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Biomimetic mineralization of carboxymethyl chitosan nanofibers with improved osteogenic activity in vitro and in vivo

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ABSTRACT

Inspired by the natural extracellular matrix, the organic-inorganic composite nanofibers are promising scaffolds for bone tissue engineering. Chitosan-based nanofibers are widely used as bone tissue engineering scaffolds with good biocompatibility but pungent solvents are frequently used for its processing. Carboxymethyl chitosan (CMCS), a water-soluble derivative of chitosan, has better biodegradability and bioactivity which allows CMCS to chelate Ca^{2+} and induce the deposition of apatite. Moreover, with water as solvent, CMCS nanofibers avoid the acidic salt removal comparing to electrospun-chitosan. In this study, we successfully prepared uniform CMCS nanofibers with the aid of polyethylene oxide (PEO) and obtained the optimized conditions with a voltage of 25 kV and PEO of molecular weight 1000 kDa. We further prepared hydroxyapatite (HA) coated electrospun CMCS nanofibers by biomimetic mineralization using 5 times simulated body fluid. The promotion of osteogenic differentiation of mouse bone marrow stromal cells (mBMSCs) in vitro was evaluated on the nanofibers scaffolds. Cell experiments revealed that CMCS-HA composite nanofibers increased the ALP activity. The gene expression level of Runx2 and ALP were about 1.6 and 4.3 folds at the 7 days, and 5.1 and 10 folds at the 14 days on CMCS-HA nanofibrous membranes than that on CMCS alone samples. The level of OCN increased by 24 and 1.5 times on the CMCS-HA scaffolds than CMCS scaffolds at the 14 and 21 days. In vivo new bone formation by nanofiber scaffolds was investigated in a critical-size rat calvarial bone defect model. Micro-CT results showed that the whole defect was covered by new bone after CMCS-HA filling the defect for 12 weeks. The results of H&E staining and Masson's trichrome staining on histological sections further confirmed that composite nanofibers promoted new bone formation and maturation.

1. Introduction

Optimal scaffolding materials are important for bone tissue engineering and bone defect repair. In order to simulate the nanofiber structure of the natural extracellular matrix of bone, the nanofiber scaffolds are prepared by a variety of methods, such as electrospinning, thermal induced phase separation and self-assembly. Electrospinning has attracted great interest with the advantages of high efficiency and high porosity. A variety of synthetic and natural polymers such as polylactic acid (Kumbar, Nukavarapu, James, Nair, & Laurencin, 2008), polycaprolactone (Song, Yu, Markel, Shi, & Ren, 2013), polyethylene (Jaeger, Bergshoef, Batlle, Schönherr, & Julius Vancso, 1998), chitosan (Klossner, Queen, Coughlin, & Krause, 2008), collagen (Matthews, Wnek, Simpson, & Bowlin, 2002) and alginate (Lu, Zhu, Guo, Hu, & Yu, 2006) nanofibers have been prepared by electrospinning for bone tissue repair. Synthetic and natural polymers have both the advantage and disadvantage for bone tissue engineering scaffolds. The synthesis and modification of synthetic polymers are easier to control but lack of the cell recognition sites. Compared with the synthetic polymers natural polymers show better biocompatibility but the processing ability and mechanical properties are poor. So the electrospun polymers are used for tissue engineering scaffolds which need to be combined with other component to improve its performance (Lee et al., 2014; Pangon, Saesoo, Saengkrit, Ruktanonchai, & Intasanta, 2016b; Van Hong Thien, Hsiao, Ho, Li, & Shih, 2013; Wang, Ding, & Li, 2013).

At present, researchers have prepared a range of scaffolds that mimic the bone extracellular matrix (ECM) from the structure and composition (Wang et al., 2013). Preparing organic-inorganic composite nanofibers to simulate the composition of ECM is an effective strategy as bone tissue engineering scaffolds. The biomimetic method,

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which based on mineralization in the natural hard tissue deposition of biologic apatite, has opened up a new way to develop biomaterials. Because of the high specific surface area of nanoparticles, it is difficult to uniformly disperse nanoparticles through blending inorganic nanoparticles into polymer nanofibers. In biomineralization process, the polymeric nanofibers can regulate the nucleation and growth of inorganic nanoparticles from the solution and enhance the interactions between organic and inorganic components as well as improve the uniform distribution of inorganic nanoparticles. Some studies have investigated the organic-inorganic composite nanofibers prepared by biomimetic mineralization as bone tissue engineering scaffolds (Nitta et al., 2017; Wei et al., 2011; Zhang et al., 2016). Zhang et al. (Zhang et al., 2016) prepared poly(L-lactide)/gelatin composite nanofibers by electrospinning techniques and studied the effect of mineralization of nanofibers on the proliferation and differentiation of MC3T3-E1. Wei et al. (Wei et al., 2011) prepared silk fibroin/nano-hydroxyapatite (nHA) composite nanofibers by means of an effective calcium and phosphate (Ca-P) alternate soaking method and found that composite nanofibers had a significant effect on the differentiation of MC3T3-E1 cells. Chitosan (CS) is a deacetylated product of chitin, the second most abundant polysaccharide in the nature. Chitosan is composed by GlcN, which is also an important component of the glycosaminoglycans (GAG) of the ECM. Owing to the positive charge from the amino groups, chitosan can bind the cell membranes (Sivashankari & Prabaharan, 2016), which are unique compared to other natural polymers and has been widely used in tissue engineering field with good biocompatibility (Chen et al., 2015; C. Yu, Bao, Shi, Yang, & Yang, 2017). Calcium phosphate modification through biomimetic mineralization to chitosan-containing materials showed good potential in bone tissue engineering in the previous studies (Lin, Fu, Lin, Yang, & Gu, 2014; Nitta et al., 2017; Pangon, Saesoo, Saengkrit, Ruktanonchai, & Intasanta, 2016a). Lin et al. (Lin et al., 2014) used chitosan to modify the surface of electrospun poly(lactic acid) (PLA) nanofibers and promoted more nucleation and growth of calcium phosphate. They have to incubate the materials in 10 times simulated body fluid (10 × SBF) and the deposited minerals was mixtures of dicalcium phosphate dehydrate (DCPD) and apatite. Nitta et al. prepared chitosan nanofibers-PEG hydrogel and CaP hybrid composites (Nitta et al., 2017). They also obtained a mixture of HA and DCPD. In addition, PEG-based hydrogels are not naturally degradable or susceptible to slow degradation in vivo (Nitta et al., 2017). Pangon et al. (Pangon et al., 2016a) prepared HA-hybridized chitosan/chitin whisker bionanocomposite fibers and the composites exhibited good osteoblast cell adhesion and proliferation. Due to poor solubility of chitosan in water, acidic solvent has to be used during electrospinning. Despite a variety of biomineralization of chitosan-containing composites, the mineralization ability of pure chitosan was generally poor. Moreover, there were few reports on the bone repair evaluation of chitosan-based composite nanofibers in vivo. Datta et al. (Datta et al., 2013) prepared N-methylene phosphonic chitosan/PVA nanofibers and tested the biocompatibility in rabbit tibial condyle defects. The preliminary in vivo evaluation after 3 weeks of implantation confirmed no adverse tissue reaction while acceleration of bone healing under radiological examination.

Chitosan has very limited affinity for alkaline and alkaline-earth metals. In contrast, the chelation with more Ca2 + was enabled due to the introduction of carboxymethyl groups in carboxymethyl chitosan (CMCS), a water-soluble derivative of chitosan (Budiraharjo, Neoh, & Kang, 2012; Mourya, Inamdar, & Tiwari, 2010; Müller et al., 2015), therefore the CMCS may have the superior biomineralization property. In addition, CMCS enhanced the biodegradability after introduction of carboxymethyl (LogithKumar et al., 2016; Upadhyaya, Singh, Agarwal, & Tewari, 2014). In particular, electrospun chitosan-based nanofiber require the use of toxic or pungent solvents such as trifluoracetic acid and acetic acid, acidic salts are generated during the electrospinning process, so the acidic salt must be removed in the following experiment (Pangon et al., 2016a; Su et al., 2017). Electrospun CMCS nanofibers are more environmentally friendly and have better biocompatibility with the water as a solvent. However, according to the previous study at least 2–2.5 times of the entanglement concentration is needed to electrospin defect-free nanofibers (McKee, Wilkes, Colby & Long, 2004) and the pure CMCS nanofibers were difficult to electrospin on its own due to lack of chain entanglements and high surface tension. Poly(ethylene oxide)(PEO) or poly(vinyl alcohol) (PVA) is normally used to decrease the surface tension and increase the chain entanglement when prepare the water-soluble polymer electrospun nanofibers. Jeong et al. prepared the chitosan–alginate nanofibers with the aid of PEO (Jeong, Krebs, Bonino, Samorezov et al., 2010). Tamizi et al. have electrospun PVA/sodium alginate composite nanofibers with water as solvent (Tamizi, Azizi, Dorraji, Dusti, & Panahi-Azar, 2017).

In this study, we prepared the CMCS nanofibers by electrospinning technique using water as solvent and with the aid of PEO. The CMCS nanofibers were mineralized through immersion in 5xSBF system. The adhesion, proliferation and differentiation behaviour of mBMSCs on the composite nanofibers was investigated. Finally, the performance of bone tissue repair by the composite nanofibers was evaluated in a critical-size rat calvarial defect model.

2. Materials and methods

2.1. Preparation of CMCS nanofibers

The CMCS (M_W: 2.0×10^5–2.5×10^5, Deacetylation Degree ≥ 90%, degree of substitution of O-carboxymethyl groups: ≥ 90%, Huamaik Biotechnology CO., LTD, China) solution was prepared by the dissolution of 4 g CMCS in 100 ml distilled water. Polyethylene oxide (PEO) (Aladdin, china) was added in the solution with different molecular weight and mass fraction, lecithin (0.3%, w/v) (Aladdin, china) was also added for reducing the surface tension of the solution. For electrospinning process, the voltage was adjusted from 13 kV–25 kV and the distance between the collector position and the needle was 15 cm. The feeding rates were adjusted to 0.6 ml/h.

Chitosan (CAS: 9012-76-4, 50–100 mPa s, 0.5% in 0.5% Acetic Acid at 20 °C, Tokyo chemical industry CO., LTD, Japan) nanofibers were also electrospun for comparing the mineralization property to CMCS nanofibers. The 2 g Chitosan was dissolved in 100 ml 0.5% (w/v) dilute acetic acid, the 2 g PEO was added after chitosan was dissolved completely, finally the lecithin 0.3% (w/v) was added into the mixture. The voltage was 30 kV and the distance between the collector position and the needle was 15 cm. The feeding rates were also adjusted to 0.6 ml/h.

The as-spun CMCS and chitosan nanofibers were further crosslinked by 15 min of exposure to 25% glutaraldehyde steam (Zhou et al., 2007).

All the nanofiber scaffold preparations were immersed in deionized water for 2 days to remove the PEO from the scaffolds.

2.2. Biomimetic mineralization of the nanofibers in 5xSBF

5xSBF was prepared by dissolving 40.176 g NaCl, 1.773 g $NaHCO_3$, 1.124 g KCl, 1.147 g $K_2HPO_4{\cdot}3H_2O$, 1.556 g $MgCl_2{\cdot}6H2O$, 0.359 g Na_2SO_4, 1.445 g $CaCl_2$ in 1L deionized water at 37 °C, the pH was controlled for 6.4 with tris(hydroxymethyl) aminomethane (Tris) and 1 mol/L HCl before adding $CaCl_2$ (Barrere, van Blitterswijk, de Groot, & Layrolle, 2002). All chemicals were analytical grade reagents and purchased from Guangzhou Chemical Corporation.

CMCS and CS nanofibrous membranes were cut into the small pieces with the diameter of 10 mm and immersed into 0.5 ml 5xSBF for 6 h, 8 h and 16 h at 37 °C to evaluate the mineralization property. All the samples were then freeze dried for further experiment and characterization. The mineralization time of the CMCS-HA samples for cell culture and animal experiment was 16 h.