Carbohydrate Polymers 113 (2014) 240–248

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

A comparison of physicochemical properties of sterilized chitosan hydrogel and its applicability in a canine model of periodontal regeneration

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 23 June 2014
Accepted 2 July 2014
Available online 16 July 2014

Keywords:
Chitosan
Periodontal tissue engineering
Thermosensitive hydrogel
Scaffold

ABSTRACT

Chitosan has previously been exploited as a scaffold in tissue engineering processes. To avoid infection, chitosan must be sterilized prior to contact with bodily fluids or blood. Previous research has shown that autoclaved chitosan solution lead to decreased molecular weight, dynamic viscosity, and rate of gelling. We prepared a thermosensitive chitosan hydrogel using autoclaved chitosan powder (121 °C, 10 min) and β-glycerophosphate (chitosan-PA/GP) and compared the physicochemical properties and biocompatibility *in vitro* with autoclaved chitosan solution/GP hydrogel. The chitosan-PA/GP hydrogel had a shortened gelation time, higher viscosity, increased water absorption, appropriate degradation time, porous structure, and no obvious cytotoxicity on human periodontal ligament cells. Scanning electron microscopy demonstrated that the cells exhibited a normal morphology. The chitosan-PA/GP hydrogel promoted periodontal tissue regeneration in dog class III furcation defects. The chitosan-PA/GP thermosensitive hydrogel displayed suitable physicochemical properties and biocompatibilities and represents a promising candidate as an injectable tissue engineering scaffold.

1. Introduction

In periodontal tissue engineering, the ultimate goal is to create a space in which functional cells can grow (Bartold, McCulloch, Narayanan, & Pitaru, 2000). To this end, different types of scaffolds, such as hydrogels, have been applied during periodontal treatment. Chitosan is widely used in medical treatments and for wound healing processes, including periodontal tissue engineering, in accordance with its non-toxic, biodegradable, biocompatible, adhesive, and antibacterial properties (Mattioli Belmonte et al., 1998; Muzzarelli., 1993; Muzzarelli, 2009, 2010). When combined with β-glycerophosphate (GP), chitosan/GP can be maintained stably in solution at room temperature for extended periods of time. At physiological temperatures (37 °C), the chitosan/GP complex turns into a hydrogel, which may improve its clinical applicability and operative convenience. This complex can also be loaded with drugs (Barreiro-Iglesias, Coronilla, Concheiro, & Alvarez-Lorenzo, 2005; Chen, Tian, & Du, 2004) that release slowly over time and may even function as a scaffold for cell growth (Ji, Khademhosseini, & Dehghani, 2011; Niranjan et al., 2013).

Sterilization of the chitosan/GP hydrogel is required prior to implantation in the body or contact with bodily fluids. Previous research has demonstrated that the physical, chemical, mechanical, and biological properties of biomaterials can be negatively affected by sterilization (Jarry et al., 2001; Jarry, Leroux, Haeck, & Chaput, 2002; Lim, Khor, & Ling, 1999). Autoclaving, dry heat, ultraviolet radiation, gamma radiation, ethylene oxidation, and immersion in alcohol aqueous solutions are all methods previously exploited to sterilize chitosan. Ethylene oxide and gamma radiation have adequate effectiveness and are compatible with a wide array of materials. However, ethylene oxide is highly flammable, produces toxic residues, and has environmental exposure risks. Gamma radiation negatively affected polymer properties and performance due to material-intrinsic sensitivities or susceptibilities. Ultraviolet radiation was previously shown to cause accelerated degradation of chitosan (Yue, He, Yao, & Wei, 2009). Because ethanol is not a true

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http://dx.doi.org/10.1016/j.carbpol.2014.07.018
0144-8617/

sterilizing agent, it does not kill the endospores of many bacteria (Holy, Cheng, Davies, & Shoichet, 2001).

Steam sterilization is a simple and effective process that does not produce toxic residues. It is currently considered to be the most practical means of sterilizing medical devices and fluids. After sterilization by autoclave or gamma radiation, the gelation time of chitosan solution was increased significantly (Peng et al., 2012; Jarry et al., 2001). Most biomedical polymers, however, are hydrolyzed, degraded and soften under the high temperature and pressure conditions of steam sterilization. Previous studies have reported the effect of different sterilization methods on the chitosan membranes and injectable chitosan hydrogel (Marreco, da Luz Moreira, Genari, & Moraes, 2004; Rao & Sharma, 1995). After sterilization, however, the chitosan membranes or chitosan/GP hydrogel have proven difficult to supplement with cells or growth factors due to thermosensitive behavior. Steam autoclaving is often used to sterilize the chitosan solution prior to the addition of GP (Jarry et al., 2001). Although the chitosan molecular weight (MW) is reduced following the autoclaving process, the hydrogels that have been prepared using autoclaved chitosan did have the desired thermosensitivity for biological-relevant applications and this autoclaving method was deemed suitable for sterilization (Jarry et al., 2002). In addition, Yang *et al.* (Yang, Zhao, Liu, Ding, & Gu, 2007) demonstrated that the steam sterilization process caused no change in the chemical structure of chitosan (powder form). For this study, we selected the steam sterilization method to treat chitosan powder, prior to solution preparation, and already-mixed chitosan solution and evaluated its effect on the physicochemical properties of the chitosan.

The proportion of chitosan to GP in the hydrogel can vary and volume:volume ratios of 9:1 (Chenite et al., 2000) and 7:1 (Wang, Yang, Duan, Li, & Zhang, 2006) have both been reported in investigative research studies. It is still necessary to establish what the optimal ratio is for cell proliferation and adhesion within the chitosan/GP gel. Ji et al. (Ji et al., 2009a, 2010) previously published a quaternized chitosan (CS-HTCC) containing α,β-GP (CS-HTCC/GP) gel and supplemented with bFGF in a canine model of periodontal regeneration. The final viscosity of their CS-HTCC/GP hydrogel, however, was low, which may limit its application in tissue engineering. The observed periodontal repair may be due in part to the co-application of bFGF. On the other hand, our previous report showed that (Peng et al., 2012) gelation time for chitosan/GP was increased significantly after sterilization by autoclave or gamma radiation (Jarry et al., 2001). The present study was designed to investigate systematically the influence of steam sterilization methods on the physicochemical properties, cytocompatibility, and growth of human periodontal ligament cells (HPDLCs) in chitosan/GP without the addition of growth factors. Additionally, sterilized chitosan/GP thermosensitive hydrogels were implanted in a canine model of periodontal defects to evaluate regenerative potential.

2. Materials and methods

2.1. Preparation of the chitosan/GP solution

The solution of autoclaved chitosan powder (chitosan-PA) was prepared by adding 200 mg autoclaved (dry cycle; 121 °C for 10 min) chitosan (median MW, 75–85% degree of deacetylation (DD); Catalogue No. 448877; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) to 9 mL hydrochloric acid (0.1 mol/L), stirring until completely dissolved. The autoclaved chitosan solution (chitosan-SA) was prepared by adding 200 mg chitosan to 9 mL hydrochloric acid (0.1 mol/L) while stirring; the samples of chitosan solution were then autoclaved at 121 °C for 10 min (Jarry et al., 2001). The primary difference between these two groups is the process sequence of the chitosan sterilization step, which occurred either before (chitosan powder) or after (chitosan solution) the gelation step. Both solutions were processed in a similar manner thereafter. β-glycerophosphate (GP) (E. Merck, Darmstadt, Germany) (560 mg) was dissolved in 1 mL distilled water and sterilized by filtration. Before use, both the chitosan and GP solutions were chilled in an ice bath for 15 min to avoid gelation. The 1 mL GP solution was then added dropwise to the 9 mL chitosan-SA with continuous stirring to form a clear homogeneous chitosan-SA/GP (9:1, volume:volume) solution. The chitosan-PA/GP (9:1) and chitosan-PA/GP (7:1) solutions were prepared as described above but with the autoclaved chitosan powder. Preparation of the chitosan-PA/GP (9:1), of the chitosan-PA/GP (7:1), and of the chitosan-SA/GP (9:1) was performed on a clean bench and all prepared solutions were sealed and stored at 4 °C until use.

2.2. Determination of chitosan/GP thermosensitive hydrogel properties

The chitosan/GP hydrogel was placed in a 37 °C water bath. Using the test tube inversion method (Chung, Simmons, Gutowska, & Jeong, 2002), we assayed the thermosensitivity of chitosan-PA/GP and chitosan-SA/GP hydrogels. The sol-to-gel behavior of chitosan/GP thermosensitive hydrogels was further studied by measuring the sample solution viscosity at predetermined time intervals using a viscometer at 37 °C.

The obtained chitosan hydrogels were frozen at −80 °C for 24 h and then lyophilized in a freeze dryer (LGJ-10; Xi'an Zhongnuo Co., Ltd., China) for 24 h at −40 °C (Yang et al., 2010). The surfaces of chitosan/GP hydrogels were coated with a gold–palladium layer and observed using scanning electron microscopy (SEM) (S-4800, HITACHI, Japan). Mean pore diameters were analyzed by digital SEM photos of sectioned samples.

2.3. Swelling studies

Chitosan/GP hydrogels were weighed and fully rehydrated in a sealed container with phosphate buffered saline (PBS; pH 7.4) as previously described (Chen et al., 2004) for 24 h at 37 °C to study the swelling characteristics. Samples were taken out of PBS and the surface was dried with filter paper and weighed again. The swelling ratio was determined gravimetrically using following equation:

$$D_s\,(\%) = \frac{W_s - W_0}{W_0} \times 100\%,$$

where W_0 is the dry weight and W_s is the saturated weight.

2.4. Biodegradability

Chitosan/GP hydrogel was prepared at a 2 mm thickness and 1 cm diameter and incubated in 2 mL of DMEM medium with 0.1% (w/v) sodium azide (Wolsen Biotechnology Co. Ltd., Shanxi, China) containing 500 μg/mL chicken egg white lysozyme (Wolsen Biotechnology Co. Ltd., Shanxi, China). As a control, hydrogel was also treated with same medium but without lysozyme. The samples were then put into a 37 °C incubator for 21 days and the medium was replenished weekly. Dry weights of the samples were measured on the 1st, 4th, 7th, 14th, and 21st days of incubation. Degradation was determined by percentage of weight loss (W_L) using following equation:

$$W_L\,(\%) = \frac{W_L - W_0}{W_0} \times 100\%,$$

where W_0 was the initial weight and W_L was the weight after degradation.