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In-situ forming biodegradable glycol chitosan-based hydrogels: Synthesis, characterization, and chondrocyte culture

Fang Li [a,b,*,1], Qiujie Ba [a,1], Sumei Niu [a], Yi Guo [a], Yankang Duan [a], Peng Zhao [b], Chao Lin [b,*], Jing Sun [a,*]

[a] Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, 200040, PR China
[b] The Institute for Advanced Materials and NanoBiomedicine, Tongji University School of Medicine, Tongji University, Shanghai, 200092, PR China

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ABSTRACT

In-situ forming hydrogels from thiolated glycol chitosan (GCH-SH) and vinyl sulfone-modified PEG (PL-VS) were designed, prepared and successfully applied as biodegradable, non-toxic bio-scaffolds for chondrocyte culture. The hydrogels could be formed in situ under physiological conditions via Michael-type addition between the GCH-SH and PL-VS at a low polymer concentration of 1–3% (w/v). Gelation times varied from 0.75 to 50 min, depending on the polymer concentration and the arm number of PEG-VS. Moreover, a high arm number and a high polymer concentration may lead to efficient network formation of GCH-SH/PEG-VS hydrogels. These hydrogels were found biodegradable in the presence of lysozyme, a cationic protein in the body, for a long period of time. Rheological studies indicated that these hydrogels generally displayed highly elastic property and had higher mechanical strength than those from thiolated hyaluronic acid/PEG-VS reported previously. SEM observation revealed that these hydrogels possessed well-interconnected microporous morphology. Besides these, the chondrocytes could be incorporated and homogeneously distributed in the hydrogel based on GCH-SH and 4-arm PL-VS. Importantly, after cell culture of 14 days, the chondrocytes in the hydrogel remained viable, as determined by a live–dead assay, and the cells kept their round chondrocytic phenotype. These results suggest that Michael-type addition is an effective method in the preparation of in-situ forming, biodegradable GCH-based hydrogels serving as bio-scaffolds for chondrocyte culture.

1. Introduction

Cartilage is avascular tissue that has very limited capability of self-healing. For the regeneration of injured cartilage tissue, tissue engineering represents a promising strategy. In a typical procedure, mature chondrocytes or progenitor cells are seeded and cultured in a tissue-engineered bio-scaffold, which is then planted into the cartilage defect site. An ideal bio-scaffold for cartilage regeneration is expected to have adequate mechanical strength and good biocompatibility, support the cell survival, and promote the cell proliferation and differentiation until neo-cartilage formation. Hydrogels are hydrated, crosslinked polymeric networks capable of absorbing and retaining a large amount of water [1]. Because of their favorable biological properties such as high water content, good biocompatibility and low inflammatory response, in the past two decades, hydrogels have been studied widely as bio-scaffolds for cartilage regeneration [2]. Particularly, in-situ forming hydrogels, also called as injectable hydrogels, are attractive for cartilage regeneration because, from a clinical point of view, they gain advantages over conventional preformed hydrogels [3,4]. For instance, flowing gel precursors can be crosslinked in-situ to fill any irregular-shaped cartilage defect via a minimally invasive injection procedure. Besides, through a simple mixing with the precursors, the cells and growth factors can be easily introduced into and homogenously distributed in hydrogels. These features thus make in-situ forming hydrogels appropriate as bio-scaffolds for cartilage tissue engineering.

Several crosslinking approaches, such as Schiff-base formation, free radical polymerization and Michael addition, have been successfully developed in the preparation of in-situ forming hydrogels [3]. By these approaches, in-situ forming hydrogels can be obtained which usually reveal stable crosslinking network and high mechanical strength. Free radical polymerization using redox- or photo-initiators is one of the most used routes to prepare in-situ forming hydrogels [5,6]. However, a major drawback of the polymerization reaction is the cytotoxicity induced by chemical initiators and crosslinking additives, which could harm the normal tissues. As an alternative method, Michael-type addition reaction offers the possibility to fabricate in-situ forming hydrogels through the addition reaction between nucleophiles and electrophiles [7]. A major advantage of Michael-type addition is of its mild conditions (e.g. physiology conditions), which is favorable for long-term survival of incorporated cells in hydrogels [8–10]. Moreover, the properties (such as gelation time and mechanical strength) of the hydrogels formed in

* Corresponding authors. Tel.: +86 21 65983706 803; fax: +86 21 65983706 0.
E-mail addresses: 09lifang@tongji.edu.cn (F. Li), chaolin@tongji.edu.cn (C. Lin), sunjing61867@yahoo.com.cn (J. Sun).
[1] Qiujie Ba and Fang Li contributed equally to this work and should be co-first authors.

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situ via Michael-type addition can be adjusted by varying the concentrations of functional groups or gel precursors [11,12]. As such, Michael-type addition was applied to produce in-situ forming hydrogels for biomedical application such as tissue regeneration. For example, Hubbell et al. described the preparation of in-situ forming hydrogels via Michael-type addition between thiolated peptides and vinyl sulfone-modified poly(ethylene glycol) (PEG) and they further examined these hydrogels for tissue engineering application [13]. A recent work from Feijen et al. showed that in-situ forming hydrogels from thiolated hyaluronic acid (HA) and PEG vinylsulfone could be served as bio-scaffolds for bovine chondrocyte culture [8,11,14]. However, these HA-based hydrogels usually had relatively low mechanical strength. More recently, we reported on a group of robust and thermosensitive in-situ forming hydrogels via Michael-type addition between thiolated dextran and thermosensitive Pluronic 127 vinyl sulfone. However, the NIH 3T3 cells encapsulated in these hydrogels showed a low cell viability, probably due to non-degradable nature [15].

Chitosan is a naturally-occurring polysaccharide composed of *N*-acetylglucosamine and glucosamine moieties. It has molecular structure similar to glycosaminoglycans in the extracellular matrices. Moreover, chitosan is biodegradable *in vivo* by lysozyme, a cationic protein in the body [16,17]. These features make chitosan a valuable material for biomedical applications such as drug release and tissue engineering [18–22]. A major drawback of chitosan is however its poor water solubility at physiological pH, which seriously limits biomedical applications of unmodified chitosan. To address this issue, chemical modification was performed to yield chitosan derivatives with improved water solubility. As a typical example, glycol chitosan reveals better solubility under physiological conditions than native chitosan due to the hydrophilicity of the glycol residues. As such, in-situ forming hydrogels based on glycol chitosan and their derivatives were investigated for potential bio-applications such as tissue engineering [23–25]. For example, Amsden et al. prepared photo-crosslinked hydrogels from methacrylated glycol chitosan and they showed that the chondrocytes can survive on the gel surface up to one week [23]. However, it should be noted that for cartilage regeneration the chondrocytes should be cultured inside hydrogels to maintain their characteristic round phenotype for cell differentiation. In another study, Ding et al. fabricated in-situ forming hydrogels from glycol chitosan and benzaldehyde-capped Pluronic copolymers via Schiff-base formation [24,25]. To the best of our knowledge, Michael-type addition is not yet examined in the preparation of in-situ forming glycol chitosan-based hydrogels for chondrocyte culture.

The purpose of this study is to prepare in-situ forming glycol chitosan-based hydrogels and investigate the possibility of the hydrogels serving as bio-scaffolds for chondrocyte culture. To this end, thiolated glycol chitosan (GCH-SH) and vinyl sulfone-modified PEG (PEG-VS) with different arm numbers were prepared and characterized by ^{1}H NMR. Hydrogels are formed in situ via Michael-type addition between GCH-SH and PEG-VS. The properties of these hydrogels were investigated in terms of gelation time, mechanical property, swelling/degradation profile and gel morphology. The viability of the chondrocytes encapsulated in the hydrogels was evaluated by live–dead assay. We show that in-situ forming hydrogels from GCH-SH and PEG-VS are suitable as bio-scaffolds to support the survival of chondrocytes and they have high potential for cartilage repair.

2. Materials and methods

2.1. Materials

Glycol chitosan (GCH, M_W ~125 kDa) was purchased from Aldrich-Sigma. The degree of deacetylation (DD) of glycol chitosan was determined from ^{1}H NMR. Dithiothreitol (DTT), 3,3′-dithiodipropionic acid di(N-hydroxysuccinimide ester) (DTSP), poly(ethylene glycol) (PEG) (M_r = 8500–11,500), divinyl sulfone (DVS), sodium hydride (NaH) and glacial acetic acid were purchased from Aldrich-Sigma. 4-arm PEG ($M_n = 1.06\times10^4$ g/mol, $M_w/M_n = 1.02$) was purchased from Jenkem Technology Co., LTD and 8-arm PEG ($M_n = 9.7\times10^3$ g/mol, M_w/M_n = 1.10) from SunBio-PEG shop. All PEGs were dried by azeotropic distillation of dried toluene. Dichloromethane (DCM) was dried with calcium hydride and distilled before use. Phosphate buffered saline (PBS, pH 7.4) was ordered from Thermo Fisher Co. Live/dead cell viability kit was obtained from Invitrogen.

2.2. Synthesis of thiolated glycol chitosan

Thiolated glycol chitosan (denoted as GCH-SH) was synthesized by a one-pot synthesis procedure. Typically, 200 mg of glycol chitosan (DD = 90% from ^{1}H NMR) was dissolved in 15 mL of water. To the solution of glycol chitosan, 5 mL DMF containing DTSP (186 mg, 0.46 mmol) was added dropwise and the reaction was stirred for 24 h. DTT (354 mg, 2.3 mmol) was then dissolved in 10 mL water and added to the GCH/DTSP mixture under nitrogen and the pH of the mixture was adjusted to 7–7.5 by the addition of 4 M NaOH. After the mixture was stirred for 24 h, the pH of reaction mixture was adjusted to pH 4–5 by the addition of 1.0 M HCl. The mixture was centrifuged and the supernatant was transferred into a dialysis tube (MWCO 12–14 k). The solution was firstly dialyzed exhaustively against dilute HCl (pH 5) containing 50 mM NaCl followed by dialysis against dilute HCl (pH 5). The conjugated GCH containing free thiol groups was obtained as white foam after freeze-drying. Degree of substitution (DS, determined from Ellman test, which is defined as the number of free thiol groups per 100 repeating units of glycol chitosan): 13. Yield: 58%. ^{1}H NMR (D_2O): δ = 4.6 (H1), 3.4–4.3 (H3, H4, H5, H6), 3.8 (OCH_2CH_2OH), 3.2 ($CO-CH_2-CH_2-SH$), 2.8 (H2), 2.7 ($CO-CH_2-CH_2-SH$), 2.1–2.2 ($NHCOCH_3$).

2.3. Synthesis of poly(ethylene glycol) vinyl sulfone

PEG vinyl sulfone (denoted as PEG-VS) was synthesized from linear or multi-arm PEG and divinyl sulfone according to a modified procedure [8]. Typically, 4-arm PEG (2 g, 0.8 mmol OH) was dissolved in 30 mL of dried DCM in nitrogen atmosphere. To the solution, 10 mL of DCM containing NaH (96 mg, 4 mmol) was added and the reaction was stirred for 1 h. After hydrogen evolution, to 20 mL of DCM solution containing DVS (4.7 g, 40 mmol), PEG/NaH mixture was added dropwise under nitrogen and the reaction was stirred for 3 days in the dark. After neutralization with glacial acetic acid, the mixture was filtrated and the filtrate was concentrated. The product was recovered by precipitation in cold diethyl ether. The precipitate was washed with diethyl ether, dissolved in water and purified by ultrafiltration (MWCO 1000). The final product was obtained by freeze-drying. The degree of end group conversion was found to be 99% from ^{1}H NMR. Yield: 75%. ^{1}H NMR (CD_3Cl): δ = 3.2–3.3 ($-O-CH_2-CH_2-SO_2-$), 3.6–3.7 ($-CH_2-CH_2-O-$), 3.8–3.9 ($-O-CH_2-CH_2-SO_2-$), 6.1 (CH_2═CH–), 6.4 (CH_2═CH–), 6.8 (CH_2═CH–).

2.4. Characterization

^{1}H NMR spectra were recorded on a Varian Inova spectrometer (Bruker AV 500 MHz). Samples of 10–20 mg each were prepared and dissolved in 0.8 mL of D_2O or CD_3Cl. The signals of solvent residues were used as a reference peak for the ^{1}H NMR chemical shifts and was set at δ 4.79 and 7.26 for water and chloroform, respectively. The degree of end group conversion of PEG-VS was calculated from ^{1}H NMR spectra by comparing the integrals of signals at δ 3.6–3.7 (PEG main chain protons) and δ 6.1–6.8 (vinyl sulfone protons). The number of free thiol groups of GCH-SH was determined by the Ellman test [21,26]. In brief, GCH-SH was mixed with the Ellman reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), in PBS buffer at room temperature for 5 min. The absorption at 412 nm of diluted GCH-SH (in PBS) was then recorded on a U-3010 UV–Visible Spectrophotometer (Hitachi) and the free thiol concentration was calculated using a calibration curve from standard solutions of mercaptoethanol.