In vitro behavior of silk fibroin-coated calcium magnesium silicate scaffolds

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ABSTRACT

Bioceramic scaffolds such as silicate bioceramics have been widely used for bone tissue engineering. However, their high degradation rate, low mechanical strength and surface instability are main challenges compromising their bioactivity and cytocompatibility which further negatively affect the cell growth and attachment. In this study, we have investigated the effects of silk fibroin coating on the tricalcium magnesium silicate scaffolds in term of biological behavior for bone tissue engineering. The microstructure, morphology, cell adhesion and chemical composition of coated scaffolds were analyzed by scanning electron microscopy and Fourier transform infrared spectroscopy. Also, MTT assay test showed that both coated and uncoated scaffolds supported growth of mouse embryonic fibroblast cell. However, the coated scaffold revealed a higher cell proliferation than uncoated one. All the results postulated that silk fibroin was successfully coated on the scaffold and improved the biological properties of scaffold indicating a promising biomaterial for bone tissue engineering application.

1. Introduction

With the growing of aging populations, there is a great demand to regenerate large bone defects. Bone grafting procedures are used worldwide to treat bone defects [1]. However, the effective regeneration of critical-sized bone defects is clinically challenging due to the limitations facing with previously used bone graft treatments [2]. It is suggested to prepare scaffolds using bioceramic materials [3]. Beta tricalcium phosphate (β-TCP) [1], hydroxyapatite (HA) [4], bioactive glass (BG) [5] and calcium silicate (CS) [6] were previously used to prepare bioactive scaffolds for bone tissue engineering applications. However, they are brittle with low mechanical strength limiting their biomedical applications. Also, the high degradation rate has negative effects on cell growth and attachment [5]. Furthermore, due to lack of fibrillar proteins in the scaffold, poor

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surface reactivity and interaction with biological agents are provided [7]. Tricalcium magnesium silicate (merwinite, $Ca_3MgSi_2O_8$) is recognized as bioactive material [8, 9] showing mechanical properties higher than conventional ceramics and similar to those of cortical bone [10]. Silk fibroin (SF) is a widely popular bone regenerative material because of its characteristics such as biocompatibility, low degradation rate, and excellent mechanical properties [11]. However, the lack of bioactivity of SF alone is a major problem [11]. Silk can be harvested from a domesticated silkworm; called *Bombyx mori* is recognized to have significant mechanical strength and toughness surpassing most of previously used polymeric materials [12]. However, the residual of organic solvents in the polymeric coating is harmful to host tissue as well as inadequately osteoconductivity. According to previous study, the bioactivity and compressive strength of bioactive glass scaffold were improved by the use of silk coating [13]. Therefore, it is reasonable to assume that the combining of calcium magnesium silicate with silk may lead to a bioactive material with improved biological properties for bone tissue engineering. The purpose of this study is to investigate the effect of SF coating on the in vitro properties of tricalcium magnesium silicate scaffold for bone tissue engineering.

2. Material and methods

Merwinite powders was prepared by sol-gel method as reported in previous study [14] and the freeze casting method was used to prepare ceramic scaffold [15]. The ceramic suspension was prepared using 10% (w/v) merwinite powder to deionized water and 3% Dolapix dispersant. Subsequently, 5% wt. polyvinyl acohol (PVA, Sigma Aldrich) was added to the prepared suspension. For scaffold fabrication, a cylindrical polyethylene (PE, Charloma Inc.) mold was provided and then 5 mL of already prepared suspension was poured into it. After entire freezing with the cooling rate of 4 °C/min, the scaffold was sintered in an electrical furnace based on a three-stage program: (1) heating at 300 °C with heating rate of 1°C/minute and kept for 1 hour (2) continued sintering process up to 600 °C with similar heating rate (3) temperature elevated to 1350 °C with heating rate of 2 °C/minute and remained for 3 hours and cooled up to room temperature. The aqueous solution of silk fibroin with concentration of 10% wt. was obtained from *Bombyx mori* cocoons [16]. The resulting SF was analyzed by X-ray diffraction (XRD) with Philips PW 3710 diffractometer. This instrument was operated with voltage and current settings of 40 kV and 40 mA, respectively and uses Cu-Kα radiation (1.540600 Å). For qualitative analysis, XRD diagrams were recorded in the interval $5^o \leq 2\theta \leq 70^o$ at scan speed of 2º/min representing the step size of 0.02º and the step time of 1s. Also, SF was examined by Fourier transform infrared (FTIR) with Thermo Nicolet spectrometer. For IR analysis, at first 1 mg of the powder samples were carefully mixed with 300 mg of KBr (infrared grade) and palletized under vacuum. Then, the pellet was analyzed in the range of 400 to 4000 cm^{-1} at the scan speed of 23 scan/min and the resolution of 4 cm^{-1}. Afterward, the ceramic scaffold was immersed in the aqueous solution 5 % (w/v) fibroin for 3 minutes to prepare SF-coated scaffold and centrifuged with the speed of 1000 rpm to remove the residual fibroin. Finally, the scaffolds were dried in air and 40 °C for 24 hour. The scaffold were immersed in ethanol for 5 minutes, dried at 40 °C in order to cross-linking of polymer layer and vacuum for 24 hour to remove the residual ethanol. The scanning electron microscopy (SEM, Carl Zeiss, Germany) and FTIR analyzes were performed.

The mouse embryonic fibroblast cell (MEFs) obtained from Pasteur Institute of Iran (IPI) and then cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) cell culture including 10U/mL penicillin, 100µg/mL streptomycin , 9% fetal bovine serum (FBS)(all from Gibco) Cells were incubated in the incubator at 37 °C, 5% CO_2. The cell seeded coated and uncoated scaffold were sterilized under ultraviolet light for 10 minutes and rinsed with phosphate buffer solution (PBS). Under sterile condition, the cell proliferation was evaluated using methyl thiazolyl tetrazolium (MTT) assay. The samples were placed in the 24 well culture plates with a density of 2×10^4 cell/cm^2 and were incubated in 1 mL DMEM-

F12 for 1 and 7 days and the extracts were collected after the centrifugation of suspension. After the cells were detached by Trypsin-EDTA (Gibco), cells were transferred to 96 well culture plates and incubated in the DMEM-F12 containing 10% FBS at 37°C for 24 hour. After, the medium was taken away and 10µL MTT (Sigma) and 100 µL DMEM medium were added to each well. After incubation for 4 hour at 37°C, 100µL dimethylsolfoxide (DMSO, Sigma) was added to dissolve crystals. Then, the absorbance was detected using ELISA-Reader in the wavelength of 560 nm. After incubation, scaffolds were rinsed with PBS containing 2.5% glutaraldehyde and fixed for 30 minutes. Finally, the samples were coated with gold for SEM analysis.

3. Results and Discussion

XRD diffractograms of fibroin before and after ethanol treatment, exhibited in Fig. 1(A) and (B), respectively. As expected, ethanol treated SF showed typical X-ray diffractograms of the β-sheet crystalline structure, since ethanol has been well known as a crystallization inducing solvent. The pattern after ethanol treatment indicated that the protein chains are more organized and the peaks associated to β-sheet structure [17, 18]. Previous study has shown that the SF before ethanol treatment reveal the FTIR spectra of 1530 cm-1 which is attributed to amide type II and 1656 cm-1 ascribed to amid type I [18]. According to FTIR spectra of SF-coated merwinite scaffold after ethanol treatment (Fig.2B), the observed peak characteristics of 3284 cm^{-1}, 1623 cm^{-1} and 1520 cm^{-1} are related to N-H group in a single bond SF, type I amide and type II amide, respectively. Also, the characteristic peaks around 1000 cm^{-1} and 500 cm^{-1} may be related to the β-sheet conformation and α-helix [19].

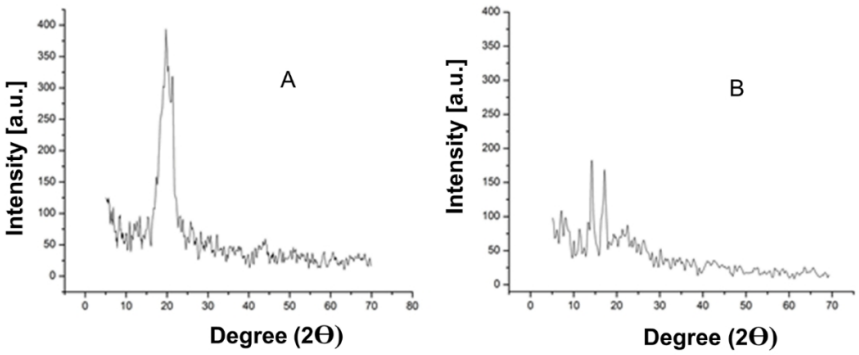

Fig 1. XRD diffractograms of SF A) untreated and (B) treated in ethanol.

Based on the results of MTT assay (Fig.2C), after 1 and 7 days of cell culture, there is no meaningful cytotoxicity difference between the coated and uncoated samples compared with control group revealing the cytocompatibility of scaffolds. Some researchers have shown that three-dimensional fibroin nets support the attachment, spread and growth of varieties of tissue cells and silk fibroin has fulfilled many essential requirements for an optimal biomaterial [20].

In addition, the morphological characteristics of MEFs cells cultured on the surface of coated and uncoated scaffolds for 7 days revealed that the cells were well attached to the surface of scaffolds (Figure 3 (a-d)). Also it should be mentioned that fig. 3e exhibited extracellular matrix (ECM) secretion. The ECM is composed of a complex mixture of structural and functional macromolecules includes collagen family, elastic fibers, glycosoaminoglycans (GAG) and proteoglycans, and adhesive glycoproteins. It has an important role in cell and tissue structure and function via acting as a reservoir for soluble signaling molecules and mediates signals from other sources to migrating, proliferating, and differentiating cells [21, 22].

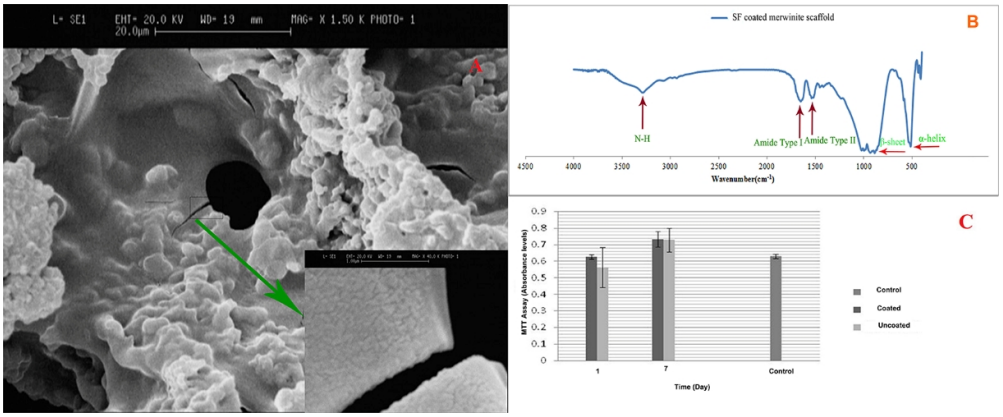

Fig 2. (A) Surface microstructure of SF coated merwinite scaffold (B) FT-IR spectra of SF coated merwinite scaffold and (C) MTT assay for MEFs cell proliferation for 1 and 7 days of cell culture with merwinite and SF coated merwinite scaffold extracts (*p *value* <0.05).

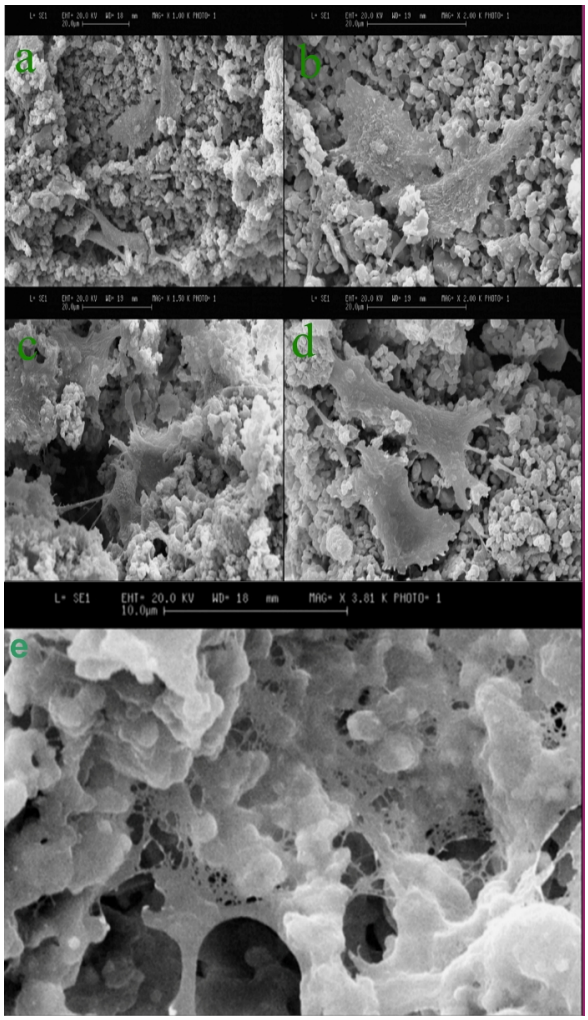

Fig 3. The morphologies of MEFs cells cultured on (a,b) merwinite scaffold with magnification of 1000 and 2000 and (c,d) SF merwinite scaffold with magnification of 1500 and 2000, d) Secretion of cells

There are two main factors playing an important role between the cells and biomaterials including (1) the ionic environment comprising ionic concentration as well as pH value of the surrounding media [23, 24] and (2) surface properties of biomaterials such as, surface stability [25, 26]. Silk is relatively stable fibroin comprising of β-sheets may provide a stable interface supporting the MEFs proliferation. Thus, it can be assumed that silk could improve the MEFs proliferation through surface changes and forming nanofibrous structure mimicking the natural ECM leading to improved cell interaction [27, 28]. Previous reports have shown that surface stability play an essential role in regulating cell response [25]. Also, the nanofibrous structures can be formed by silk which is able to mimic the natural ECM [27, 28].Previous studies have shown that silk coating may improve the attachment, and proliferation of bone marrow stromal cells (BMSCs) seeded on mesoporous bioactive glass scaffold. Also silk layer formed on the scaffold retained an uniform and continuous pore network indicating that the required pore architecture for better bone tissue ingrowth may be met [29]. In other study, a combination of PCL and silk layer was coated on biphasic calcium phosphate and improved the bioactivity of scaffold [30]. All the results showed that silk-modified merwinite scaffold may be used as suitable scaffold for bone tissue engineering. However, the mechanical properties of the scaffold also should be evaluated.

4. Conclusion

In this study, we have successfully prepared SF-coated merwinite scaffolds and cells were well attached to its surface which indicating that this could be a potential candidate material for tissue engineering. However, further *in vitro* and *in vivo* studies are needed to be performed.

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