Biomaterials 30 (2009) 2499–2506

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Injectable *in situ* forming biodegradable chitosan–hyaluronic acid based hydrogels for cartilage tissue engineering

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ARTICLE INFO

Article history:
Received 1 December 2008
Accepted 29 December 2008
Available online 23 January 2009

Keywords:
Chitosan
Hyaluronic acid
Biodegradable hydrogel
Bovine articular chondrocytes
Tissue engineering

ABSTRACT

Injectable, biodegradable scaffolds are important biomaterials for tissue engineering and drug delivery. Hydrogels derived from natural polysaccharides are ideal scaffolds as they resemble the extracellular matrices of tissues comprised of various glycosaminoglycans (GAGs). Here, we report a new class of biocompatible and biodegradable composite hydrogels derived from water-soluble chitosan and oxidized hyaluronic acid upon mixing, without the addition of a chemical crosslinking agent. The gelation is attributed to the Schiff base reaction between amino and aldehyde groups of polysaccharide derivatives. In the current work, *N*-succinyl-chitosan (S-CS) and aldehyde hyaluronic acid (A-HA) were synthesized for preparation of the composite hydrogels. The polysaccharide derivatives and composite hydrogels were characterized by FTIR spectroscopy. The effect of the ratio of S-CS and A-HA on the gelation time, microstructure, surface morphology, equilibrium swelling, compressive modulus, and *in vitro* degradation of composite hydrogels was examined. The potential of the composite hydrogel as an injectable scaffold was demonstrated by the encapsulation of bovine articular chondrocytes within the composite hydrogel matrix *in vitro*. The results demonstrated that the composite hydrogel supported cell survival and the cells retained chondrocytic morphology. These characteristics provide a potential opportunity to use the injectable, composite hydrogels in tissue engineering applications.

1. Introduction

Various hydrogels and microspheres have been employed as injectable scaffolds for a variety of biomedical applications [1–5]. Injectable, biodegradable hydrogels could be utilized as delivery systems, cell carriers, and scaffolds for tissue engineering [6–8], which allow easy and homogenous drug or cell distribution within any defect size or shape. Recently, many methods have been employed for the preparation of injectable *in situ* forming hydrogels, including photopolymerization of their custom-made monomers [9,10] and chemical crosslinking with agents such as carbodiimide, glutaraldehyde, genipin, and adipic dihydrazide [11–15]. However, photopolymerization often requires a photosensitizer and prolonged irradiation, thus limiting their applications. The chemical crosslinking agents are the major obstacles in the use of injectable *in situ* forming polymer scaffolds, due to their toxicity to cells [16,17].

Presently, several polysaccharides such as dextran [18], gum arabic [19], chondroitin sulfate [20] and hyaluronic acid [21,22] are partially oxidized and employed for possible medical applications such as drug release and peritoneal adhesion prevention. However, little has been reported on the use of oxidized polysaccharides for the preparation of hydrogels as cell carriers for tissue engineering applications. Herein, we describe a new injectable, *in situ* forming biodegradable hydrogel by self-crosslinking of water-soluble chitosan and oxidized hyaluronic acid, without employing any extraneous chemical crosslinking agents.

Chitosan, a partially deacetylated derivative from chitin composed of glucosamine and *N*-acetylglucosamine, is structurally similar to glycosaminoglycan (GAG) and its analogs. Chitosan has been widely applied in drug delivery, gene therapy and tissue engineering because of its biocompatibility and biodegradability [23–25]. However, chitosan has poor solubility in physiological solvents due to its strong intermolecular hydrogen bonding, thereby greatly limiting further biomedical applications, particularly as an injectable scaffold. *N*-Succinyl-chitosan (S-CS), a water-soluble

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doi:10.1016/j.biomaterials.2008.12.080

chitosan derivative, was synthesized via introduction of succinyl groups at the *N*-position of the glucosamine units of chitosan. It is attractive as a drug carrier as it shows biocompatibility and long-term retention *in vivo* [26–28]. By incorporating with other polysaccharides such as hyaluronic acid, S-CS can create a more biomimetic microenvironment with improved biocompatibility and biodegradation for tissue regeneration.

Hyaluronic acid is a linear high-molecular weight polysaccharide, composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid [29,30]. In ECM, hyaluronic acid is the backbone of GAG superstructure complexes, mostly associated with other polysaccharides such as chondroitin sulfate [31]. Due to its good biocompatibility, biodegradability, as well as excellent gel-forming properties, hyaluronic acid shows potential in biomedically-relevant hydrogel systems. Hyaluronic acid can be oxidized, and the carbon–carbon bonds of the cisdiol groups in molecular chain are cleaved and generate reactive aldehyde functions (aldehyde hyaluronic acid, A-HA), which can develop chemical crosslinking action with amino functions via Schiff's base linkage [21,22].

The aim of this work was to prepare a non-toxic *in situ* forming biodegradable S-CS/A-HA composite hydrogel, and to study the effects of varying the ratio of S-CS and A-HA on gelation time, microstructure, morphology, equilibrium swelling, compressive modulus and degradation *in vitro*. Bovine articular chondrocytes were encapsulated within the hydrogels *in vitro* to assess their biological performance and applicability as cell carriers.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree: 85%, M_η: 4×10^5), hyaluronic acid sodium, succinic anhydride, sodium periodate, ethylene glycol, *t*-butyl carbazate, ninhydrin and L-lactic acid were purchased from Sigma–Aldrich, 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 S-CS

S-CS was synthesized according to an already reported procedure slightly modified [27]. 0.5 g of chitosan was dissolved in 40 mL 5% (v/v) lactic acid solution and then 160 mL methanol was added to dilute the solution. 1.5 g of succinic anhydride was added to this solution with stirring at room temperature. After 24 h, the succinyl modified chitosan was precipitated by adjusting the solution pH to 6–7. The precipitate was filtered, re-dissolved in H_2O, and dialyzed for 3 days. The purified product was freeze-dried and stored at 4 °C. The substitution degree of S-CS was determined by the ninhydrin assay [32].

2.3. Synthesis of A-HA

A-HA was synthesized according to an already reported procedure slightly modified [21,22]. 1.0 g HA (~2.5 mmol) was dissolved in 100 mL nanopure H_2O at a concentration of 10 mg/mL. An aqueous solution of sodium periodate (0.5 M, 5 mL) was added dropwise, and the reaction was stirred for 2 h at room temperature in the dark. 1 mL Ethylene glycol was then added to inactivate any unreacted periodate. The reaction was stirred for 1 h at ambient temperature and the solution was purified by exhaustive dialysis against H_2O for 3 days, and the dry product was obtained by freeze-drying. The percentage oxidation of A-HA was quantified by measuring the number of aldehydes in the polymer using *t*-butyl carbazate [33].

2.4. Fabrication of composite hydrogels

S-CS and A-HA were dissolved in PBS separately at a concentration of 20 mg/mL. The crosslinked composite hydrogels were formed by mixing S-CS and A-HA solutions at various volume ratios of 1/9, 3/7, 5/5, 7/3 and 9/1 at room temperature. The gelation time of composite hydrogels was monitored.

2.5. Characterization of composite hydrogels

2.5.1. Morphologies

Morphologies of composite hydrogels were characterized by utilizing scanning electron microscopy (SEM) after gelation. The hydrogels were freeze-dried and then gold-coated using a Cressington 108 Auto (Cressington, Watford UK). The surface and cross-sectional morphologies were viewed using a JSM-6330F SEM (JEOL, Peabody, MA) operated at 10 kV accelerating voltage.

2.5.2. Infrared (IR) spectroscopic measurement

Fourier transformed infrared (FTIR) spectra of polysaccharides and hydrogel membranes were measured to confirm the expected pendant functionalities. Various samples were recorded with FTIR spectrometer (Nicolet Avatar 360, USA) against a blank KBr pellet background.

2.5.3. Equilibrium swelling

The known weights of freeze-dried hydrogels were immersed in DMEM/F12/10% FBS and PBS, respectively, and kept at 37 °C for 2 h until equilibrium of swelling had been reached. The swollen hydrogels were removed and immediately weighed with a microbalance after the excess of water lying on the surfaces was absorbed with a filter paper. The equilibrium swelling ratio (ESR) was calculated using the following equation:

$$\mathrm{ESR} = (\mathrm{Ws} - \mathrm{Wd})/\mathrm{Wd}$$

where Ws and Wd are the weights of the hydrogels at the equilibrium swelling state and at the dry state, respectively.

2.5.4. Compressive modulus

Mixtures of solutions described above were injected into a 12-well culture plate for 15 min to obtain columned hydrogels (22 mm diameter, 6 mm height). Compressive modulus of elasticity was measured in the elastic region of hydrogel

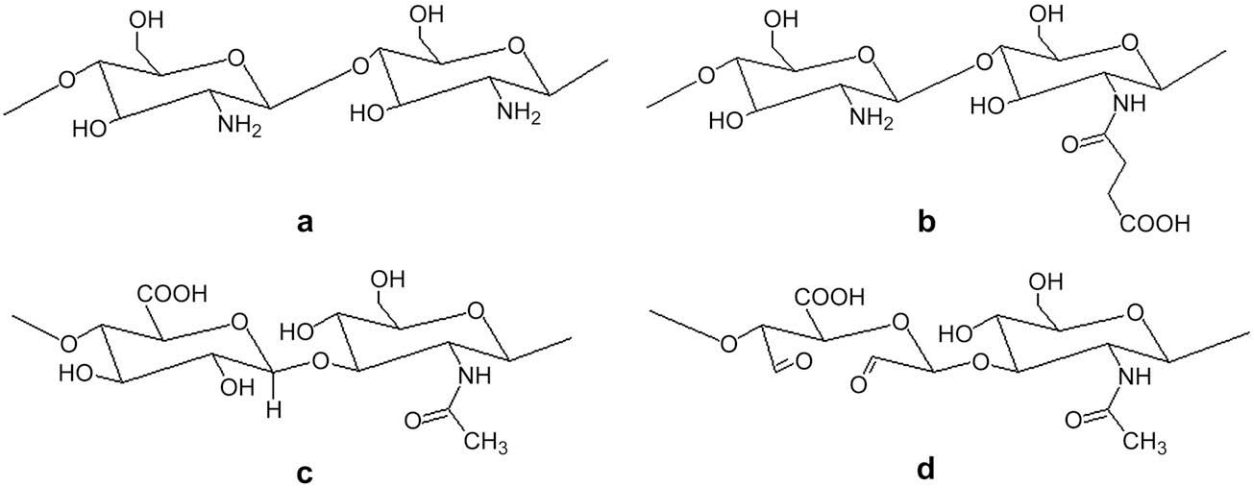

Fig. 1. Chemical structures of chitosan (CS) (a), *N*-succinyl-chitosan (S-CS) (b), hyaluronic acid (HA) (c) and aldehyde hyaluronic acid (A-HA) (d).