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Injectable dextran hydrogels fabricated by metal-free click chemistry for cartilage tissue engineering

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ABSTRACT

Injectable dextran-based hydrogels were prepared for the first time by bioorthogonal click chemistry for cartilage tissue engineering. Click-crosslinked injectable hydrogels based on cyto-compatible dextran (Mw = 10 kDa) were successfully fabricated under physiological conditions by metal-free alkyne-azide cycloaddition (click) reaction between azadibenzocyclooctyne-modified dextran (Dex-ADIBO) and azide-modified dextran (Dex-N_3). Gelation time of these dextran hydrogels could be regulated in the range of approximately 1.1 to 10.2 min, depending on the polymer concentrations (5% or 10%) and ADIBO substitution degree (DS, 5 or 10) of Dex-ADIBO. Rheological analysis indicated that the dextran hydrogels were elastic and had storage moduli from 2.1 to 6.0 kPa with increasing DS of ADIBO from 5 to 10. The in vitro tests revealed that the dextran hydrogel crosslinked from Dex-ADIBO DS 10 and Dex-N_3 DS 10 at a polymer concentration of 10% could support high viability of individual rabbit chondrocytes and the chondrocyte spheroids encapsulated in the hydrogel over 21 days. Individual chondrocytes and chondrocyte spheroids in the hydrogel could produce cartilage matrices such as collagen and glycosaminoglycans. However, the chondrocyte spheroids produced a higher content of matrices than individual chondrocytes. This study indicates that metal-free click chemistry is effective to produce injectable dextran hydrogels for cartilage tissue engineering.

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1. Introduction

Articular cartilage has poor ability of autonomous healing due to its avascular property. Although orthopedics implants based on ceramics and metals have been applied clinically in cartilage surgery, they have no capability to facilitate cartilage tissue repair. Cartilage tissue engineering offers an innovative strategy for cartilage regeneration [1]. For successful cartilage tissue engineering, three major components are generally required which include cells, bio-scaffold materials and growth factors. Among these, bio-scaffolds are essential to provide bio-mimic environments for chondrocyte growth and neocartilage formation [2]. Water-containing polymeric hydrogels represent typical bio-scaffolds for cartilage tissue engineering [3], because they can be designed and prepared with flexible properties to simulate tissue micro-environments, thus inducing chondrocyte proliferation and matrix production. Particularly, in situ forming (injectable) hydrogels for cartilage tissue regeneration have received much attention in the past few years [4–6]. A major advantage is that, in comparison with pre-formed hydrogels, injectable hydrogels can be constructed in any contour by filling the gel precursor in an irregular-shape cartilage defect. Such minimally invasive operation is desired by the doctors in cartilage surgery. Thus, the availability of injectable hydrogels is a critical issue in cartilage tissue engineering [7].

Different methods were developed to design and prepare injectable hydrogels for cartilage tissue engineering. Photo-initiating crosslinking with ultraviolet (UV) curing represents a regular strategy to prepare injectable hydrogels [8]. By this way, a lot of UV-crosslinked hydrogels were fabricated and applied for chondrocyte culturing. However, clinical translation of the hydrogels was hampered by the adverse effects of UV light on cellular metabolic activity and potential cytotoxicity of photo-initiators [9]. Alternatively, the chemical reactions such as Schiff-base reaction [10–12] and Michael-type addition reaction [13–15] were applied to produce injectable hydrogels. However, most of the reactive groups involved in the reactions might non-specifically react with tissues (or proteins). For example, in the synthesis of dextran hydrogels by Schiff-base reaction between aldehyde and hydrazide groups, the aldehyde can also react with primary amine of membrane proteins. Such side reaction causes high cytotoxicity of aldehyde-modified dextran in mesothelial cells [16]. Moreover, Michael-type addition between thiol and acrylate groups is another approach widely used to prepare injectable hydrogels. However, free thiol group could degrade the disulfide bond of growth factors, which leads to diminished

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bioactivity of the factors encapsulated in the hydrogels. Thus, as these chemical reactions are applied to prepare injectable hydrogels, it is difficult to circumvent non-specific reactions of the reactive chemical groups with normal tissues (proteins), probably compromising the functions of injectable hydrogels and hampering their clinical usage. More recently, novel injectable hydrogels were prepared using bio-specific enzyme-catalyzed reactions involving tyrosinase [17] and horseradish peroxidase (HRP) [18,19] However, the compatibility of exogenous HRP in human body would be a worrying issue. Although photo-crosslinked poly(ethylene glycol) (PEG)-based hydrogels were applicable for human cartilage repair in a pilot clinical trail [20], most of the injectable hydrogels reported so far were not advanced for clinical translations.

An ideal injectable polymeric hydrogel for cartilage tissue engineering are expected to be biocompatible with and highly inert to human tissues. These crucial requirements promote the development of bioorthogonal chemical reactions for hydrogel fabrication. The azide-alkyne cycloaddition reaction, also referred to as "click" reaction, is a new focus because alkyne and azide are bio-inert groups against tissues but they can specifically conjugate to form triazole. Particularly, over the past few years, strain-promoted click chemistry has received increasing interest for bio-applications [21]. A major reason is that such click reaction occurs in a mild environment (e.g. physiological conditions) by strain-promoted cyclooctyne-azide cycloaddition instead of cytotoxic copper-catalyzed cycloaddition. Such metal-free click chemistry was thus attractive to design injectable hydrogels. For example, an injectable PEG hydrogel was prepared by metal-free click reaction between monofluoro-tagged cyclooctyne-modified PEG and azide-terminated PEG [22]. In another study, an injectable hyaluronic acid (HA) hydrogel was fabricated by metal-free click crosslinking between cyclooctyne-modified HA and azide-modified HA [23]. An injectable HA-chitosan composite hydrogel was yielded by metal-free click crosslinking of azide-modified HA and oxanorbornadiene-modified chitosan [24]. More importantly, these injectable hydrogels possessed good cyto-compatibility, thereby supporting the growth of fibroblasts and stem cells. However, to our best knowledge, such injectable hydrogels by metal-free click chemistry are not well elucidated for cartilage tissue engineering.

In this work, we aim to fabricate injectable hydrogels by metal-free click chemistry and investigate their feasibility for cartilage tissue engineering. As such, two dextran conjugates were prepared which comprise azadibenzocyclooctyne (ADIBO) and azide residue, respectively. It is assumed that injectable dextran hydrogels would be formed in situ by strain-promoted click chemistry between ADIBO-modified dextran and azide-modified dextran, and these hydrogels would be applicable as bio-scaffolds for chondrocyte growth. We herein describe the synthesis and characterization of the dextran conjugates and preparation method of the injectable dextran hydrogels. The properties of the hydrogels in terms of gelation time, mechanical strength, and swelling profile were examined. The possibility of the hydrogels for cartilage tissue engineering was evaluated in vitro after incubating rabbit chondrocytes and the chondrocyte spheroids in the hydrogels. The viability of the chondrocytes was assessed after the incubation. The production of neocartilage matrices (glycosaminoglycan and collagen) in the hydrogels was also investigated.

2. Materials and methods

2.1. Materials

Dextran ($M_w = 10$ kDa) and *p*-nitrophenyl chloroformate (PNC) were ordered from Sigma-Aldrich Co. (USA). The chemicals, 2-aminoethyl *N*-azadibenzocyclooctyne amide (ADIBO-NH_2, $C_{18}H_{16}N_2O$) and 2-[2-(2-azidoethoxy)ethoxy]-ethanamine (N_3-PEG_2-NH_2, $C_6H_{14}N_4O_2$) were ordered from PurePEG, LLC. (USA). Two dextran derivatives, i.e. *p*-nitrophenyl carbonated dextran (Dex-PNC), at substitution degree (DS) of 5 and 10 (3 and 6 PNC residues per dextran chain) was synthesized by the previous method [25]. DS of PNC moieties is defined as the number of PNC moieties per 100 anhydroglucose unit of dextran. DMEM medium, fetal bovine serum (FBS) and Live-dead assay kit were ordered from ThermoFisher scientific, USA.

2.2. Synthesis and characterization of dextran derivatives

Two dextran conjugates with azadibenzocyclooctyne (ADIBO) and azide residues were synthesized by conjugating Dex-PNC with ADIBO-NH_2 and N_3-PEG_2-NH_2, respectively, thus giving ADIBO-modified dextran (Dex-ADIBO) and azide-modified dextran (Dex-N_3), as shown in Scheme 1. In a typical example for Dex-ADIBO preparation, Dex-PNC at a DS of 10 (300 mg) and ADIBO-NH_2 (100 mg) were added into the flask and dissolved in DMSO (5 mL) containing the pyridine (0.5 mL). The mixture was stirred at 40 °C for 3 days and then diluted in deionzed water and purified by ultrafiltration operation (1 kDa molecular weight cut-off). Dex-ADIBO was obtained as solid powder after freezing-dry (370 mg, yield: 90%). In a similar way, Dex-N_3 was prepared by reacting Dex-PNC at a DS of 10 (1 g) and N_3-PEG_2-NH_2 (155 mg) in DMSO (5 mL) at 40 °C for 3 days, and obtained as a solid powder after freezing-dry (1.15 g, ~90% yield). Chemical compositions of Dex-ADIBO and Dex-N_3 were characterized by ^1H NMR (300 MHz) recorded on a Varian Inova spectrometer (Varian, USA) and functional groups of the conjugates were analyzed by a Spectrum 1000 FT-IR spectrometer (Bruker, USA).

2.3. Preparation of injectable click-crosslinked dextran hydrogels

To prepare injectable dextran hydrogels, Dex-ADIBO DS 10 and Dex-N_3 DS 10 were respectively dissolved in PBS buffer (pH 7.4, 200 μL) at a polymer concentration of 10% (*w*/*v*). Herein, the molar ratio of N_3 to ADIBO residues was set at 1.1/1. Next, the two solutions were mixed together in a vial followed by gentle vortexing. The polymer concentration of the gels is defined as the total dry mass of both two polymers per total volume of solution (i.e. 400 μL). Moreover, when observing the gelation process, the time to form a gel (denoted as gelation time) was recorded with a timer by tilting the vial. Gelation time for the hydrogel formation is defined when no liquid flows in the vial.

2.4. Property characterization of dextran hydrogels

Gel content of the hydrogels was measured using the previous method [26]. In brief, the hydrogel sample (400 mL) was prepared and freeze-dried. The dried gel was weighed with the mass W_1. Next, the gel was immersed in PBS (3 mL) for 5 days and the buffer was replaced every day. After freeze-dried, the gel was weighted again with the mass W_2. Gel content was calculated as $W_2 / W_1 \times 100\%$. The experiments were run in triplicate tests.

Swelling ratio of the hydrogels was measured according to our previous report [26]. Briefly, the hydrogel (400 mL) prepared in a vial was weighed with the mass W_1. Next, cell culture medium (3 mL) was added onto the top of the hydrogel sample and incubated at 37 °C during a few days. At regular time intervals, the medium solution was removed from the samples and the hydrogel sample was weighed with the mass W_2. The swelling ratio of the gels is calculated as $W_2 / W_1 \times 100\%$. The cell culture medium was replaced twice a week and the experiments were done in triplicate.

Mechanical property of the hydrogels was measured using a rheometer (MCR 301, Anton Paar) using a parallel plate (25 mm diameter, 0°) at 37 °C at the oscillatory mode. The gap size between the plate and the chamber is set with 0.5 mm. The measurement was run to record the value of storage (G′) and loss (G″) modulus as a function of time at a frequency of 0.5 Hz and a strain of 0.1%.

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