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Injectable hyaluronic acid/poly(ethylene glycol) hydrogels crosslinked *via* strain-promoted azide-alkyne cycloaddition click reaction

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ABSTRACT

This paper reports injectable hyaluronic acid (HA)-based hydrogels crosslinked with azide-modified poly(ethylene glycol) (PEG) *via* the strain-promoted azide–alkyne cycloaddition (SPAAC) between cyclooctyne and azide groups. Cyclooctyne-modified HA (Cyclooctyne-HA) is prepared by the reaction of HA with 2-(aminoethoxy)cyclooctyne. To crosslink the modified HA, quadruply azide-terminated poly(ethylene glycol) (Azide-PEG) is designed and prepared. The mixture of Cyclooctyne-HA and Azide-PEG gelates in a few minutes to form a strong HA-PEG hydrogel. The hydrogel has fast gelation time, good strength, and slow degradation rate, because of the high reactivity of SPAAC, high crosslinking density originated from the quadruply-substituted Azide-PEG, and the good stability of the crosslinking amide bonds. *In vitro* cell culturing within the hydrogel demonstrated an excellent cell-compatibility. The bioorthogonality of SPAAC makes the hydrogel injectable. With good mechanical properties and biocompatibility, the hydrogel would be useful in a wide range of applications such as injection filling materials for plastic surgery.

1. Introduction

In situ injectable hydrogels have gained increasing importance in recent years as they are able to avoid the tissue damage caused by complex surgical implantation (Führmann et al., 2016; Johnson & Christman, 2013; Macaya & Spector, 2012; Rahman et al., 2011). Injectable hydrogels are formed by either covalent crosslinking or non-covalent crosslinking (Hennink & Van Nostrum, 2012; Peppas, Bures, Leobandung, & Ichikawa, 2000). The formation of non-covalent crosslinking interactions such as hydrogen bonds (Kenawy, Kamoun, Eldin, & El-Meligy, 2014), coordination bonds (Berger et al., 2004), electrostatic coupling (Mann, Kremer, Lenz, & Holm, 2011), hydrophilic and hydrophobic interactions or Van der Waals forces (Van Oss, Good, & Chaudhury, 1986) are usually biocompatible. However, the sensitivity of some non-covalent interactions to physiological environments makes them unstable *in vivo* (Peppas et al., 2000). On the other hand, covalently crosslinked hydrogels have controlled degradation rate and stable mechanical property(Wu, Wu, Mutschler, & Chu, 2012; Wu, Zhao, Wu, & Chu, 2014), but effective formation of covalent crosslinking *in situ* often requires crosslinking agents, UV-light or radiation ray exposure. The biosafety of the crosslinking reactions should be taken into account. For example, the toxicity of crosslinking agents, the slow gelation kinetics and the complexity of the reaction system should not be underestimated (Hennink & Van Nostrum, 2012; Patterson, Nazarova, & Prescher, 2014; Qiu & Park, 2012). Rays can cause damage to cells and tissues in radiation crosslinking (Bessho, Kojima, Okuda, & Hara, 2007; Razzak & Darwis, 2001). UV-light or free radicals may affect the cells or drugs encapsulated (Kennedy et al., 2014; Zheng et al., 2002). A simple, efficient and biologically friendly reaction system is preferred in the preparation of injectable hydrogels.

Strain-promoted azide–alkyne cycloaddition (SPAAC) click reaction has become one of the best choices for biomaterials preparation, thanks to its bioorthogonality, high reactivity, and high yield (Agard, Prescher, & Bertozzi, 2004; Roy, Mondal, Hatai, & Bandyopadhyay, 2014). The superiority of SPAAC in comparison with other click reactions is that it proceeds efficiently even in mild biophysical environment without needing of catalyst or UV-light exposure (Jiang et al., 2015; Su et al., 2016; Takahashi et al., 2013). Some biomedical applications of SPAAC reaction have been reported like patterning of surface (Orski et al., 2010), labeling of biomolecules (Baskin et al., 2007), rapid and efficient DNA ligation (Jung & Yi, 2013), imaging of cancer cells (Subramanian et al., 2014). Unfortunately, the synthesis process of the functional cyclooctynes for SPAAC has always been complex, involving problematic steps,

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rigor react conditions, and low overall yield. A tractable synthesis of cyclooctynes remains one of the major challenges to the applications of SPAAC, especially in the cases that a large quantity of materials is required (Hodgson, Bakaic, Stewart, Hoare, & Adronov, 2016; Jewett & Bertozzi, 2010; Martin, Parameswarappa, O'Dorisio, Pigge, & Schultz, 2010).

Hyaluronic acid (HA) is a main component of the extracellular matrix found in various tissues throughout the body. HA-based hydrogel has attracted great attention in biomedical areas because it is biocompatible, biodegradable, bioactive, non-immunogenic and non-thrombogenic. However, hydrogels formed by high molecular weight HA at high concentrations through the viscoelastic and entangled molecular networks in solution do not have long-lasting mechanical integrity and exhibit a fast degradation and clearance within the body (Garg & Hales, 2004). To provide a mechanically robust hydrogel, HA must be covalently cross-linked. Although HA can be directly crosslinked by crosslinking agents such as bisepoxides, divinyl sulfone derivatives and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and so on, the residual toxic reagents and harsh conditions are harmful (Xu, Jha, Harrington, Farach-Carson, & Jia, 2012). In order to prepare injectable HA hydrogels through covalent cross-linking biocompatibly, chemical modification of the native HA is a necessary prerequisite (Testa et al., 2009). For instance, furan-modified HA derivatives were synthesized and cross-linked *via* dimaleimide poly(ethylene glycol) to produce HA-PEG hydrogels *via* Diels-Alder click chemistry (Nimmo, Owen, & Shoichet, 2011). Tan, Chu, Payne, and Marra, (2009) fabricated hydrogels *via* the Schiff-base reaction using aldehyde modified HA. Dubbini et al. (2015) developed an approach for the Michael addition cross-linking between vinylsulfone-modified PEG and thiol-modified HA.

Herein, we present a protocol for the preparation of injectable HA-PEG hydrogel using cyclooctyne-modified HA and azide-modified PEG as two gel precursors. HA is modified by attaching cyclooctyne groups through amide bonds by the amino/carboxyl coupling reaction of HA with 2-(aminoethoxy)cyclooctyne. The amino cyclooctyne is the choice for modifying HA because the high reactivity of the amino group endows the selectivity and efficiency of the coupling reaction in the presence of plenty of HA hydroxyl groups in an aqueous solution. To ensure the efficiency and density of crosslinking, PEG is multiply functionalized by introducing four azide groups on the PEG chain (with two on each end). By this strategy, stable and strong hydrogels are formed by simply mixing of the two precursors at physiological conditions, owing to the high activity of SPAAC reaction, the stability of amide crosslinking bond, and high crosslinking density.

2. Experimental

2.1. Materials

Hyaluronic acid (HA) with a nominal molecular weight of 35000 was purchased from Bloomage Freda Biopharm Co., Ltd. Gel permeation chromatography (GPC) measurement in 0.1 M $NaNO_3$ water solution showed that it has an M_n of 44300 and M_w of 58300. Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen Corp. Hyaluronidase was purchased from Shanghai Ryon Biological Technology Co., Ltd. Live-Dead cell staining kit was purchased from Biovision. Poly(ethylene glycol) (PEG) with a nominal molecular weight of 6000 was purchased from Sinopharm Chemical Reagent Co., Ltd. GPC measurement in THF showed that it has an M_n of 7180 and M_w of 7830. Hydroxyethyl trifluoroacetamide (Dykhuizen & Kiessling, 2008; Skrzypczynski & Wayland, 2004) and 8,8-dibromobicyclo[5.1.0]octane (Kent, Spiropulos, & Heemstra, 2013) were synthesized by the literature procedures.

2.2. Synthesis of azide-PEG

2.2.1. Synthesis of diepoxy PEG (**1**)

PEG (20.2 g, 3.37 mmol) was dried in vacuum for 3 h at 120 °C in a 500 mL Schlenk flask and then purged using three cycles of Ar and vacuum. Then, 200 mL of dried THF was added by a syringe. The flask was heated to 40 °C to aid solvation. After being cooled to room temperature, NaH (1.35 g, 33.7 mmol) was added under Ar atmosphere. The solution was stirred at room temperature overnight. Then epichlorohydrin (5.3 mL, 67.4 mmol) was injected and the reaction was left stirring for 20 h. Afterward, the reaction mixture was filtered through a pad of celite to remove the excess NaH and salt. The filtered solutions were concentrated and then precipitated in ethyl ether. The precipitation was dried under vacuum, yielding 19.0 g of a white powder in 94% yield. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 3.57–3.74 (m, 545H), 3.39 (m, 4H), 3.1 (s, 2H), 2.72, 2.54 (d, 4H); ^{13}C NMR (100 MHz, $CDCl_3$, TMS), δ 70.5, 72.8, 50.4, 44.2.

2.2.2. Synthesis of tetrahydroxyl PEG (**2**)

In a 250 mL flask, a mixture of diepoxy PEG (12.2 g, 2.03 mmol) and 120 mL of 0.1 M NaOH aqueous solution was stirred at 60 °C for 10 h. Then the system was neutralized with hydrochloric acid. After the water was evaporated under vacuum, 50 mL of dichloromethane was added. The mixture was dried with anhydrous magnesium sulfate and then filtered. Tetrahydroxyl PEG was obtained as a white solid (11.8 g, 97%) after the DCM was evaporated under vacuum. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 3.53-3.79 (m, 555), 2.65 (OH). ^{13}C NMR (100 MHz, $CDCl_3$, TMS): δ 70.5, 72.8, 63.7.

2.2.3. Synthesis of tetrakis(methylsulfonyl) PEG (**3**)

Tetrahydroxyl PEG (2.77 g, 0.46 mmol) and triethylamine (1.4 mL, 18.5 mmol) were dissolved in anhydrous DCM (20 mL). Methanesulfonyl chloride (2.8 mL, 18.5 mmol) was then added to the mixture drop by drop. After stirring for 5 h at room temperature, the mixture was evaporated under vacuum to remove the organic solvent and then 100 mL of ethyl acetate was added. Afterward, the reaction mixture was filtered and the filtrate was concentrated and then precipitated in ethyl ether. The precipitation was dried under vacuum, yielding 2.72 g of a yellow powder in 98% yield. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ 4.90–4.95 (m, 2H), 4.33–4.46 (m, 4H), 3.76–3.79 (m, 4H), 3.41–3.74 (m, 545H), 3.11 (s, 6H), 3.06 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, TMS): δ 70.5, 72.8, 69.2, 67.8, 38.6, 37.6.

2.2.4. Synthesis of azide-PEG

Tetrakis(methylsulfonyl) PEG (2.59 g, 0.42 mmol) and NaN_3 (560.0 mg, 8.61 mmol) were added to a 25 mL round bottom flask. Then 5 mL of dry DMF was added. The mixture was stirred overnight at 80 °C. After cooling down to room temperature and filtration, the crude product was poured into a large amount of ether. The precipitation was dried under vacuum, dissolved in water and then dialyzed against distilled water for 2 days (MW cutoff 1000 Da). Water was removed by lyophilization to obtain 2.27 g of white powder in 87% yield. ^{1}H NMR (400 MHz, DMSO, TMS): δ 3.89–3.94 (m, 2H), 3.37–3.71 (m, 553H). ^{13}C NMR (100 MHz, $CDCl_3$, TMS): δ 70.5, 72.77, 72.57, 53.29, 50. GPC: M_n = 7300, M_w = 8170 (measured in THF, calibrated with PEG standards).