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Injectable glycopolypeptide hydrogels as biomimetic scaffolds for cartilage tissue engineering

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ABSTRACT

Glycopolypeptides are an emerging class of bioinspired polymers that mimic naturally occurring glycopeptides or glycoproteins, and therefore are expected to exhibit great potential for biomedical applications. In this study, a glycopolypeptide was synthesized by conjugation of poly(γ-propargyl-L-glutamate) (PPLG) with azido-modified mannose and 3-(4-hydroxyphenyl) propanamide (HPPA), *via* click chemistry. Injectable hydrogels based on the glycopolypeptide were developed through enzymatic crosslinking reaction in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). The physicochemical properties of the hydrogels, such as gelation time, storage modulus, swelling and degradation time, could be controlled by varying the concentrations of HRP and H_2O_2. The glycopolypeptide copolymer as well as the extracts of the glycopolypeptide hydrogels displayed good cytocompatibility *in vitro*. After subcutaneous injection into rats, the glycopolypeptide hydrogels were rapidly formed *in situ*, and exhibited acceptable biocompatibility accompanying the degradation of the hydrogels *in vivo*. The rabbit chondrocytes inside the glycopolypeptide hydrogels showed spherical morphology with high viability during the incubation period of 3 weeks *in vitro*, and exhibited a higher proliferation rate than within the hydrogel counterparts of PPLG grafted with 2-(2-(2-methoxyethoxy)ethoxy)ethane (MEO_3) and HPPA. Biochemical analysis demonstrated that the production of glycosaminoglycans (GAG) and type II collagen were significantly enhanced after incubation for 2 and 3 weeks *in vitro*. Moreover, the chondrocyte-containing glycopolypeptide hydrogels in subcutaneous model of nude mice maintained chondrocyte phenotype and produced the cartilaginous specific matrix. These results indicated that the biomimetic glycopolypeptide-based hydrogels hold potential as three-dimensional scaffolds for cartilage tissue engineering.

1. Introduction

Articular cartilage is an organized tissue that allows for smooth motion in diarthrodial joints. This tissue possesses distinct load-bearing and low-friction capabilities, which benefit from its extracellular matrix (ECM) composed of water, collagen, and glycosaminoglycans (GAGs) [1–3]. Damage to articular cartilage can be caused by trauma, disease, or sports-related injury, leading to disability of joint function. Without access to blood supply, abundant nutrients and progenitor cells, the damaged articular cartilage lacks the ability to repair and regenerate itself. Current clinical therapies for articular cartilage defects include autologous chondrocyte transplantation (ACI), osteochondral auto- and allografts, and microfracture [4–6]. Although these techniques have shown some efficacy in remodeling cartilage defects, the therapies have limitations such as the lack of donors, poor integration to the surrounding cartilage tissue and formation of fibrous tissue instead of hyaline cartilage [7].

Over the past decades, tissue engineering provides a promising approach for the regeneration of cartilage defects. This approach involves the combination of chondrocytes or progenitor cells with three-dimensional scaffolds, which serve as the temporary ECM [6,8,9]. An ideal three-dimensional scaffold for cartilage regeneration is expected to have adequate mechanical strength, possess suitable degradability, promote cell survival and differentiation, facilitate the diffusion of nutrients and metabolites, and integrate with the surrounding cartilage tissue. Recently, injectable hydrogels acting as three-dimensional scaffolds have received much

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attention for cartilage tissue engineering [10–12]. Injectable hydrogels generally retain a large amount of water, exhibit excellent permeability for nutrients and metabolites, and show good biocompatibility [13–17]. They can be administrated *via* a minimally invasive procedure, and are able to appropriately fill irregular-shaped defects. Meanwhile, cells and bioactive molecules can be homogeneously incorporated in the hydrogels. Due to their physical properties that resemble the native ECM, injectable hydrogels may be suitable platforms for supporting the survival, proliferation and differentiation of incorporated chondrocytes or progenitor cells, and promoting the regeneration of cartilage tissue.

Various crosslinking approaches have been applied to prepare injectable hydrogels under physiological conditions for cartilage tissue engineering, such as Michael-addition reactions [18,19], enzyme-catalyzed crosslinking reactions [20–23] and thermo-induced gelation [24–26]. Among the gelation methods used for injectable hydrogels, enzyme-mediated crosslinking strategy with the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) has shown advantages in tissue engineering applications, attributed to their fast gelation behavior, tunable mechanical properties and good biocompatibility [27]. The intermolecular linkages are generally formed by the coupling of phenol or aniline moieties through carbon–carbon or carbon–oxygen/nitrogen bonds during enzyme-catalyzed crosslinking reaction [28]. Additionally, the physical properties of hydrogels can be conveniently controlled by varying the HRP and H_2O_2 concentrations.

In recent years, synthetic glycopolypeptides have emerged as a new class of bioinspired polymers due to their unique molecular compositions and secondary conformations that mimic natural glycopeptides or glycoproteins [29–32]. Glycoproteins are ubiquitous in nature and are involved in a wide range of biological functions including cell–cell recognition, adhesion, immune response and hormone interaction [33]. Accordingly, synthetic glycopolypeptides may serve as biomimetic materials for biomedical applications, such as scaffolds for tissue repair or carriers for drug delivery. Nevertheless, to-date, the hydrogels based on glycopolypeptides applied for tissue engineering are seldom reported.

In the present work, we developed a novel kind of glycopolypeptide-based injectable hydrogels as biomimetic scaffolds for cartilage tissue engineering. The glycopolypeptide hydrogels were designed to act as an analog of proteoglycans present in the ECM of native cartilage [23]. The glycopolypeptide hydrogels were rapidly formed *in situ* through enzymatic crosslinking reaction in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). The physicochemical properties of the hydrogels, such as gelation time, mechanical properties, swelling and degradation behavior, were studied. The cytocompatibility of the glycopolypeptide copolymer as well as the extracts of the glycopolypeptide hydrogels were measured by MTT assay against L929 cells *in vitro*. The biodegradability and biocompatibility of the hydrogels *in vivo* were evaluated by subcutaneous injection of the hydrogels into rats. To assess the feasibility of the glycopolypeptide hydrogels as 3D scaffolds for cartilage tissue engineering, rabbit chondrocytes were incorporated in the hydrogels, and the cell viability and proliferation of chondrocytes within the hydrogels were examined by live-dead assay and cell counting kit-8 method *in vitro*. Additionally, the morphology of chondrocytes and formation of the cartilaginous specific matrix within the glycopolypeptide hydrogels were investigated both *in vitro* and in the subcutaneous model of nude mice.

2. Experimental section

2.1. Materials

γ-Propargyl-L-glutamate-N-carboxyanhydride (PLG NCA) was synthesized according to our previous method [34]. *n*-Hexylamine (Aldrich, 99%), D-(+)-mannose (Alfa Aesar, 98%), 3-(4-hydroxyphenyl)-propionic acid (Aladdin, 98%), 2-(2-(2-methoxyethoxy)ethoxy)ethanol (MEO_3OH, 97%, Sigma), sodium azide (NaN_3, Multipoint Chem, 98%), 3-chloropropylamine hydrochloride (Aladdin, 98%), N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, GL Biochem, 98%) and N-hydroxysuccinimide (NHS, GL Biochem, 98%) were used as received. 2′-Azidoethyl-*O*-α-D-mannopyranoside and 1-(2-(2-methoxyethoxy)ethoxy)-2-azidoethane (MEO_3-N_3) were prepared according to the literature method [35,36]. The chemical structures of 2′-azidoethyl-*O*-α-D-mannopyranoside and MEO_3-N_3 were confirmed by ^1H NMR spectra (Fig. S1 and S2 in the Supplementary Information). Copper (I) bromide (CuBr, Sinopharm, 98%) was purified by stirring in acetic acid, washing with methanol and then drying under vacuum. N,N,N′,N′N″-pentamethyldiethylenetriamine (PMDETA, Aldrich, 99%) was freed from its inhibitor (phenothiazine) *via* distillation under reduced pressure. Horseradish peroxidase (HRP, 200 units/mg), papain, dimethylmethylene blue (DMMB) and chondroitin sulfate A were purchased from Sigma–Aldrich and used without further purification. 1,4-Dioxane was refluxed with sodium and distilled under nitrogen prior to use. All the other reagents and solvents were of analytical grade and used as obtained.

2.2. Characterization

^1H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer. The molecular weight and polydispersity index (PDI) of the glycopolypeptide were determined by gel permeation chromatography (GPC) using a Waters linear Ultrahydrogel colume and a Waters 515 isocratic HPLC pump with a Waters 2414 refractive index detector. The eluant was 0.2 M phosphate buffer (PB) containing 0.1 M NaN_3 at a flow rate of 1.0 mL min^{-1} at 25 °C. Monodispersed poly(ethylene glycol) (PEG) standards obtained from Waters Co. with the molecular weights ranging from 3×10^3 to 1.0×10^5 were used to generate the calibration curve. The content of conjugated HPPA was measured by using an ultraviolet-visible (UV-Vis) spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan). The copolymer was dissolved in deionized water at 2 mg/mL and the absorbance at 275 nm was measured. The content of conjugated HPPA was calculated from a calibration curve obtained by measuring the absorbance of 3-(4-hydroxyphenyl)-propionic acid at different concentrations in deionized water. The circular dichroism (CD) spectrum of glycopolypeptide aqueous solution (0.5 mg/mL, pH = 7.4) was obtained on a JASCO J-810 spectrometer at 25 °C.

2.3. Synthesis of 3-(4-hydroxyphenyl)-N-(3-azidopropyl) propanamide

First, 3-azidopropylamine was synthesized by the substitution reaction of 3-chloropropylamine hydrochloride with NaN_3. Briefly, 3-chloropropylamine hydrochloride (13 g, 0.1 mol) and NaN_3 (13 g, 0.2 mol) were dissolved in water, and the resulting solution was stirred for 48 h at 80 °C. The product was extracted with diethyl ether, and the organic layer was evaporated to yield a transparent liquid.

3-(4-Hydroxyphenyl)-N-(3-azidopropyl) propanamide was then synthesized by coupling 3-(4-hydroxyphenyl)-propionic acid with 3-azidopropylamine *via* EDC/NHS activated amidation reaction. 3-(4-Hydroxyphenyl)-propionic acid (3.3 g, 0.020 mol) was dissolved in 30 mL of DMF. EDC (4.6 g, 0.024 mol) and NHS (2.8 g, 0.024 mol) were added, and the mixture was stirred for 4 h to activate the carboxyl groups of 3-(4-hydroxyphenyl)-propionic acid. 3-Azidopropylamine (2.4 g, 0.024 mol) was then added and the resulting solution was reacted for 24 h at room temperature. The product was purified by precipitating into water. The product was collected as a yellow solid with a yield of 53%. The chemical structure of 3-(4-hydroxyphenyl)-N-(3-azidopropyl) propanamide was confirmed by ^1H NMR spectrum, as shown in Fig. S3 (Supplementary Information).

2.4. Synthesis of poly(γ-propargyl-L-glutamate) grafted with mannose and 3-(4-hydroxyphenyl) propanamide (PPLG-g-Man/HPPA) and poly(γ-propargyl-L-glutamate) grafted with 2-(2-(2-methoxyethoxy)ethoxy)ethane and 3-(4-hydroxyphenyl) propanamide (PPLG-g-MEO_3/HPPA)

Poly(γ-propargyl-L-glutamate) (PPLG) was prepared according to our previously reported method [36]. Typically, PLG NCA was dissolved in anhydrous 1,4-dioxane at ~10% (w/v) under nitrogen atmosphere, followed by adding the *n*-hexylamine solution in 1,4-dioxane as an initiator. After stirring for three days at room temperature, the reactive solution was precipitated into cold diethyl ether, followed by filtration. The obtained product was further purified by precipitation into diethyl ether again and dried under vacuum. PPLG was obtained with a yield of over 70%.

The glycopolypeptide was then synthesized by copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction. A typical procedure was described as follows: PPLG (1.67 g, 0.01 mol of PLG units), 2′-azidoethyl-*O*-α-D-mannopyranoside (7.47 g, 0.03 mol) and 3-(4-hydroxyphenyl)-N-(3-azidopropyl) propanamide (0.248 g, 0.001 mol) were dissolved in 70 mL of DMSO. The mixture was then degassed three times through freeze-pump-thaw cycles, followed by adding CuBr (0.144 g, 1.0 mmol) and PMDETA (210 μL, 1.0 mmol) into the mixture. The reaction was carried out at 40 °C for 1 day. DOWEXHCR W2 resins were added and stirred over night to exchange the copper ions. After filtration of the resin, the solution was dialyzed against deionized water for 3 days (MWCO 7000 Da). The product, PPLG-g-Man/HPPA, was collected by lyophilization with a yield of 89%.