue, angiogenesis, modulation of inflammation and wound healing [10-12]. Because of its unique features, HA has been widely investigated and applied as biomaterials for drug and protein delivery [13-15], three-dimensional (3D) scaffold for cells culture [16-18], cartilage and bone regeneration [12, 19, 20], wound healing [21, 22], bio-printing [23, 24], and so on. However, as a natural polymer, rapid degradation *in vivo* and poor biomechanical properties limited its biomedical applications to some extent [2, 12]. Furthermore, HA-based biomaterials are reported to inhibit cell attachment due to its hydrophilic and polyanionic nature, since cells were more willing to selective adhereion to neutral, hydrophobic or polycationic surfaces of materials [6, 9, 25].

Hydrogels are desirable biomaterials for tissue engineering because of high water content, good mechanical property, promising biocompatibility and high permeability to oxygen, nutrients and other water-soluble metabolites [26, 27]. For obtaining appropriate mechanical properties and stable metabolic characterizations, a variety of chemical crosslinking methods and crosslinking agents have been developed to produce amphiphilic and gel-like HA materials by forming covalent networks [1], such as photo polymerization [28], Michael-type addition reaction [29], Schiff-base reaction [30], thiol-ene reaction [31], oxidizing reaction of tyramine [32, 33] and other reactive groups [34, 35]. Although these methods could effectively prepare HA derivatives hydrogels for application in biomedicine field, it still remained some problems for researchers to dissolve, such as the exogenous cytotoxicity derived from the additive initiator, crosslinking agents or byproducts and poor gelation efficiency due to use of light, radiation to initiate the crosslinking reaction. Moreover, uncontrollable gelation process and lack

of responses to stimuli resulted in many limitations for reasonable clinical applications [36, 37].

In recent years, disulfide bond crosslinked hydrogel has attracted many researchers' interests because of its simple synthesis protocol, convenient gelation method, in-situ gelation property, stimulating response to reductant and low-risk of crosslinking agent and byproduct. Prestwich's groups developed disulfide crosslinked HA–gelatin hydrogel film and sponge, and then co-cultured with cells to investigate the biocompatibility. The results showed that addition of gelatin into the HA-DTPH hydrogel could obviously increase the attachment and spreading of BALB/c 3T3 murine fibroblasts, which was seeded on the surface of the materials. Meanwhile, the subcutaneous implantation of thiolated HA films in rat peritoneal cavity showed that the films were tolerated with modest inflammatory response [38]. Yu et al synthesized thiol-modified chitosan and prepared disulfide bond crosslinked hydrogel with pore size ranging from 5 to 30 μm to simulate the native extracellular matrix (ECM). The co-culture of cells and the hydrogels indicated that disulfide bond crosslinked chitosan hydrogels exhibited good potential for encapsulating cells and delivering protein *in vitro* [39]. Giammona and his coworkers synthesized a thiolated HA derivative, and then obtained a disulfide bond crosslinked hydrogel through the auto-oxidization of free thiol groups. Human derm fibroblasts were encapsulated into the hydrogel. The results suggested that the cells could proliferate effectively, which showed the potential application for tissue engineering [40]. Although these reported disulfide bond crosslinked hydrogels had good biocompatibility and were responsive to reductive condition, HA hydrogels with controllable characters and injectable properties have always been intense requirements as biomaterials applied in different biomedical fields.

In this research, a novel self-crosslinking smart hydrogel with in-situ gelation property was prepared from a single component, thiolated hyaluronic acid derivatives (HA-SH), which was obtained through simple chemically modification of HA. HA-SH hydrogels have excellent biocompatibility because HA was a kind of natural polymer and sulfhydryl compounds were widely distributed in animal tissues [41, 42]. The disulfide bond of HA-SH hydrogels could be dissociated by reductant glutathione (GSH), which could be synthesized in cells and secreted in and out of cells. Disulfide bond

crosslinked HA-SH hydrogels with encapsulated cells could be decomposed via a stepwise cells proliferation process because of the cleavage of the disulfide bond, triggered by GSH secreted in cells. Based on these characteristics, a series of HA-SH hydrogels were prepared by regulating molecule weight of HA, degrees of thiol substitution and gelation concentrations of HA-SH polymer. Their in-situ gelation properties were investigated, the change of volume and quality of the hydrogels in PBS immersion solution were measured, the mechanical property was evaluated by dynamic modulus analysis (DMA) and the smart degradation behavior with the existence of the reductant D, L-dithiothreitol (DTT) was investigated. Furthermore, the hydrogel was applied as a three-dimensional scaffold to mimic native extracellular matrix (ECM) for the culture of fibroblasts cells (L929) and chondrocytes to further appraise the potential application capacity in tissue engineering and regenerative medicine.

2. Materials and methods

2.1 Materials

Hyaluronic acid (HA, sodium salt, Cosmetic grade, Mw = 0.1M Da, 0.3M Da and 1.0M Da) was obtained from Bloomage Freda Biopharm Corporation (Shandong, China). 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDCI, 99%), N-hydroxysuccinimide (NHS, 99%), cysteamine hydrochloride (CSA·HCl, 99%), and dithiothreitol (DTT, 99%) were purchased from Best-reagent Corporation (Chengdu, China). 5, 5′-Dithiobis-(2-nitrobenzoic acid) (DTNB, 99%) was obtained from Aladdin Corporation (Shanghai, China). RPMI-1640, DMEM, α-MEM medium and Phosphate Buffered Saline (PBS, 0.0067M) were purchased from Thermo Fisher Scientific Corporation (USA). Fetal bovine serum (FBS, Gibco, Australia origin) was bought from Life Technologies Corporation (USA).

2.2 Synthesis of thiolated hyaluronic acid (HA-SH)

Thiolated hyaluronic acid derivative (HA-SH) was synthesized through the following process as a representative example. Firstly, HA (400 mg) and NHS (230 mg, 2 mmol) was dissolved thoroughly in 80 mL of deionized water under stirring at room temperature. Then, EDCI (383 mg, 2 mmol) in solid

form was added into the mixture solution and kept reacting for 2h to activate the carboxylic group of HA. Subsequently, CSA·HCl (114 mg, 1 mmol) was dissolved in 10ml of deionized water and added into the mixture solution. The pH value of mixture solution was adjusted to 4.75 by using 1.0 M NaOH or 1.0 M HCl during the reaction process. After stirring the mixture solution for 24h, the solution was transferred to the dialysis tubing and dialyzed exhaustively against dilute HCl solution (pH = 3.5) containing 100 mM NaCl for 48 h. At last, the acidified solution was lyophilized to yield the HA-SH solid. According to the method as described above, nine kinds of HA-SH polymers with different degrees of thiol substitution and molecule weights were synthesized through adjusting molar ratio of EDCI and CSA·HCl (molecular ratio: HA/EDCI/CSA·HCl = 1/2/1, 1/3/2, 1/4/4) and molecule weight of HA (Mw=0.1M Da, 0.3M Da and 1.0M Da). The chemical structures of thiolated HA and HA were determined by ^{1}H NMR (400 MHz, Bruker AMX-400, USA) using D_2O as a solvent.

2.3 The fabrication of HA-SH self-crosslinking hydrogel

HA-SH solution could crosslink automatically to form the hydrogel through the oxidation reaction of free thiol groups to form disulfide bond. For example, HA-SH polymer was dissolved into water at pH 3.5. And then the pH value of the solution was regulated to 7.4 by addition of 1.0 M NaOH. After that, the freshly prepared HA-SH solution was immediately injected into a glass tube before exposured to the air at room temperature for 1h. As a result, the hydrogel was formed by self-crosslinking of free thiol groups. Afterwards, the hydrogel was frozen in liquid nitrogen and immediately lyophilized, and then cross-sections of the hydrogel were further sputter-coated with a layer of gold for observing the morphology and interior structure of HA-SH hydrogel [43] by scanning electron microscopy (SEM, HITACHI S-800, Japan).

2.4 The gelation time of HA-SH self-crosslinking hydrogel

The solution (flowable)-gel (non flowable) transition was determined by a flow test utilizing a test tube inverting method reported by Jeong et al [44]. As mentioned above, freshly prepared HA-SH solution was injected into a test tube at certain temperature, and then was inverted to confirm gel formation. If no fluidity was visually observed in 1 minute when the test tube was inverted, it can be

considered that the hydrogel had formed. For investigating the influence of molecular weight of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da), degree of thiol substitution (ranging from 10% to 60%), polymer concentration (w/v = 1.0%, 2.0% and 3.0%) and gelation temperature (4 and 37 °C) on the gelation time of the hydrogel, a series of HA-SH solutions were prepared and the gelation time was recorded.

2.5 The swelling ratio of HA-SH self-crosslinking hydrogel

The equilibrium swelling experiment was conducted to study the swelling property of HA-SH hydrogel. The disk shaped hydrogel with concentration of 3.0% (w/v) was prepared by the method described above. Briefly, freshly prepared HA-SH solution (pH = 7.4) was immediately covered a cylindrical mould (8.5 mm in diameter and 3.0 mm in height). After exposure to the air for 1h at 37°C, the transparent hydrogel formed. At pre-determined time, HA-SH hydrogel was washed in distilled water, freeze-dried and weighed (Wd). And then the freeze-dried hydrogel was immersed into 10 mL PBS buffer (pH = 7.4) and placed in a constant temperature shaker (ZHWY-2012C, Shanghai Zhicheng, China) to shake at 90 rpm at 37°C. At regular time intervals, 5ml aliquot of PBS was replaced by fresh PBS, and the hydrogel was weighted again (Ws). The diameters of hydrogel were also measured. Hydrogels with different swelling ratio by adjusting molecular weight of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da) and degree of thiol substitution (ranged from 10% to 60%) were investigated. Three hydrogels were measured for every sample in three replicates.

The equilibrium swelling ratio is defined as:

$$\text{Swelling ratio} = (Ws – Wd) / Wd.$$

2.6 The degradation behavior of HA-SH self-crosslinking hydrogel

The disk shaped HA-SH hydrogel with concentration of 3.0% (w/v) was prepared by the method described above. At pre-determined time, the hydrogel was washed in distilled water, freeze-dried and weighed (Wo). Subsequently, the hydrogel was immersed in 10 mL PBS containing different concentrations of DTT (0.1 mM, 1 mM, 10 mM and 100 mM) and placed in a constant temperature shaker at 90 rpm at 37°C. Then the hydrogel was taken out and washed in distilled water, freeze-dried

and weighed again (Wr) at regular time intervals. As a result, the degradation behavior of HA-SH hydrogel was expressed as percentage of weight loss (Wt %) and calculated according to the equation:

$$Wt\ \% = (Wo\text{-}Wr)/Wo \times 100\%.$$

Just as the swelling experiment previously mentioned, this test also used hydrogels with different molecular weights of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da) and the degrees of thiol substitution (ranging from 10% to 60%) to investigate intelligent controllable degradation behavior of HA-SH hydrogel. Three hydrogels were measured for every sample in three replicates.

2.7 Dynamic mechanical analysis (DMA)

The preparation of HA-SH self-crosslinking hydrogel with concentration of 3.0% (w/v) was the same as mentioned above. The storage modulus (G′) and loss modulus (G″) of the hydrogel were measured by dynamic mechanical analyzer (TA Instruments Q800, USA) equipped with a parallel-plate compression clamp, and HA-SH hydrogel was tested in the multi frequency mode and at strain of 1% in the compression mode. Following this way, nine kinds of hydrogels with different molecular weights of HA and degrees of thiol substitution were tested and the variation curves of storage modulus (G′) and tan δ of hydrogels were obtained with the frequency ranging from 0.5 to 10 Hz respectively. These hydrogels were measured for every sample in three replicates.

2.8 Cytotoxicity in vitro

In vitro cytotoxicity of HA-SH polymer was evaluated by MTT assay. In brief, chondrocytes (rabbit), L929 cells and 3T3 cells were separately cultured on a 96-well culture plate (100 μL/well) with concentration of 5×10^4 cells/mL in α-MEM containing 10% FBS and 1% Vc, RPMI-1640 and DMEM containing 10% FBS. Then the three kinds of cells were incubated at 37°C in 5% CO_2 for 24h. Subsequently, HA-SH polymer was sterilized by UV radiation for 12 h and solved in α-MEM, RPMI-1640 or DMEM, and then was diluted to obtain a series of solutions with gradient concentrations (4, 20, 100, 500 and 2500 μg/mL). After that, 100μL of each solution was added into the individual well respectively to replace the original medium and the viability of cells cultured in α-MEM, RPMI-1640 or DMEM was used as control. At last, after culture for 48h, the cells viability was evaluated by MTT

assay and the absorbance at 490 nm was measured by a Multiscan Spectrum (Varioskan Flash, Thermo Fisher Scientific, USA). Ten wells were used for each sample test and replicated three times.

2.9 Cell viability and proliferation in vitro

Firstly, 33 mg HA-SH polymer (Mw = 0.3M Da; Ds = 55.44%) was sterilized by UV radiation and dissolved in 1mL α-MEM medium (w/v = 3.0%) at pH 7.4. Then 100 μL chondrocytes (rabbit) suspension solution containing 5.5×10^6 cells was added to HA-SH polymer solution and mixed well to achieve the mixture solution with concentration of 5×10^6 cells/mL. Subsequently, the freshly prepared mixture solution was immediately injected into a cylindrical mould (8.5mm in diameter and 3.0 mm in length). After exposure to the air at 37°C for 0.5 h, the hydrogel encapsulating living cells formed. At last, the hydrogel was detached from the mould and immersed into α-MEM containing 10% FBS and 1% Vc on 6-well tissue culture plate, and then incubated at 37 C in 5% CO_2 circumstance (MCO-15AC, SANYO, Japan). Similarly, L929 cells were encapsulated in HA-SH hydrogel by this method as described above and the hydrogel was immersed into RPMI-1640 containing 10% FBS.

After 3, 7, 14 and 21 days culture *in vitro*, HA-SH hydrogel containing chondrocytes and L929 cells were observed using inverted phase contrast microscope (IPCM, Leica DMI4000B, Germany). The morphological change and viability of chondrocytes and L929 cells encapsulated in the hydrogel were determined by a staining procedure using fluorescein diacetate (F-DA), and the living cells were observed by a confocal laser scanning microscope (CLSM, Leica TCS SP5, Germany).

Besides, the morphology of chondrocytes and L929 cells encapsulated in HA-SH hydrogel was observed using scanning electron microscope (SEM). After 3, 7, 14 and 21 days culture, HA-SH hydrogel encapsulating living cells was fixed in 2.5% glutaraldehyde for 24 hours at room temperature and dehydrated in ethanol/water solutions in the range of 10%-100% (v/v), and followed by replacement using tertbutyl-alcohol and CO_2 critical dying method [45]. The cross-sections of the hydrogel were further sputter-coated with a layer of gold for SEM observation.

2.10 Biocompatibility studies in vivo

Firstly, HA-SH polymer (Mw = 0.3M Da, Ds = 55.44%, 3.0% (w/v)) was sterilized by UV radiation

for 12 h and dissolved in α-MEM medium to prepare HA-SH solution. Then HA-SH hydrogel was prepared by the method as described above. Subsequently, the hydrogel (0.1mL) without cells (as control) or containing chondrocytes (mice, 5×10^5 and 2.5×10^6 cells, respectively) was subcutaneously injected into BALB/c mice back to investigate the proliferation of chondrocytes *in vivo*, and then the hydrogel encapsulated with chondrocyte was cultured for 4 weeks. In addition, one day after injection, the mice were euthanized and the hydrogel was taken out to observe the morphology, and then the animals were euthanized and implanted hydrogel were harvested and observed by CLSM at 1 and 4 weeks after injection. BALB/c mice in this research were provided by the Experimental Animal Center of Sichuan University, raised in housing of laboratory with a constant temperature of 20-22℃, relative humidity of 50-60% and 12 h light-dark cycles, and fed with water and commercial rat pellet diet. All animal experiments were permitted by the Sichuan Provincial Committee for Experimental Animal Management.

3. Results and discussion

3.1 The preparation of thiolated hyaluronic acid (HA-SH)

A desired synthesis protocol of the thiol-modified hyaluronic acid was shown in Fig. 1A. At first, the carboxylic group of HA was reacted with NHS to prepare HA-NHS active ester using EDCI as condensing agent [46]. Then, HA-NHS was reacted with CSA•HCl to synthesize HA-SH polymer. Subsequently, for obtaining pure product, HA-SH raw product was dialyzed exhaustively against dilute HCl aqueous (pH=3.5) which prevented the thiol group from reacting with each other to form the disulfide bond. At last, the solution was lyophilized to get the solid thiolated HA (HA-SH). In this reaction, the amidation reaction between HA-COOH and CSA-NH_2 was strongly affected by feeding molar ratio of HA, EDCI and CSA•HCl. As shown in Table. 1, nine kinds of HA-SH polymers with different degrees of thiol substitution and molecule weights were synthesized through adjusting molar ratio of HA, EDCI and CSA•HCl (molar ratio: HA/EDCI/CSA•HCl = 1/2/1, 1/3/2, 1/4/4) and molecule weight of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da). The degree of thiol substitution ranged from

11.28% to 60.56%, which was measured by a modified Ellman method [47]. Following this method, DTNB reacted with the free thiol group and the product had characteristic absorption peak at 412 nm with UV test, which could be used for the quantitative determination of the free thiol group. The results revealed that the degree of thiol substitution went up with the increasing molar ratio of EDCI and CSA•HCl, and the higher the molecule weight of HA was, the lower the degree of thiol substitution was. The possible reason was that the high viscosity for high molecular weight HA would result in lower liquidity, which might decrease the opportunity for HA and CSA to touch with each other and cause lower reaction activity. The ^{1}H NMR spectrum was used to confirm the existence of the conjugated thiol groups and the typical spectra of native HA and thiolated HA were shown in Fig. 1B. Compared with the spectrum of native HA, it is obviously seen that new resonant peaks of HA-SH appeared at 2.45 ppm and 2.63 ppm, which represented the methylene protons of the -CH_2CH$_2$SH and -CH$_2$CH_2SH in spectrum of HA-SH polymer, respectively.

3.2 The preparation, characterization and biocompatibility of HA-SH self-crosslinking hydrogel

HA-SH hydrogel was acquired by self-crosslink of HA-SH polymer in neutral or basic condition and kept stable morphological characterizations. In the gelation process, the self-crosslinking derived from the oxidation reaction of two thiol groups to form disulfide bond with the existence of oxygen. In this process, no chemical additives, cytotoxic crosslinking agents and byproducts were introduced or produced, which was conducive to keep good biocompatibility. In this research, it was demonstrated that regulating the degree of thiol substitution, molecule weight, concentration of the solution and gelation temperature would controlled the gelation time of HA-SH hydrogel. As shown in Table. 2, when the gelation temperature was defined at 37°C, the gelation time of HA-SH hydrogel with different molecular weights and thiol contents could be controlled to range from 5 minutes to 80 minutes. Meanwhile, the gelation time was extended for more than 2 hours at 4°C. Furthermore, it was worthy to note that the higher molecular weight of HA, degree of thiol substitution and concentration of the solution were, the shorter the gelation time was. As a result, the suitable gelation time for co-culture with cells could be obtained.

HA-SH polymer solutions (Mw = 0.3M Da, Ds = 55.44%) with lower concentration were co-cultured with 3T3 cells, L929 cells and chondrocytes, respectively, to investigate the cytotoxicity through MTT test method (Fig. 1C). At these very low concentrations (from 4 μg/mL to 2500 μg/mL), HA-SH polymer existed in liquid-state rather than gel-state because very low crosslinking density of thiol groups was not enough to form a stable gel structure. In this way, the cells could contact with HA-SH polymer adequately and the results could effectively display the cytotoxicity of the material. From these results, it can be concluded that the viability of the three kinds of cells were all more than 90% when co-cultured with different concentrations of HA-SH polymer for 48h, which revealed that this material had desirable biocompatibility.

HA-SH hydrogel was flash-frozen in liquid nitrogen prior to freeze drying in order to better preserve the morphology and microstructure. The SEM pictures of the hydrogel presented the interconnected porous structure and the diameter of the pore was about 35 μm as shown in Fig. 1D. Pore size, an important parameter of 3-D porous scaffolds, had great influence on exchange of substance inside and outside scaffolds and tensile force of scaffold skeletons, which affected cells spreading and adjusted cells physiology [48]. Scaffolds with small pore size were benefit of maintaining cells morphology and secretion of extracellular matrix [49]. The SEM pictures indicated that the structure and the diameter of pores were mainly associated with the concentration of HA-SH solution, while had only slight correlation with the molecular weight of HA and degree of thiol substitution.

3.3 The controllable swelling ratio of HA-SH self-crosslinking hydrogel

The equilibrium swelling ratio played an important role in maintaining defined structure of hydrogel and retaining sufficient amount of water. The swelling results were shown in Fig. 2A (the diamater change) and Fig. 2B (the swelling ratio). From the change in diameter of HA-SH hydrogel, it was obviously seen that the diameter was closely relative to the thiol content of HA-SH polymer with certain concentration. Moreover, there was not significant change in size for the hydrogel with high degree of thiol substitution. The high degree of thiol substitution meant high cross-linking density,

which strengthen the stability of the hydrogel. Relatively, the lower the degree of thiol substitution was, the more obvious increase tendency of HA-SH hydrogel diameter was. When the degree of thiol substitution was more than 50%, the diameter of the hydrogel kept almost constant as time going on to 28 days, while the hydrogel with low thiol substitution (less than 15%) quickly swelled in 14 days. These results indicated that the higher degree of thiol substitution was conducive to obtain the desirable stability of HA-SH hydrogel.

In addition, the swelling ratio was also essential to investigate the stabilization of the hydrogel. Fig. 2B showed the change in swelling ratio of HA-SH hydrogel. It could be concluded that all of HA-SH hydrogels reached the swelling equilibrium within 25 minutes, which was indicative for the high exchange speed of the water-soluble substance. Meanwhile, the higher the molecule weight of HA and degree of thiol substitution were, the shorter the time for reaching the swelling equilibrium was, and the smaller the swelling ratio was. These results were similar with the previous conclusion about the change in diameter of HA-SH hydrogel. From the above description, it was obviously that increasing the degree of thiol substitution would effectively maintain the structure and improve the stability of the hydrogel.

3.4 The intelligent degradation behavior of HA-SH self-crosslinking hydrogel in vitro

As shown in Fig. 1E, F and G, HA-SH polymer kept the liquid state when dissolved in water, and then formed the hydrogel by adjusting the value of pH to 7.4 with the addition of NaOH. On the other hand, the hydrogel decomposed to solution by addition of DTT. To confirm the intelligent degradation behavior of HA-SH hydrogel via reversible disulfide linkage, the hydrogel was immersed in PBS with different concentrations of DTT (0.1mM, 1 mM, 10 mM and 100 mM) at 37℃. The correlative results were shown in Fig. 3. When the concentration of DTT was 0.1 mM, the weight loss of the hydrogel was lower than 5% in 4 hours. While increasing the concentration of DTT to 1 mM, the weight loss increased to above 67% and even reached 100% with the degree of thiol substitution less than 15% in 4 hours. Moreover, when the concentration of DTT was increased to 10 mM, the hydrogel was fully degraded within 25 minutes with the degree of thiol substitution being less than 40%, while the weight

loss was also achieved to above 75% in 40 minutes despite that the degree of thiol substitution was more than 50%. Furthermore, the hydrogel decomposed to solution exhaustively within 5 minutes with 100 mM DTT (the data was not shown). The principle of the intelligent reductant-responsive degradation behavior of HA-SH hydrogel resulted from the self-crosslinking of free thiol groups to form disulfide bond with the existence of oxygen and the decomposition of disulfide bond to free thiol groups with the existence of reductant. From these results, it was clear that the lower degree of thiol substitution and the higher concentration of the reductant DTT were, the higher degradation speed of the hydrogel was. In addition, the molecular weight of HA had no prominent influence on the degradation behavior of HA-SH hydrogel.

On the other hand, bioengineered proteolytically-degradable hydrogels with protease-sensitive substrates could locally degrade in response to the presence of cell-derived proteases and degraded gradually with the proliferation of cells, representing a step forward compared to other biodegradation mechanisms [50, 51]. Disulfide bond cross-linked hydrogels had similar degradation strategy with the protease-sensitive hydrogels because the disulfide bond was reductant-sensitive and decomposed by cell-derived GSH, which was a kind of reductant [41, 42]. As a result, when co-cultured with cells, the hydrogel could be degraded gradually by GSH produced by cells and provided enough space for cells proliferation.

3.5 *Dynamic mechanical analysis (DMA)*

As a powerful technique, dynamic mechanical analysis (DMA) was widely applied in material science to detect viscoelasticity [52, 53]. In recent years, the measurement of DMA has become indispensable in the research of tissue engineering scaffold materials [54, 55]. Frequency sweep dynamic rheology data of HA-SH hydrogel were shown in Fig. 4. The storage modulus (G′) and loss modulus (G″) of the hydrogel represented the energy stored or dissipated per unit strain respectively. As shown in Fig. 4A, with the same molecular weight of HA, the higher the degree of thiol substitution was, the bigger the storage modulus (G′) of HA-SH hydrogel was. Meanwhile, with the same degree of thiol substitution, the higher the molecular weight of HA was, the bigger the storage modulus (G′) was.

Remarkably, the storage modulus (G′) increased with the increasing frequency. For example, the storage modulus (G′) of HA-SH hydrogel (Mw = 1.0M Da, Ds = 51.47%, 3% (w/v)) was 38.75 kPa under 0.5 Hz. When raising the frequency to 10 Hz, the storage modulus (G′) increased to 42.85 kPa, which increased more than 10% compared with the initial value at 0.5 Hz. Moreover, the storage modulus of HA-SH hydrogel (Mw = 0.3M Da, Ds = 55.44%, 3% (w/v)) at frequency 10 Hz increased more than 16% compared with that at frequency of 0.5 Hz.

On the other hand, as an indication of the dissipated energy (G″) by the viscous mechanism relative to the stored energy (G′), the tan δ played an important role in the long-term degradation of mechanical stability, which was shown in Fig. 4C. Because of the stored energy rising along with the increase of the molecule weight and thiol substitution, HA-SH hydrogel with higher molecule weight and degree of thiol substitution had higher storage modulus (G′), which generated big value of tan δ. Interestingly, with the frequency rising from 0.5 Hz to 10 Hz, the value of tan δ expressed significant decreasing tendency. The reduced value of tan δ at 10 Hz was more than 20% and 40% compared with the initial value of 0.5 Hz respectively, for the representative HA-SH hydrogel (Mw = 1.0M Da, Ds = 51.47%, 3% (w/v) and Mw = 0.3M Da, Ds = 55.44%, 3% (w/v)). Combined with the results shown in Fig. 4A and B, it can be concluded that the dissipated energy (G″) decreased significantly when the stored energy (G′) apparently increased and the speed of shift of the former was obviously faster than that of the latter with the frequency rising from 0.5 Hz to 10 Hz. The variation trends indicated that HA-SH hydrogel could actively increase the storage modulus (G′) and decrease loss modulus (G″) when received higher frequency shock. These results implied that higher degree of thiol substitution and molecular weight would strengthen the mechanical property of the hydrogel and the mechanical strength of the hydrogel could be controlled by regulating the molecular weight of HA and the degree of thiol substitution. In addition, these features were beneficial for HA-SH hydrogel to apply as the scaffold material for bone or cartilage tissue engineering because of its effective energy storage (G′) self-enhancement and the dissipated energy (G″) self-reduction characterizations with the increasing frequency [56].

3.6 *Cell viability and proliferation in vitro*

HA-SH polymer had good biocompatibility based on its natural material source and non-exogenous additive composition. In order to further investigate its application as a scaffold material, the HA-SH hydrogel (Mw = 0.3M Da, Ds = 55.44%, 3% (w/v)) was chosen as a representative sample because of its suitable gelation time about 11 minutes, desirable swelling character and appropriate mechanical and degradation properties, to encapsulate chondrocytes and L929 cells, respectively, and cultured at 37°C in incubator with 5% CO_2 to study the cells viability and proliferation. After culture for 3, 7, 14, 21 days, the hydrogels were observed by IPCM, CLSM and SEM to explore the physiological morphology of the cells, and the results were summarized in Fig. 5.

The CLSM pictures indicated that the cells encapsulated into HA-SH hydrogel could proliferate normally along with the increase of culture days *in vitro*. After 7 days of culture, the pairs of cells can be observed and the morphology of cell clusters, which composed by tens of cells, appeared after 14 days of culture for both of chondrocytes and L929 cells. Previous research has shown that the cells which were seeded on the surface of materials with unfavorable cell attachment would migrate and aggregate to form spherical clusters [57]. HA-based biomaterials inhibited cell attachment because of hydrophilic and polyanionic surfaces of hyaluronic acid [6, 9, 25]. As a result, the clusters of chondrocytes and L929 cells encapsulated in HA-SH hydrogels were observed in this research. Moreover, it could be observed in Fig. 5 A2-A4, B2-B4, C2-C4 and D2-D4 that the number and size of cells clusters increased with the incubation time prolonged. The result might indicate that HA-SH hydrogels could effectively promoted the encapsulated cells aggregation and proliferation.

In addition, it could also be observed in Fig. 5 E2-E4, F2-F4, G2-G4 and H2-H4 that the amount of cells increased as the culture time went on. For chondrocytes, the cells contacted with each other closely with ECM spreading on the surfaces of the cells after culture for 14 days and 21 days, but for L929 cells, the cell clusters were surrounded by the membrane composed of ECM after culture for 14 days and 21 days. The phenomenon of matrix secretion showed that chondrocytes and L929 cells still maintained the normal physiological characterizations. The IPCM pictures showed in Fig. 5A1, E1, B1,

F1, C1, G1 and D1, H1 indicated the same phenomena as described above.

3.7 Biocompatibility studies in vivo

Compared with traditional hydrogels, the in-situ forming hydrogels or injectable hydrogels had received more and more attentions as desirable biomaterials for clinical biomedical applications because they could reduce trauma and surgery costs [58, 59]. So in-situ gelation property of HA-SH self-crosslinking hydrogel made it became a desirable material for application in tissue engineering and regenerative medicine. In this research, the gelation time of HA-SH hydrogel (Mw = 0.3M Da, Ds = 55.44%, 3% (w/v)) could be controlled from few minutes to 2 hours depending on the gelation temperature ranging from 37°C to 4°C, which meant that there was enough time to mix the cells with HA-SH solution well at 4°C and the hydrogel via subcutaneous injection could be formed within few minutes at the body temperature of 37°C. Moreover, the viscosity of HA-SH solution with pH = 7.4 at 4°C made it possible to inject the hydrogel into the mice by injection syringe, which could reduce the surgical trauma and infection significantly. As shown in Fig. 6F1-F3, the stable hydrogel formed at 1 day after injection. Furthermore, the hydrogel still existed and unobvious degradation was observed after 28 days since injection, which indicated the desirable stability and mild degradation of HA-SH hydrogel *in vivo*.

In addition, at 1 and 4 weeks after injection, HA-SH hydrogel encapsulating chondrocytes was taken out and stained using fluorescein diacetate (FDA) to observe the living cells by CLSM. As shown in Fig. 6A1-E1 and A2-E2, a large number of living cells could be observed, and the density of the cells increased significantly after 4 weeks of culture (Fig .6C1, C2 and D1, D2) compared with 7 days of culture (Fig. 6A1, A2, B1 and B2), which illustrated the proliferation of the cells and the good biocompatibility of the hydrogel. While only sparse in-growth of cells was observed in HA-SH hydrogel containing no cells (Fig. 6E1, E2). Interestingly, when the density of chondrocytes was 5×10^6 cells/mL, the polygonous morphology of chondrocytes (Fig. 6A1, A2) at 1 week after injection changed to the circular morphology at 4 weeks (Fig. 6C1, C2) after injection. On the other hand, the morphology of chondrocytes with density of 2.5×10^7 cells/mL maintained roundness during 4 weeks

(Fig. 6B1, B2 and D1, D2) culture *in vivo*. The observation showed that the density of cells had some influence on the cytological behavior of chondrocytes *in vivo*. The possible reason was that the increasing density of chondrocytes resulted in increasing interaction among the newly-formed cell clusters and decreasing direct contact with HA-SH hydrogel in the process of the cells migration and proliferation, while higher number of cells meant higher quantity of excreted ECM and GSH which might affect the cells behavior and degradation of HA-SH hydrogel, respectively. The phenomena described above had great difference with morphology of chondrocytes cultured *in vitro*, which might be resulted from the difference of degradation behavior of HA-SH hydrogel and microenvironment surrounding in the cells between *in vivo* and *vitro*. In conclusion, the results indicated that HA-SH hydrogel was conducive to facilitate the encapsulated chondrocytes proliferation based on its superior biocompatibility and biodegradation, which, to some extent, might benefit from the self-crosslinking character and intelligent degradation behavior.

4、Conclusion

A disulfide crosslinked HA-based hydrogel was designed as a three-dimensional scaffold for biomedical applications. HA-SH hydrogel was obtained by a single component, thiolated hyaluronic acid (HA-SH), and formed via the self-crosslinking process of disulfide bond derived from two free thiol groups. In the process of preparation, no chemical additives, cytotoxic crosslinking agent and byproduct were added or produced. HA-SH polymer could be synthesized easily and the degree of thiol substitution could be controlled. The gelation time, swelling property, mechanical property and degradation behavior of the hydrogel was also controllable. HA-SH hydrogel showed good biocompatibility when co-cultured with cells. The proliferation of cells and secretion of extracellular matrix (ECM) were obvious during culture for 21 days *in vitro*. Large amounts of living cells were also observed and the density of living cells increased significantly during culture for 28 days *in vivo*. In conclusion, this disulfide bond crosslinking method was a desirable way to prepare self-crosslinking HA hydrogels with in-situ forming property, controllable characterizations, intelligent degradation

behavior and promising biocompatibility. It has great potential to meet the requirement of biomaterials applied in different fields of biomedicine.

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Captions

Figure 1

(A) Synthesis protocol of thiol modified hyaluronic acid (HA-SH). (B) 1H-NMR (D2O) spectra of HA and HA-SH (Mw = 0.1M Da, Ds = 37.47%). (C) Biocompatibility of HA-SH polymers (Mw = 0.3M Da, Ds = 55.44%) for chondrocytes, 3T3 cells and L929 cells after 48h of co-culture (n=3). (D) The SEM micrograph of HA-SH hydrogel (Mw = 0.3M Da, Ds = 55.44%, 3.0% (w/v)). (E) Picture of HA-SH solution (Mw = 0.3M Da, Ds = 55.44%, 3.0% (w/v)). (F) Picture of HA-SH hydrogel. (G) Picture of HA-SH solution derived from the decomposition of HA-SH hydrogel with DTT (100 mM).

Figure 2

The diameter (A) and equilibrium swelling ratio (B) change of HA-SH hydrogels with different molecular weights of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da, respectively) and degrees of thiol substitution (Ds ranging from 11.28% to 60.56%) in PBS with pH 7.4 at 37℃ using 3.0% (w/v) solutions of HA-SH polymers (n = 3).

Figure 3

In vitro degradation behavior of HA-SH hydrogels with different molecular weights of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da, respectively) and degrees of thiol substitution (Ds ranging from 11.28% to 60.56%) using 3.0% (w/v) solutions of HA-SH polymers (n = 3) in various PBS medium (pH 7.4, 37℃) containing different concentrations of DTT (0.1mM, 1mM, 10mM).

Figure 4

The mechanical property of HA-SH hydrogels with different molecular weights of HA (Mw = 0.1M Da, 0.3M Da and 1.0M Da, respectively) and degrees of thiol substitution (Ds ranging from 11.28% to 60.56%) using 3.0% (w/v) solutions of HA-SH polymers (n = 3) in various PBS medium (pH 7.4, 37℃). (A) Dependence of storage modulus on the frequency. (B) Dependence of loss modulus on the frequency. (C) Dependence of average tan δ (G''/G') on the frequency.

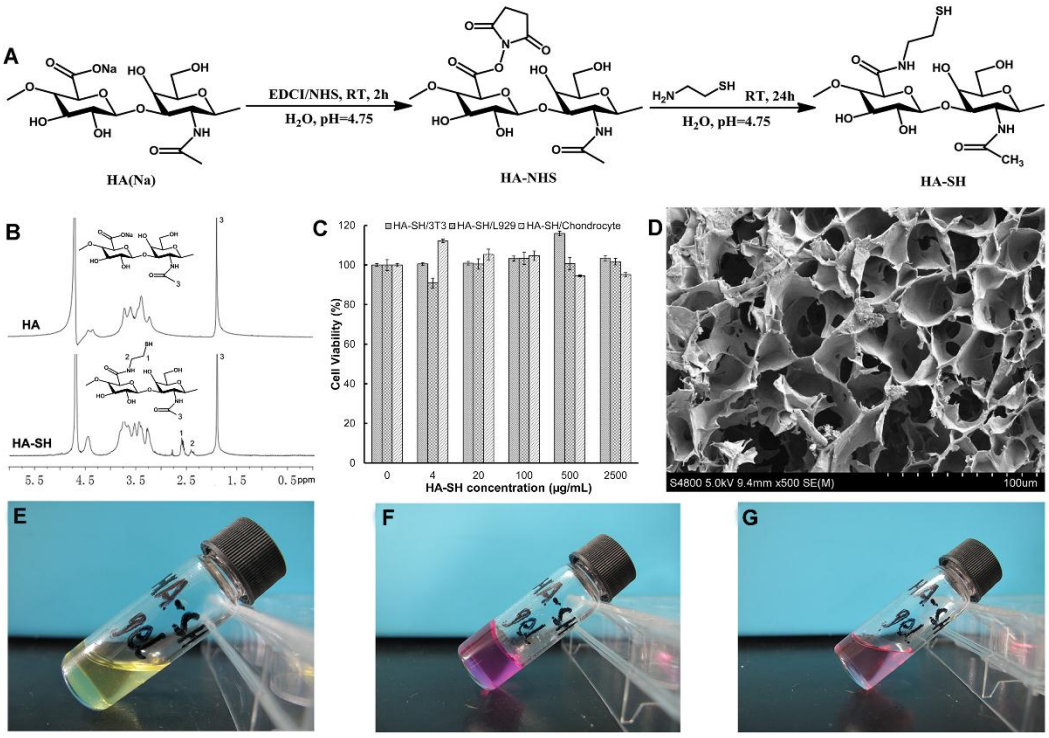
Figure 5

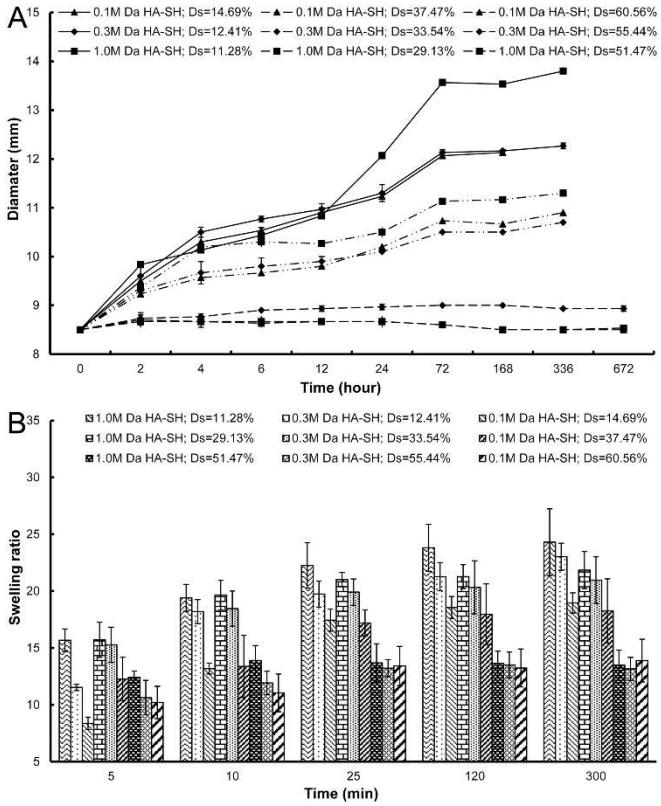
The HA-SH hydrogel (Mw = 0.3M Da, Ds = 55.44%, 3.0% (w/v)) co-cultured with L929 cells (5×106 cells/mL) and chondrocytes (5×106 cells/mL) *in vitro*. The inverted phase contrast micrographs of L929 cells encapsulated in HA-SH hydrogel after co-culture for: (A1) 3 days, (B1) 7 days, (C1) 14 days and (D1) 21 days. The CLSM micrographs of L929 cells encapsulated in HA-SH hydrogel after co-culture for: (A2 and A3) 3 days, (B2 and B3) 7 days, (C2 and C3) 14 days and (D2 and D3) 21 days. The SEM micrographs of L929 cells encapsulated in HA-SH hydrogel after co-culture for: (A4) 3 days, (B4) 7 days, (C4) 14 days and (D4) 21 days. The

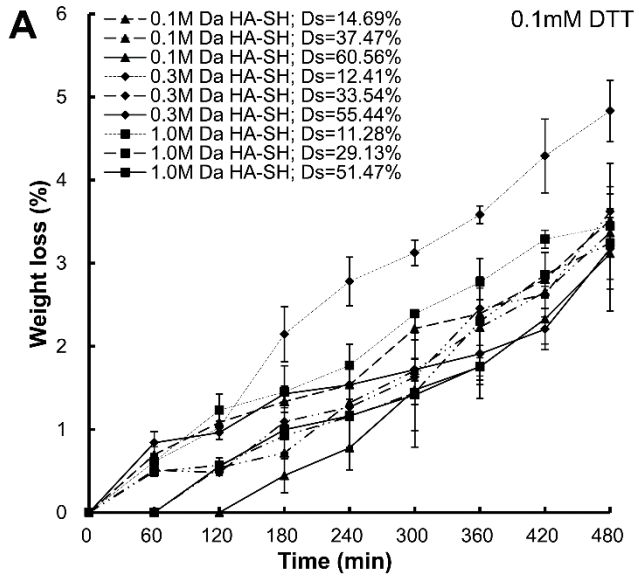
inverted phase contrast micrographs of chondrocytes encapsulated in HA-SH hydrogel after co-culture for: (E1) 3 days, (F1) 7 days, (G1) 14 days and (H1) 21 days. The CLSM micrographs of chondrocytes encapsulated in HA-SH hydrogel after co-culture for: (E2 and E3) 3 days, (F2 and F3) 7 days, (G2 and G3) 14 days and (H2 and H3) 21 days. The SEM micrographs of chondrocytes encapsulated in HA-SH hydrogel after co-culture for: (E4) 3 days, (F4) 7 days, (G4) 14 days and (H4) 21 days. Note: The scale bars were shown in micrographs, respectively.

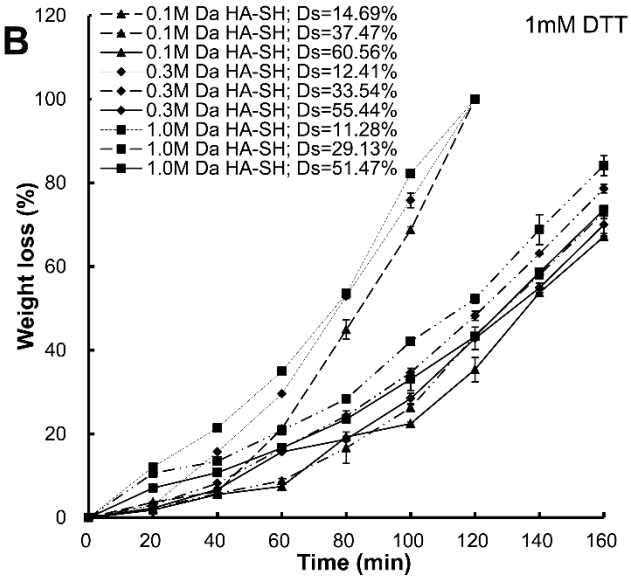
Figure 6

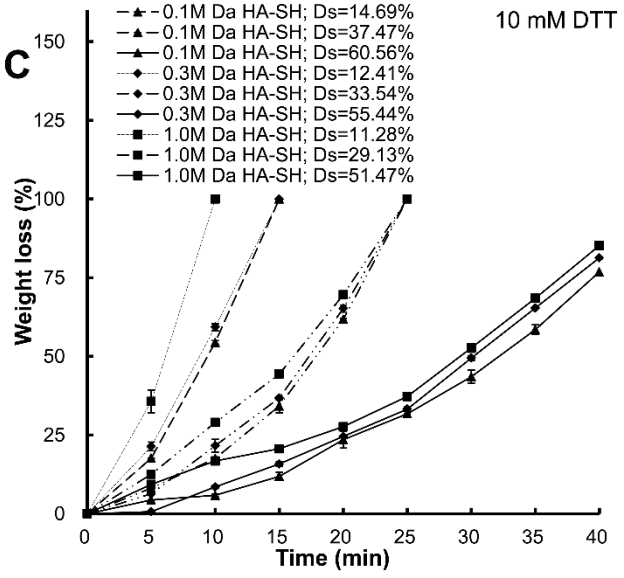
The HA-SH hydrogel (Mw = 0.3M Da, Ds = 55.44%, 3.0% (w/v)) co-cultured with chondrocytes (A1 and C1, 5×106 cells/mL, B1 and D1, 2.5×107 cells/mL, E1 as control, 0 cells/mL) *in vivo*. The co-culture time of chondrocytes encapsulated in HA-SH hydrogel for: 1 week (A1, A2, and B1, B2) and 4 weeks (C1, C2, D1, D2 and E1, E2). The Pictures (F1, F2 and F3) showed the visual morphology of HA-SH hydrogel after one day with subcutaneous injection the HA-SH polymer solution into the back of mice.

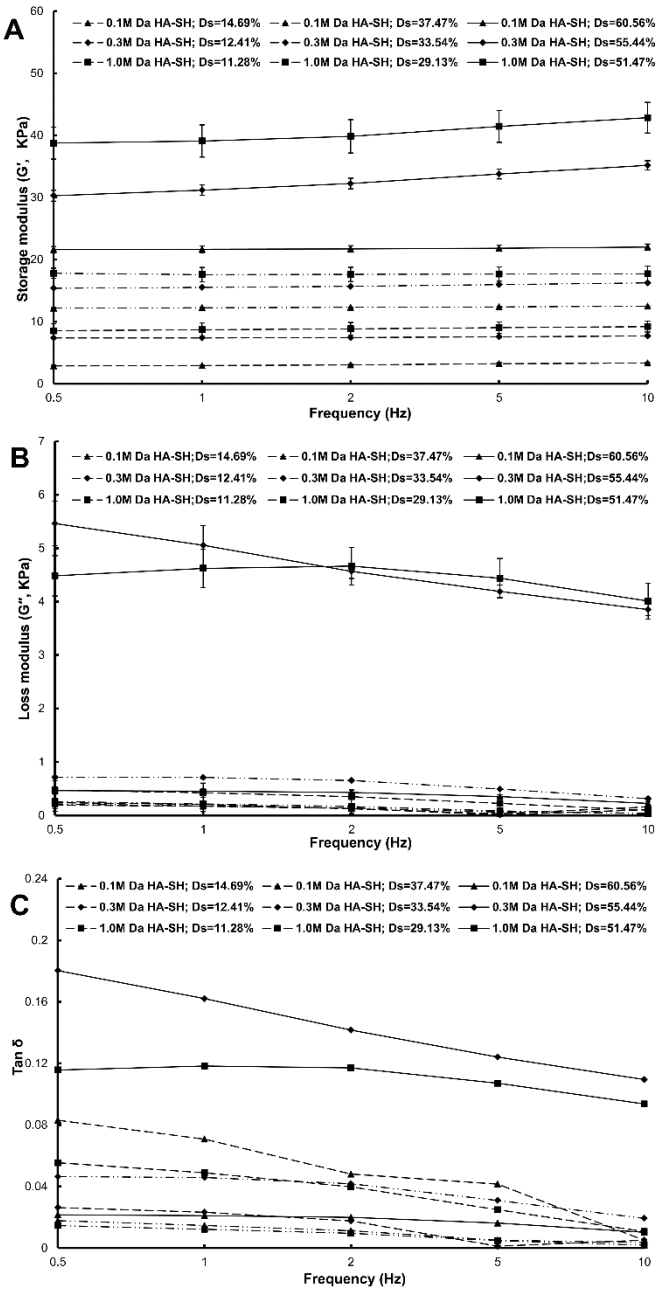

A
EDCI/NHS, RT, 2h
H2O, pH=4.75
RT, 24h
H2O, pH=4.75
HA(Na)
HA-NHS
HA-SH
B
HA
HA-SH
5.5
4.5
3.5
2.5
1.5
0.5ppm
C
HA-SH/3T3
HA-SH/L929
HA-SH/Chondrocyte
Cell Viability (%)
HA-SH concentration (μg/mL)
D
S4800 5.0kV 9.4mm x500 SE(M)
100um
E
F
G

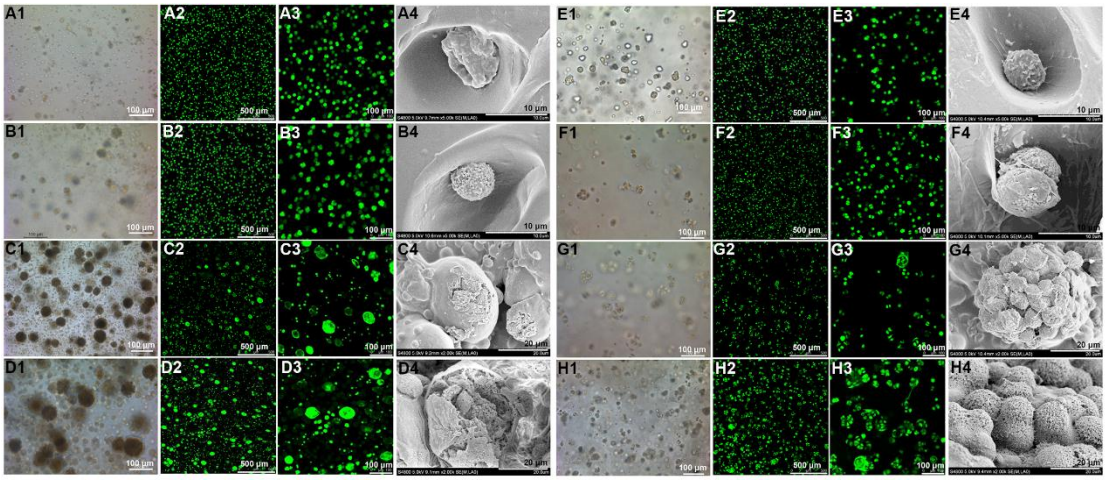

A
0.1M Da HA-SH; Ds=14.69%
0.1M Da HA-SH; Ds=37.47%
0.1M Da HA-SH; Ds=60.56%
0.3M Da HA-SH; Ds=12.41%
0.3M Da HA-SH; Ds=33.54%
0.3M Da HA-SH; Ds=55.44%
1.0M Da HA-SH; Ds=11.28%
1.0M Da HA-SH; Ds=29.13%
1.0M Da HA-SH; Ds=51.47%
Diamater (mm)
Time (hour)
B
1.0M Da HA-SH; Ds=11.28%
0.3M Da HA-SH; Ds=12.41%
0.1M Da HA-SH; Ds=14.69%
1.0M Da HA-SH; Ds=29.13%
0.3M Da HA-SH; Ds=33.54%
0.1M Da HA-SH; Ds=37.47%
1.0M Da HA-SH; Ds=51.47%
0.3M Da HA-SH; Ds=55.44%
0.1M Da HA-SH; Ds=60.56%
Swelling ratio
Time (min)

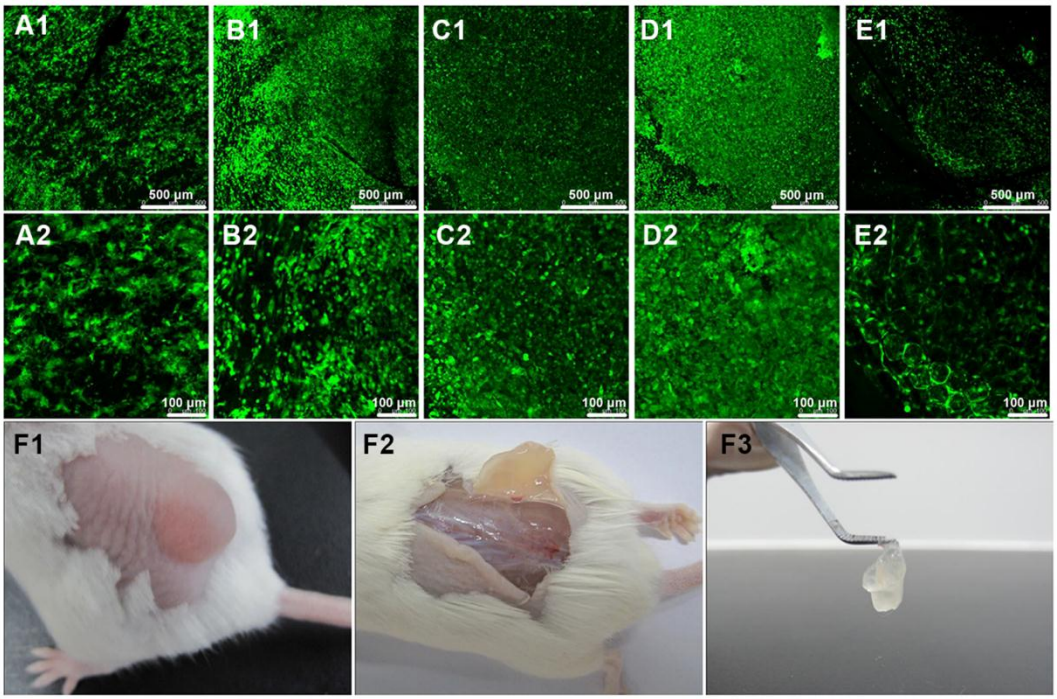

A
0.1mM DTT
0.1M Da HA-SH; Ds=14.69%
0.1M Da HA-SH; Ds=37.47%
0.1M Da HA-SH; Ds=60.56%
0.3M Da HA-SH; Ds=12.41%
0.3M Da HA-SH; Ds=33.54%
0.3M Da HA-SH; Ds=55.44%
1.0M Da HA-SH; Ds=11.28%
1.0M Da HA-SH; Ds=29.13%
1.0M Da HA-SH; Ds=51.47%
Weight loss (%)
Time (min)
0
1
2
3
4
5
6
0
60
120
180
240
300
360
420
480


B
1mM DTT
0.1M Da HA-SH; Ds=14.69%
0.1M Da HA-SH; Ds=37.47%
0.1M Da HA-SH; Ds=60.56%
0.3M Da HA-SH; Ds=12.41%
0.3M Da HA-SH; Ds=33.54%
0.3M Da HA-SH; Ds=55.44%
1.0M Da HA-SH; Ds=11.28%
1.0M Da HA-SH; Ds=29.13%
1.0M Da HA-SH; Ds=51.47%
Weight loss (%)
Time (min)
0
20
40
60
80
100
120
0
20
40
60
80
100
120
140
160


C
10 mM DTT
0.1M Da HA-SH; Ds=14.69%
0.1M Da HA-SH; Ds=37.47%
0.1M Da HA-SH; Ds=60.56%
0.3M Da HA-SH; Ds=12.41%
0.3M Da HA-SH; Ds=33.54%
0.3M Da HA-SH; Ds=55.44%
1.0M Da HA-SH; Ds=11.28%
1.0M Da HA-SH; Ds=29.13%
1.0M Da HA-SH; Ds=51.47%
Weight loss (%)
Time (min)
0
25
50
75
100
125
150
0
5
10
15
20
25
30
35
40


A
0.1M Da HA-SH; Ds=14.69%
0.1M Da HA-SH; Ds=37.47%
0.1M Da HA-SH; Ds=60.56%
0.3M Da HA-SH; Ds=12.41%
0.3M Da HA-SH; Ds=33.54%
0.3M Da HA-SH; Ds=55.44%
1.0M Da HA-SH; Ds=11.28%
1.0M Da HA-SH; Ds=29.13%
1.0M Da HA-SH; Ds=51.47%
Storage modulus (G', KPa)
Frequency (Hz)
B
Loss modulus (G", KPa)
Frequency (Hz)
C
Tan δ
Frequency (Hz)


A1
A2
A3
A4
E1
E2
E3
E4
B1
B2
B3
B4
F1
F2
F3
F4
C1
C2
C3
C4
G1
G2
G3
G4
D1
D2
D3
D4
H1
H2
H3
H4


A1
B1
C1
D1
E1
500 μm
A2
B2
C2
D2
E2
100 μm
F1
F2
F3

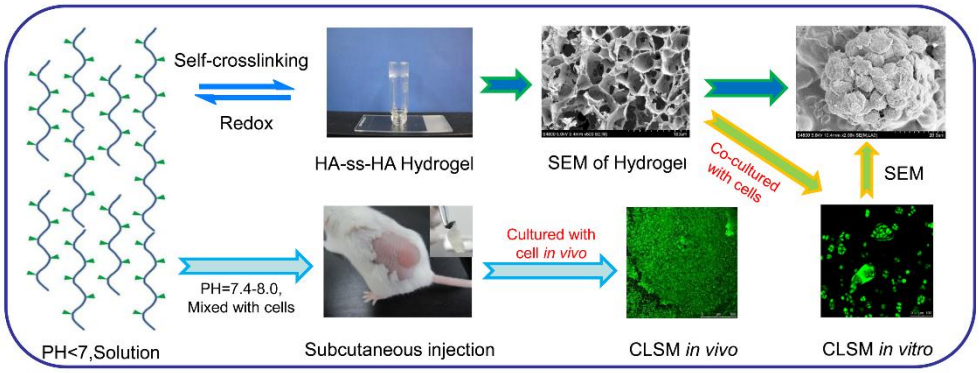

Self-crosslinking
Redox
HA-ss-HA Hydrogel
SEM of Hydrogel
Co-cultured with cells
SEM
PH=7.4-8.0, Mixed with cells
Cultured with cell in vivo
PH<7,Solution
Subcutaneous injection
CLSM in vivo
CLSM in vitro

Table 1 The composition and correlated characterization of thiolated hyaluronic acid (HA-SH) polymer

Molecular weight of HA (Da)	Molar ratio of HA/NHS/EDCI/CSA·HCl	Degree of thiol substitution (%)
0.1 M	1:2:2:1	14.69
	1:2:3:2	37.47
	1:2:4:4	60.56
0.3 M	1:2:2:1	12.41
	1:2:3:2	33.54
	1:2:4:4	55.44
1.0 M	1:2:2:1	11.28
	1:2:3:2	29.13
	1:2:4:4	51.47

Table 2 The auto-crosslinking gelation time of different HA-SH solutions determined by a test tube inversion method.

molecular weight of HA (Da)	degree of thiol substitution (%)	concentration of solution (w/v)	gelation time (minute)
0.1 M	14.69	1%	78
		2%	45
		3%	34
	37.47	1%	62
		2%	41
		3%	28
	60.56	1%	51
		2%	33
		3%	18
0.3 M	12.41	1%	58
		2%	34
		3%	25
	33.54	1%	51
		2%	26
		3%	18
	55.44	1%	39
		2%	20
		3%	11
1.0 M	11.28	1%	53
		2%	31
		3%	24
	29.13	1%	39
		2%	21
		3%	15
	51.47	1%	31
		2%	15
		3%	6