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Chitosan microparticles based polyelectrolyte complex scaffolds for bone tissue engineering *in vitro* and effect of calcium phosphate

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ABSTRACT

Chitosan microparticles were mixed with chitosan and carboxymethyl cellulose solution to achieve a good binding between the microparticles. Three different compositions of scaffolds were made by varying the calcium phosphate (CaP) amount: 0%, 10%, and 20%. Potassium chloride was used as salt, to make pores inside the scaffolds after leaching out when immersed in phosphate buffer saline (PBS). Compressive strength and compressive modulus of both non-porous (before leaching out), and porous (after leaching out) scaffolds were measured according to the ASTM standards. The highest compressive strength of 27 MPa was reported on 10% CaP scaffolds while 20% CaP scaffolds showed the lowest. The increasing CaP content reduces the compressive strength of the scaffolds. The highest wet state compressive strength was reported on 0% CaP scaffolds with 0.36 MPs and 0.40 MPa at day 1 and day 3 respectively. *In vitro* cell culture studies showed good cell adhesion and cell proliferation on 10% CaP scaffolds.

1. Introduction

In bone tissue engineering, biodegradable scaffolds play an important role, since it serves as a temporary skeleton for the lost bone or site of defective bone to support and stimulate the tissue growth and bone regeneration while the scaffold gradually degrades and replaced by the new bone tissue (Li, Ramay, Hauch, Xiao, & Zhang, 2005; Persidis, 1999; Petite et al., 2000; Vacanti & Langer, 1999). Bone is a heterogeneous composite which consists of inorganic phase, organic phase, and water in decreasing order. Inorganic phase mainly contains hydroxyapatite, and organic phase contains type I collagen, non-collagenous protein, and lipids (Boskey, 2013). Therefore, a combination of both ceramic and polymer provides a better-suited scaffold for bone tissue engineering. Moreover, the porous materials are most suited with bone tissue applications, as porosity allows osteogenesis into the pores, which strengthen the union between the host bone and the implant (Langer & Vacanti, 1993).

Biopolymers have been used over the decades as scaffolding materials for bone grafts due to its favorable biological properties, such as biodegradability and biocompatibility. Natural polymers have shown more favorable biological properties compared with the synthetic polymers. Synthetic polymers demonstrate lower cell adhesion due to their hydrophobic nature and lack of functional groups for further surface modification (Cai et al., 2009; Li et al., 2005). Chitosan, a natural cationic copolymer of β-[1→4]-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose, is one of the most studied natural polymers in the field of tissue engineering due to its appealing intrinsic properties, such as biodegradability, bioactivity, non-toxicity. At physiological pH, chitosan is positively charged and hence susceptible to surface modifications and proper cell adhesions (Berger, Reist, Mayer, Felt, & Gurny, 2004). Also, the biodegradability of chitosan is due to the enzymes in the human body, such as lysozyme (Berger et al., 2004; Seda Tiğli, Karakeçili, & Gumusderelioglu, 2007). The degradation rate of chitosan by lysozyme is inversely related to the molecular weight, and degree of crystallinity of the chitosan (Nwe, Furuike, & Tamura, 2009). Also, chitosan with a higher degree of deacetylation (DD) shows higher degradation (Thein-Han & Kitiyanant, 2007; VandeVord et al., 2002). Because of the *N*-acetylglucosamine repeating units, chitosan has some similarity to the glycosaminoglycan (GAG), the major component of the extracellular matrix of bone and cartilage (Khor & Lim, 2003), and thus binds to the growth factors (Muzzarelli et al., 1994). Also, chitosan scaffolds are osteoconductive, and enhance osteogenesis has been shown in both *in vitro* and *in vivo* conditions.

Carboxymethylcellulose (CMC), an anionic hydrophilic polymer derived from cellulose, is readily soluble in water due to the presence of carboxymethyl group. This negatively charged carboxymethyl group allows making complexation with positively charged polymers, such as

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chitosan (Gaihre & Jayasuriya, 2016; Kassem, Negm, Shukry, & El-Kalyouby, 2014; Rosca, Popa, Lisa, & Chitanu, 2005). Several authors have studied this strong polyelectrolyte complex due to its improved biological functions (Chen & Fan, 2007; Kawasaki, Nakaji-Hirabayashi, Masuyama, Fujita, & Kitano, 2016; Sainitya et al., 2015). The primary concern associated with the biopolymer scaffold is their low mechanical strength and shape retention problems due to excessive swelling. The polyelectrolyte complex formation reduces the swelling. Adding bioactive ceramics is one of the standard methods to increase the mechanical properties of the polymer scaffolds. Calcium phosphate (CaP) bioceramics, including hydroxyapatite (HA), are the favored bioceramic types used in bone tissue engineering due to its chemical and crystallographic similarities to the human bone. These CaP ceramics showed good osteoconductivity, and hence these are heavily used in numerous craniofacial and orthopedic procedures (Xu & Simon, 2005).

Number of attempts have been taken to improve the mechanical properties of the chitosan-based scaffolds by incorporating bioceramics, such as nano-HA (nHA) (Cai et al., 2009; Oliveira et al., 2006; Thein-Han & Misra, 2009), calcium phosphate (Sendemir-Urkmez & Jamison, 2007; Xu & Simon, 2005), and β-tricalcium phosphate (Yin et al., 2003). These studies indicated a significant improvement in the compressive strength of chitosan-based scaffolds. Jiang et al. (2008) reported 3.54 MPa of compressive strength in nHA/chitosan/CMC scaffolds. Also, it was reported that the highest tensile strength of 40 MPa in the dry state and 12 MPa in the wet state (Liuyun, Yubao, & Chengdong, 2009). Moreau and Xu (2009) reported that chitosan-calcium phosphate scaffolds with the flexural strength of 10 MPa in the dry state. All of these reported mechanical strength values are higher than the polymer scaffolds without bioceramics (Chung et al., 2002; Li et al., 2005). Wan, Wu, Cao, and Dalai (2008) reported that the incorporation of synthetic polymer, poly(caprolactone) into chitosan was increased the mechanical properties.

The main objective of this research work was to develop a natural polymer-based scaffold which can exhibit better mechanical properties in both dry and wet state. According to the published literature, the mechanical stability of the polymer-based scaffolds in the wet state is not in the acceptable level for using as bone grafts. Therefore, we investigated a method to enhance the mechanical stability by incorporating chitosan microparticles (MPs). Even though chitosan MPs were extensively studied as a drug delivery system, insufficient studies have been carried out to incorporate MPs into the scaffolds. In this study, commonly used salt leaching method was used to fabricate the porous scaffolds with chitosan MPs. CS solution and CMC solution were used as a binder to aggregate chitosan MPs. Also, Calcium phosphate (CaP) only scaffolds were previously studied in our lab, and low mechanical properties and poor stability in wet conditions were reported (Aryaei, Liu, Jayatissa, & Champa Jayasuriya, 2015). CaP was added as an osteoconductive material to enhance the cell attachment and proliferation. So, we examined the compressive strength and mechanical stability of Chitosan MPs based scaffold with the addition of the CaP and further, the cytotoxicity of the scaffolds were checked on murine pre-osteoblast cells.

2. Materials and methods

2.1. Materials

Low MW chitosan (MW: 50,000–190,000 kDa) with a DD of 85%, potassium chloride (ACS reagent 99%), sodium tripolyphosphate (TPP), acetic acid (99.7%), sodium carboxymethylcellulose (CMC - MW: 90,000), hexamethyldisilazane (HMDS), and cell proliferation reagent WST-1 (Roche diagnostic) were all purchased from Sigma Aldrich Chemicals (St. Louis, MO, USA). Calcium phosphate tribasic (CaP) and calcium chloride dehydrate were obtained from Fisher Scientific (USA). Alpha minimum essential media (α-MEM), Fetal Bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's phosphate buffered saline (DPBS), and penicillin/streptomycin were purchased from Gibco, life technologies (Thermo Fisher Scientific, USA). Live/Dead cell viability/cytotoxicity kit was purchased from the Invitrogen (USA). 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution was purchased from electron microscopy sciences (Hatfield, PA, USA).

2.2. Fabrication of microparticles and scaffolds

2.2.1. Preparation of microparticles (MPs)

Simple coacervation method was used to prepare the chitosan MPs. 20 ml of 2% (w/v) chitosan solution was made using 2% (v/v) acetic acid and the solution was filtered through a nylon mesh with 52 μm pores to remove any undissolved chitosan. Then the solution was added dropwise to the 300 ml of 1% (w/v) TPP solution while under continuous stirring at 300 rpm. The mixture was kept for 4 h under stirring for proper crosslinking between chitosan and TPP. After 4 h, MPs were washed with deionized (DI) water and air-dried overnight.

2.2.2. Preparation of scaffolds

Scaffolds were prepared using three different compositions of the CaP, 0%, 10%, and 20% (w/w). All the scaffolds contained varying amount of chitosan MPs according to the CaP content and fixed 20% (w/w) KCl salt to make pores after leaching out. The weight percentages were calculated according to the solid portion of the scaffold. All the solid portions were mixed well and then 2% (w/v) CMC solution and 2% (w/v) chitosan solution with 2% (w/v) $CaCl_2$ were added to the mixture. For 1 g of solid portion, 1 ml of chitosan and 1 ml of CMC solutions were used. For 1 g of solid portion, 800 mg of MPs and 200 mg of KCL was added to make the 0%CaP scaffolds. 100 mg of CaP was added to 10% CaP scaffolds while reducing the MPs content to 700 mg, and 200 mg of CaP and 600 mg of MPs were added to make the 20% CaP scaffolds. After proper mixing, the mixture was added to the stainless steel cylindrical molds, and the molds were properly closed using two glass slides, as shown in the Fig. 1, and air-dried for two days. After drying, the scaffolds were immersed in PBS solution for three days to leach out the KCl salt. To distinguish the effect of MPs on the mechanical stability, CaP only scaffolds were prepared by using 80%(w/w) of CaP and 20%(w/w) of KCl and mixing with same amount of CS and CMC solutions.

2.3. Porosity of scaffolds

The porosity of scaffolds was calculated using the following equation with a measured volume of scaffolds.

$$\text{Porosity} = \left(\frac{\text{Volume of Pores}}{\text{Total Volume}}\right) \text{x } 100\% = \left(\frac{\text{Total Volume} - \text{Volume of Solids}}{\text{Total Volume}}\right) \text{x } 100\% \quad (1)$$

2.4. Testing of mechanical properties

Cylindrical test samples were used for all the mechanical tests according to the ASTM standard. Four types of scaffold conditions were used to understand the mechanical behavior under various conditions: (i) non-porous dry scaffolds before leaching out of the KCl salt, (ii) porous dry scaffolds after leaching out of the KCl salt, (iii) wet scaffolds at day 1- immersed in 200% (w/w) PBS for one day, and (iv) wet scaffolds at day 3 – immersed in 200% (w/w) PBS for three days. All the tests were performed using the ADMET eXpert 2600 series Universal testing machine, and constant rate of 0.01 mm/s was used. The compressive strength and compressive modulus of the scaffolds were calculated using the 0.2% offset method and the linear portion of the stress-strain curve respectively.