Carbohydrate Polymers 151 (2016) 335–347

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Carboxymethyl cellulose enables silk fibroin nanofibrous scaffold with enhanced biomimetic potential for bone tissue engineering application

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ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 23 May 2016
Accepted 24 May 2016
Available online 25 May 2016

Keywords:
Silk fibroin
Electrospinning
Carboxymethyl cellulose
Calcium phosphate
Tissue engineered scaffold

ABSTRACT

Novel silk fibroin (SF) and carboxymethyl cellulose (CMC) composite nanofibrous scaffold (SFC) were developed to investigate their ability to nucleate bioactive nanosized calcium phosphate (Ca/P) by biomineralization for bone tissue engineering application. The composite nanofibrous scaffold was prepared by free liquid surface electrospinning method. The developed composite nanofibrous scaffold was observed to control the size of Ca/P particle (≤100 nm) as well as uniform nucleation of Ca/P over the surface. The obtained nanofibrous scaffolds were fully characterized for their functional, structural and mechanical property. The XRD and EDX analysis depicted the development of apatite like crystals over SFC scaffolds of nanospherical in morphology and distributed uniformly throughout the surface of scaffold. Additionally, hydrophilicity as a measure of contact angle and water uptake capacity is higher than pure SF scaffold representing the superior cell supporting property of the SF/CMC scaffold. The effect of biomimetic Ca/P on osteogenic differentiation of umbilical cord blood derived human mesenchymal stem cells (hMSCs) studied in early and late stage of differentiation shows the improved osteoblastic differentiation capability as compared to pure silk fibroin. The obtained result confirms the positive correlation of alkaline phosphatase activity, alizarin staining and expression of runt-related transcription factor 2, osteocalcin and type1 collagen representing the biomimetic property of the scaffolds. Thus, the developed composite has been demonstrated to be a potential scaffold for bone tissue engineering application.

1. Introduction

The development of an ideal scaffold for bone tissue engineering application has been an extensive area of research in the last decades. Various biopolymers have emerged through time to meet the required specification for bone tissue regeneration. The polymer blends and composite are more efficient than the individual biopolymer as scaffold material (Li et al., 2014; Lü et al., 2013). *Bombyx mori* silk fibroin (SF), a naturally occurring biopolymer, has excellent tuneable mechanical properties which make it an important scaffold entity for hard as well as soft tissue engineering applications (Meinel et al., 2005; Minoura, Tsukada & Nagura, 1990; Omenetto & Kaplan, 2010; Santin, Motta, Freddi & Cannas, 1999). The two major components of *Bombyx mori* silk are silk fibroin, a hydrophobic structural protein consisting of equimolar ratio of heavy and light chain of protein and sericin, a hydrophilic component (Ochi, Hossain, Magoshi & Nemoto, 2002; Tanaka et al., 1999; Zhou et al., 2000). In the last decade, apart from silk various other proteins and polysaccharides based biopolymers such as cellulose, starch, chitosan, alginate and their derivatives have been chosen for their potential applications in many areas such as pharmacy, medicine, tissue engineering and biotechnology (Gombotz & Wee, 2012; Nguyen & West, 2002). Carboxymethylcellulose (CMC), a highly hydrophilic semi-synthetic natural polymer has significant super absorbing, defoaming and chelating abilities and finds widespread applicability in pharmaceutical industries (Ghanbarzadeh & Almasi, 2011; Wang & Wang, 2010; Whistler, 2012). The natural structural matrix of bone consists of type-I collagen fibers, which is reinforced with hydroxyapatite like inorganic phase by biomineralization (Fratzl, Gupta, Paschalis & Roschger, 2004). Hydroxyapatite, the main mineral phase of bone and skeletal systems have several favourable properties namely biocompatibility, osteoconductivity, osteoinductivity and bioactivity (Cao & Hench, 1996; Jiang et al., 2013; Suchanek & Yoshimura, 1998). Thus, it is significant to mimic the biomineralized organic phase of natural bone. The aim of this present work is

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http://dx.doi.org/10.1016/j.carbpol.2016.05.088
0144-8617/

to bring several advantageous properties of both silk fibroin (high mechanical strength, biocompability) and carboxymethylcellulose (hydrophilicity, chelating capacity) at the same template to develop a biomaterial for bone tissue engineering application. We consider the development of SF/CMC based composite scaffolds as a promising strategy to overcome the limitations of the pure SF scaffolds. In fact, there are published reports on SF/CMC based composite films and hydrogels being put into various biotechnology and biomedical applications (Ju et al., 2014; Kundu, Mohapatra & Kundu, 2011). The present research has however, focused on the processing of the composite material into nanofibers through free liquid surface electrospinning technique with an aim of creating a close juxtaposition between bone and the scaffold for enhanced osteointegration. Electrospun nanofibrous scaffold closely resembles the nanofibrous structure of natural extracellular matrix (ECM) and thus, promotes better cell attachment and proliferation (Ma & Zhang, 1999). SF and CMC have negligible inflammatory reactions and are biocompatible(Miyamoto, Takahashi, Ito, Inagaki & Noishiki, 1989; Santin, Motta, Freddi & Cannas, 1999). Moreover, the chelating ability of CMC can coordinate bonding between $-COO^-$ and Ca^{2+}, and elicit the homogeneous Ca/P crystal nucleation which is a vital osteogenic property that an ideal scaffold should demonstrate for bone regeneration. This paper not only presents a comprehensive understanding of the morphological and physicochemical characteristics of the SF/CMC nanofibrous scaffold, but also accounts a thorough investigation of their biological performance for bone regeneration through the use of seeded human mesenchymal stem cells (hMSCs) that are derived from the human umbilical cord blood. In this study, the novelty and the superiority of the SF/CMC nanofibrous scaffold over other polymeric/composite scaffolds has been explored to enhance osteogenic differentiation of the cells. The osteogenic response of MSCs to biomimetic SF/CMC composite nanofibrous scaffolds was assessed by expression analysis of osteogenic genes (RUNX2 transcription factor, Osteocalcin and type1 collagen), alkaline phosphatase activity, and calcium deposition.

2. Materials and methods

2.1. Materials

Bombyx mori silk cocoons were purchased from Central Tasar Research and Training Institute (Jharkhand, India). Lithium bromide (LiBr), sodium carbonate (Na_2CO_3) and formic acid (98%) were procured from Merck, India. Dulbecco's Modified Eagle Medium (DMEM), Phosphate-buffered saline (PBS), Fetal bovine serum (FBS), Trypsin (0.25%), Alexa-Fluor 488 conjugated phalloidin, Live/Dead staining kit, Antibiotic-Antimycotic solution, anti-RUNX2 antibody, anti-osteocalcin antibody and FITC-conjugated secondary antibodies, TRIzol, High-capacity cDNA Reverse Transcription Kit and SYBER Green RT-PCR kit were purchased from Invitrogen, USA. The dialysis cassette was obtained from Thermo fisher, USA. Bovine serum albumin (BSA), SIGMAFAST™ *p*-nitrophenyl phosphate (pNPP) tablets, paraformaldehyde, Triton X-100, 4′6-Diamidino-2-phenyindole (DAPI), Dimethyl Sulfoxide (DMSO), MTT assay kit, Alizarin red S (ARS) solution, CMC as sodium salt (molecular weight ≈700 kDa and degree of substitution ~0.65–0.85) ammonium hydroxide, cetylpyridinium chloride (CPC) were obtained from Sigma.

2.2. Preparation of regenerated silk fibroin

Silk fibroin (SF) was extracted from Bombxy mori silk cocoon and the regenerated aqueous SF solution was prepared following the previously established protocol (Rockwood, Preda, Yücel, Wang, Lovett & Kaplan, 2011; Sah & Pramanik, 2010). Briefly *Bombyx mori* silk cocoons were chopped and degummed in 0.02 M aqueous Na_2CO_3 for 20 min at 100 °C, followed by washing in distilled water to remove sericin and other impurities. After degumming, silk fibers were dried at 37 °C for overnight. Degummed silk fibers were then dissolved in 9.3 M LiBr aqueous solution at 45 °C for 2hr to 3hr resulted in a 10 wt% solution. The obtained solution was then dialyzed against deionized water for 2–3 days to remove LiBr ions. The obtained solutions were then centrifuged at 5000 rpm to remove un-dissolved impurities and aggregates. Finally SF solutions were freeze dried and used for further study.

2.3. Generation of SF/CMC nanofibers

Nanofibers of *SF/CMC* blends were fabricated by the electrospinning method. Four different compositions of SF/CMC (100/0, 99/1, 98/2 and 97/3 (w/w)) blend solutions (10 wt%) were prepared by dissolving appropriate amount of SF and CMC powder in 98% formic acid with stirring to make well dispersed homogenous solutions. A 10 wt% pure SF solution was used as control. The electrospinning of the solutions was done by using a free liquid surface electrospinning machine (NS Lab 200, ELMARCO), at 4.25 $kVcm^{-1}$ voltage, 40% relative humidity of electrospinning chamber and 12 rpm of wire based spinning electrode at 20 ± 2 °C. Multiple Taylor cones were developed over the spinning electrode in response to the potential difference created between spinning and collector electrode separated apart at 16 cm. The different composite scaffolds are designated as SF, SFC1A, SFC2 B and SFC3C for 100/0, 99/1, 98/2 and 97/3 (w/w) compositions respectively. The gelatin nanofibrous scaffold fabricated by electrospinning of 10 wt% solution of gelatin under similar electrospinning conditions was used as control.

2.4. Post treatment of electrospun scaffolds

The electrospun SF and SFC nanofibrous scaffolds were then cross-linked with 3 wt% EDC-NHS [2:1 (w/w)] in ethanol: water (95:5 v/v)] solution and were further treated with 0.1 M $CaCl_2$ solution overnight at 40 °C. The cross-linked scaffolds were rinsed thoroughly with deionized water to remove ions like chlorine and residual cross-linking reagent, and dried under vacuum at room temperature for 24 h. The different composite scaffolds treated with $CaCl_2$ were designated as SF1, SFC1 and SFC2 corresponding to SF, SF/CMC (99:1 w/w) and SF/CMC (98:2 w/w) respectively.

2.5. In vitro mineralization

The *in vitro* mineralization of the scaffold was performed by incubating 1 × 1 cm^2 sizes of scaffolds in 20 ml simulated body fluid (SBF) prepared following the earlier reported method (Kokubo, Kim & Kawashita, 2003) for 7 days. The pH of the fluids was adjusted to 7.4 at 36.5 °C. After 7 days, the scaffolds were taken out, gently rinsed in distilled water and dried at room temperature for further analysis.

2.6. Morphological characterization

Characterization of the developed scaffold was done before and after the *in-vitro* mineralization. To scaffolds with 0.5 × 0.5 cm^2 sizes of scaffolds were coated with gold, and observed under field emission scanning electron microscope (FESEM) (NOVA NANO SEM, USA) for morphological assessment. The average fiber diameters were measured from ten different FESEM images of ≥5000 magnification using Image J software. Energy dispersive X-ray analysis (EDX) was used to ensure mineral deposition on the scaffolds.