



# Cytocompatible *in situ* forming chitosan/hyaluronan hydrogels via a metal-free click chemistry for soft tissue engineering



Ming Fan, Ye Ma, Jiahui Mao, Ziwei Zhang, Huaping Tan \*

School of Materials Science and Engineering, Nanjing University of Science and Technology, Nanjing 210094, China

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## ABSTRACT

Injectable hydrogels are important cell scaffolding materials for tissue engineering and regenerative medicine. Here, we report a new class of biocompatible and biodegradable polysaccharide hydrogels derived from chitosan and hyaluronan via a metal-free click chemistry, without the addition of copper catalyst. For the metal-free click reaction, chitosan and hyaluronan were modified with oxanorbornadiene (OB) and 11-azido-3,6,9-trioxadecan-1-amine (AA), respectively. The gelation is attributed to the triazole ring formation between OB and azido groups of polysaccharide derivatives. The molecular structures were verified by FT-IR spectroscopy and elemental analysis, giving substitution degrees of 58% and 47% for chitosan-OB and hyaluronan-AA, respectively. The *in vitro* gelation, morphologies, equilibrium swelling, compressive modulus and degradation of the composite hydrogels were examined. The potential of the metal-free hydrogel as a cell scaffold was demonstrated by encapsulation of human adipose-derived stem cells (ASCs) within the gel matrix *in vitro*. Cell culture showed that this metal-free hydrogel could support survival and proliferation of ASCs. A preliminary *in vivo* study demonstrated the usefulness of the hydrogel as an injectable scaffold for adipose tissue engineering. These characteristics provide a potential opportunity to use the metal-free click chemistry in preparation of biocompatible hydrogels for soft tissue engineering applications.

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## 1. Introduction

Soft tissue fillers are needed for soft tissue defects resulting from congenital abnormalities, trauma, or tumor resections. Autografts and lipotransfer have been studied for soft tissue fillers, however, their success is unpredictable, with 20%–90% graft resorption within months [1]. This leaves significant problems for the patient and thus the regeneration of soft tissue defects remains an unmet clinical need. Injectable hydrogels have been clinically desired as delivery systems for soft tissue engineering resulting in minimally invasive surgeries [2–5]. Biodegradable hydrogels could be utilized as cell carriers and scaffolds for soft tissue regeneration, which allow easy and homogenous drug or cell distribution within any defect size or shape [6–10]. Many methods including physical conjugation and chemical crosslinking have been employed for the preparation of *in situ* forming biodegradable hydrogels [11–15]. Recently, the copper-catalyzed click chemistry has received much attention in hydrogel fabrication due to its high

chemoselectivity in mild reaction conditions with a variety of functionalizations [16–20]. Typically, 1,3-dipolar cyclo-addition catalyzed by Cu (I) can be accomplished with high efficiency, reliability, and no by-products under the physiological condition [21–29]. Some macromolecular derivatives, such as poly(vinyl alcohol) (PVA) [18], polyethylene glycol (PEG) [21,23,26,28] and hyaluronan [20,27], were functionalized with pendant acetylene and azide groups to form hydrogels by the 1,3-dipolar cyclo-addition reaction. Generally, these hydrogels prepared via this cyclo-addition reaction have controlled architectures and improved mechanical properties.

Although the versatility of copper-catalyzed click chemistry has been broadly exploited for hydrogels, it is important to note that a major limitation is the intrinsic toxicity of copper and the inability to translate these approaches into tissue engineering. In that case, the risks of metal toxicity will prevent its use in clinical applications. Therefore, many attempts have been devoted toward exploiting efficient metal-free click conjugation for molecule ligation. In the past, an example of metal-free click reaction called “the Strain-Promoted Azide-Alkyne Cycloaddition (SPCC)”, the strain-promoted [3 + 2] cycloaddition using cyclooctyne derivatives, was reported by Agard et al. [30], which enabled the click reaction without copper catalysis under physiological condition

\* Corresponding author at: School of Materials Science and Engineering, Nanjing University of Science and Technology, 200 Xiao Ling Wei St., Nanjing 210094, China. Tel.: +86 25 84315840.

E-mail address: [hptan@njust.edu.cn](mailto:hptan@njust.edu.cn) (H. Tan).

[31,32]. Presently, the SPPC has been employed to graft bioactive peptides to PEG hydrogels for molecular patterning [33,34]. Also, Takahashi et al. chemically modified hyaluronan with azide and cyclooctyne groups to prepare an *in situ* crosslinked hyaluronan hydrogel [35]. Although the formed hydrogel was biocompatible, it showed fast hydrolytic degradation that may prevent its use in tissue engineering.

More recently, oxanorbornadiene (OB) moieties have been considered to be effective functional molecules to bring in a reaction with azido resulting in triazole linkages [36,37]. Krause and Jirawutthiwongchai et al. have successfully modified alginate and chitosan via OB for a metal-free click ligation, respectively [38,39]. Their results indicated that OB with a certain chain length is necessary as it might help minimize the steric hindrance in the reaction. The most attractive point of OB is that the reaction can progress at room temperature without any additives or catalysts. For this reason, this metal-free click conjugation would be a good pathway to design bio-structured hydrogels, for example, on the basis of biopolymers. To the best of our knowledge, the OB mediated click conjugation in preparation of injectable polysaccharide hydrogels as cell scaffolds for tissue engineering has not yet been reported.

Herein, we have for the first time described a new *in situ* forming biocompatible hydrogel by metal-free click chemistry for adipose tissue engineering, without employing extraneous copper catalyst. Hydrogels derived from naturally occurring polysaccharides can mimic many features of natural extracellular matrix (ECM), including structure and properties, and thus have the potential to direct the growth and organization of transplanted cells during tissue regeneration. In order to mimic the natural ECM of adipose, which is composed of glycosaminoglycans (GAGs), chitosan and hyaluronan were chosen to prepare a composite hydrogel. Chitosan, a partially deacetylated derivative from chitin composed of glucosamine and *N*-acetylglucosamine, is structurally similar to GAGs [40,41]. Hyaluronan is composed of repeating disaccharide units of *N*-acetyl-*D*-glucosamine and *D*-glucuronic acid, which is the backbone of GAGs superstructure complexes in ECM, mostly associated with other polysaccharides [42,43]. By incorporating with chitosan, hyaluronan can create a more biomimetic microenvironment with improved properties for tissue regeneration. For the metal-free click reaction, chitosan and hyaluronan were modified with OB and 11-azido-3,6,9-trioxundecan-1-amine (AA), respectively. The aim of this work was to examine the *in vitro* gelation, microstructure, morphology, equilibrium swelling, compressive modulus and degradation of the metal-free click hydrogel. Human adipose-derived stem cells (ASCs) were encapsulated within the hydrogels *in vitro* to assess their biological performance and applicability as cell carriers. A preliminary *in vivo* study of the injectable efficacy in the metal-free click hydrogel was also administered.

## 2. Materials and methods

### 2.1. Materials

Chitosan (deacetylation degree: 92%, MW: 15 kDa), hyaluronan sodium (MW:  $1.6 \times 10^6$  Da), 11-azido-3,6,9-trioxundecan-1-amine (AA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid (MES), 1-hydroxybenzotriazole hydrate (HOBt), diisopropylethylamine (DIPEA) and ninhydrin were purchased from Sigma–Aldrich, St. Louis, USA. CyQuant Cell Proliferation Assay Kit was purchased from Invitrogen, Eugene, Oregon, USA. All chemicals and reagents were used as received.

### 2.2. Synthesis of CS–OB

CS–OB was synthesized according to an already reported procedure slightly modified [39]. In our study, a NHS-active ester oxanorbornadiene derivative, 2,5-dioxopyrrolidin-(3-(trifluoromethyl)-7-oxabicyclo-2,5-diene-2-carboxamido) butanoate (DTCOB), was prepared prior to obtaining CS–OB in alkaline condition without EDC. Typically, the reaction condition was performed by dissolving chitosan in water containing 1.2 equiv HOBt. Briefly, chitosan (0.1 g, 0.6 mmol) and HOBt (0.1 g, 0.7 mmol) were dissolved in 100 mL water. Then, CS/HOBt solution was added into the DTCOB (706 mg, 1.8 mmol) which was dissolved in the mixture of THF (25 mL) and water (75 mL). Additionally, DIPEA (235 mg, 1.8 mmol) was added to the mixture. The reaction was cooled to 0 °C and stirred overnight. The mixture was purified by dialysis (MWCO 8,500) against NaCl solution followed by ultrapure water for 3 days. CS–OB was obtained by lyophilization at –50 °C for 24 h. The percentage of substitution which was quantified as 58% ( $n = 5$ ) by  $^1\text{H}$  NMR (300 MHz, Bruker Avance, Switzerland).

### 2.3. Synthesis of HA–AA

HA–AA was synthesized by amidation of carboxyl groups of HA with amine groups of AA [27]. Briefly, 500 mg of EDC and 200 mg of NHS were successively added to 100 mL of 0.2% HA solution with magnetic stirring. After 30 min, 1 g of MES and 350  $\mu\text{L}$  of AA were successively added to the solution. The reaction was maintained for 24 h at room temperature and purified by dialysis (MWCO 10,000) against ultrapure water for 3 days. HA–AA was obtained by lyophilization at –50 °C for 24 h. The AA degree of substitution was quantified as 47% ( $n = 5$ ) from the C and N contents by elemental analysis (Eager 300, Thermo, USA).

### 2.4. Fabrication of CS–OB/HA–AA hydrogels

CS–OB and HA–AA were dissolved in PBS separately at a concentration of 2 wt%. The composite hydrogels were formed by mixing of CS–OB and HA–AA solutions ( $n = 5$ ) at various volumes ratio of 1/9, 3/7, 5/5, 7/3 and 9/1 at 37 °C. Fourier transformed infrared (FT–IR) spectra of polysaccharide derivatives and hydrogels were measured to confirm the gel formation and expected pendant functionalities. Samples were recorded with a FT–IR spectrometer (Nicolet Avatar 360, USA) against a blank KBr pellet background.

### 2.5. Characterization of hydrogels

#### 2.5.1. Mechanical properties

Mixtures of CS–OB and HA–AA solutions in PBS (2 wt%) described above were injected into a 12-well culture plate at 37 °C to obtain columned hydrogels (22 mm diameter, 5 mm height,  $n = 5$ ). Compressive modulus of elasticity was measured in the elastic region of hydrogel using a dynamic mechanical analyzer (ELF3200, Endura TEC, Bose, USA) in unconfined compression at a constant stress rate of 40  $\text{mN min}^{-1}$  up to 20% strain at 37 °C. The rheological analysis of mixtures of CS–OB and HA–AA solutions was investigated on a strain-controlled rheometer (AR2000, TA instrument, USA) using a parallel plate (diameter, 40 mm) at 37 °C. Mixtures were injected onto the plate and each injection was 5 mL in volume. The angular frequency  $\omega$  was set at 6.28 rad/s. The gelation time was recorded when the value of the storage modulus  $G'$  equals that of the loss modulus  $G''$ .

#### 2.5.2. Degradation *in vitro*

Degradation of composite hydrogels (volume ratio 5:5) was examined with respect to weight loss. Weight loss of initially

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