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Research Paper

Rheological, mechanical and degradable properties of injectable chitosan/silk fibroin/hydroxyapatite/glycerophosphate hydrogels

*Jingjing Wu, Jiaoyan Liu, Yanmei Shi, Ying Wan**

College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, PR China

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ABSTRACT

Silk fibroin (SF) and hydroxyapatite (HA) were incorporated into chitosan/glycerophosphate (GP) system to prepare new types of hydrogels. The formulated chitosan/SF/GP and chitosan/SF/HA/GP solutions were found to be injectable at room temperature, and able to form into hydrogels at near-physiological temperature and pH. Rheological measurements showed that elastic modulus of certain chitosan/SF/GP and chitosan/SF/HA/GP gels could reach around 1.8 and 15 kPa, respectively, and was much higher than their respective viscous modulus. Compressive measurements revealed that some chitosan/SF/GP and chitosan/SF/HA/GP gels had 8 and 20-fold modulus and strength higher than the chitosan/GP gel, respectively, confirming that compressive properties of these gels were greatly improved. Results obtained from *in vivo* degradation demonstrated that degradation endurance of the optimized chitosan/SF/GP and chitosan/SF/HA/GP gels was significantly enhanced as compared to the chitosan/GP gel, and the degradation rate of the gels could be regulated by the SF component alone or by the combination of SF and HA components.

1. Introduction

Articular cartilage damage often occurs as a result of joint-related injuries, tumor extirpation and diseases such as osteoarthritis, rheumatoid arthritis and congenital defects (Chiang and Jiang, 2009). Adult articular cartilage has a limited capacity for natural regeneration because of its low mitotic activity, avascular nature and sparse cell population (Thomas et al., 2009). The current treatment modalities mainly include arthroscopic lavage with or without corticosteroids, microfracture, subchondral bone drilling, abrasion arthroplasty and mosaicplasty (Hao et al., 2010; Madeira et al., 2015). In addition, autologous implantation involving chondrocytes and periosteum is also used for repairing articular cartilage defects (Remya and Nair, 2013). Although various improvements have been reported in clinical practice, these strategies have intrinsic limitations. They appear to be inadequate to long-term articular cartilage repair, and often lead to the formation of inferior fibro-cartilaginous tissue in the cartilage defect void. As a result, progressive deterioration of the regenerated cartilage tissue frequently occurs in the later periods (Chiang and Jiang, 2009; Hunziker, 2002; Matsiko et al., 2013).

*Corresponding author. Fax: +86 27 87792234.
E-mail address: ying_x_wan@yahoo.ca (Y. Wan).

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Injectable biomaterials have drawn increased attention in tissue engineering over the last decade due to their advantages of minimal invasive injection procedure (Hou et al., 2004; Hu et al., 2008; Madeira et al., 2015; Vermonden and Klumperman, 2015) and convenient filling of irregular tissue defects (Sheehy et al., 2015). So far, different types of hydrogels based on natural polymers have been produced for the injectable applications (Berthiaume et al., 2011; Fu et al., 2013; Kretlow et al., 2007; Tan and Marra, 2010). Among them, the chitosan/glycerophosphate (GP) hydrogel has received strong interest (LaPorta et al., 2012; H.Y. Zhou et al., 2015). In recent years, chitosan/GP gels have already been used for repairing cartilage, bone and nerves; and also, functioned as injectable vehicles for delivering different drugs or bioactive molecules (Tan and Marra, 2010; H.Y. Zhou et al., 2015).

However, the chitosan/GP gel exhibits low mechanical strength and has fast *in vivo* degradation rate, which limits its applications. To date, efforts have been made to enhance the chitosan/GP gel using additional natural polymers, such as collagen, starch and chopped silk fibers, respectively. The resulting gels show improvements in strength while having lowered incipient gelation temperatures (Ngoenkam et al., 2010; Mirahmadi et al., 2013; Wang and Stegemann, 2010). Nevertheless, the modified chitosan/GP gels involving the use of collagen or starch may not be able to maintain necessitated strength for a long enough period of time *in vivo* due to the rapid degradation of collagen or starch. The concern of chopped silk fibers is that the fibrous component could be unfavorable for the injection of the gel. Hence, the chitosan/GP gel still needs to be modified to increase its strength and degradation tolerance while maintaining its injectability.

Silk fibroin (SF) is a type of natural fibrous protein and has been commonly used for cartilage repair (Hardy and Scheibel, 2010; O'Brien, 2011; Vepari and Kaplan, 2007). SF scaffolds in wet state show robust mechanical properties and have significantly slower *in vivo* degradation rate as compared to that made from other natural polymers such as collagen, starch and chitosan (Bhardwaj and Kundu, 2011; Kluge et al., 2010; Vepari and Kaplan, 2007). Therefore, SF could be the additional component to enhance the strength and degradation tolerance of the chitosan/GP gel.

Histological analysis shows that articular cartilage can be approximately divided into four identifiable layers, commonly named as the superficial layer, intermediate layer, deep layer and calcified layer (Castro et al., 2012; Hunziker, 2002). Among these layers, the calcified layer functions as a crucial layer to intimately connect the articular cartilage with the subchondral bone (Castro et al., 2012; Levingstone et al., 2014). Therefore, layered structures containing a calcified layer with required compositions and functions are essential for the regenerated articular cartilage tissue. Some iterative layering techniques have been developed, and varied materials, including collagen, polysaccharides and nano-hydroxyapatite (HA), have been used to build layered scaffolds to achieve improved results in cartilage repair (Jeon et al., 2014; Levingstone et al., 2014). Given that incorporating SF and HA into the chitosan/GP gel is able to generate a new type of injectable gel with improved strength and degradation tolerance, it will be feasible to use the gel to build different layers for cartilage repair in an injectable manner by regulating the compositions of the gel. In addition, this type of gel would also be quite useful in periodontal repair or reconstruction (Foss et al., 2014; Jhaveri-Desai and Khetarpal, 2011; Susin and Wikesjo, 2013).

In the present study, a type of injectable hydrogel was prepared by incorporating SF and HA into the chitosan/GP system. The rheological and mechanical properties as well as the degradation of the resulting gels were investigated. It was found that optimal chitosan/SF/HA/GP gels indeed showed well-defined phase-transition characteristics at near-physiological temperature and pH with significantly enhanced strength and increased degradation tolerance in comparison to the chitosan/GP gel.

2. Experimental

2.1. Materials

Chitosan was purchased from Aladdin, China. Viscosity-average molecular weight of chitosan was measured as 2.2 ($\pm$0.12) $\times 10^3$ kDa with Ubbelohde-type viscometer by using a solvent composed of 0.25 M CH_3COOH/0.25 M CH_3COONa (Wan et al., 2004). Degree of deacetylation for chitosan was determined as 94.2 ($\pm$1.6)% based on the first derivative UV spectra of chitosan (Wan et al., 2004). All other reagents and chemicals were of analytical grade and purchased from Sinopharm, China.

SF was isolated from *Bombyx mori* cocoons according to methods mentioned elsewhere (Zhang et al., 2005; T. Zhou et al., 2015). The SF solution was dialyzed against distilled water for 3 days using membrane tubes (MW cutoff: 3500). The achieved SF solution with a concentration of around 1.2 wt% was further concentrated by immersing the SF-solution-loaded membrane tube in a PEG20000 solution for 2 h. The concentrated SF solution with a concentration of about 2.0 wt% was sucked out of the membrane tube and lyophilized at $-65\ ^{\circ}C$ for further use.

HA was synthesized using a coprecipitation method similar to that described in our previous study (T. Zhou et al., 2015). In brief, to 350 mL of 0.2 M $Ca(NO_3)_2 \cdot 4H_2O$ solution in ethanol, 210 mL of 0.2 M $(NH_4)_2HPO_4$ solution in distilled water were added dropwise. The pH of the mixture was adjusted to around 11 using a 25% $NH_3 \cdot H_2O$ solution. After that, the mixture was stirred for 24 h, and stored at room temperature for additional 24 h, followed by filtration under reduced pressure. The product was washed repeatedly with distilled water to neutral pH and dried at 60 °C in an oven. The content of calcium in HA was determined by titration of its ethylene diamine tetraacetic acid complexes (Kim and Vipulanandan, 2003). The phosphorus content in HA was measured following a phosphomolybdate-quinoline precipitation method (Xie et al., 2015). The Ca/P ratio in the so-produced HA was about 1.67.

2.2. Preparation of composite solutions

Chitosan/SF/GP composite solutions were prepared as follows. Briefly, 200 mg of chitosan were dissolved in 9 mL of 0.1 M HCl. To this solution, prescribed amounts of SF were