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A novel chitosan-*tussah* silk fibroin/nano-hydroxyapatite composite bone scaffold platform with tunable mechanical strength in a wide range

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ABSTRACT

Currently, great efforts have been made to enhance the mechanical strength of bone tissue engineering (BTE) scaffolds, which are composed of biopolymeric matrices and inorganic nano-fillers. But the tunability of mechanical strength in a wide range for BTE scaffolds has seldom been investigated in spite of the great importance of this performance. In this work, a chitosan-*tussah* silk fibroin/hydroxyapatite (CS-TSF/HAp) hydrogel was synthesized by using a novel *in situ* precipitation method. Through *in situ* inducing the conformation transition of TSF in the CS-TSF/HAp hydrogel, which could be monitored by XRD, FT-IR, TGA, and DTA, the elastic modulus and fracture strength of the final CS-TSF/HAp composite could be tailored in a wide range without changing its composition, morphology, roughness, and crystal structures. The elastic modulus of the CS-TSF/HAp composite ranged from ~250 to ~400 MPa while its fracture strength ranged from ~45 to ~100 MPa. In order to clarify the rationale behind this process, a speculative explanation was provided. *In vitro* cell culture indicated that MC3T3-E1 cells cultured on the CS-TSF/HAp composite had positive adhesion, proliferation, and differentiation potential. We believed that the CS-TSF/HAp composite could be used as an ideal scaffold platform for cell culture and implantation of bone reconstruction.

1. Introduction

Currently, nanocomposites composed of biopolymeric matrices and nano-hydroxyapatite (HAp) fillers are considered as attractive alternatives for conventional materials (*e.g.,* autografts, allografts, dense ceramic biomaterials, and metallic orthopedic biomaterials) to be used as scaffolds for 3D cell culture and transplantation of bone reconstruction [1–5]. Among the requirements which bone scaffolds should satisfy, mechanical strength is of great significance [6–8]. To date, great efforts have been made to enhance the mechanical strength of bone scaffolds [9], while the tunability of mechanical strength in a wide range for bone scaffolds has seldom been investigated [10].

In fact, tunability of mechanical strength in a wide range for a bone scaffold is very important. For one thing, by tuning the mechanical strength of bone scaffolds, the behaviors of adherent cells (*e.g.,* migration, proliferation, differentiation, protein expression, and apoptosis) could be mediated [11,12]. Through specific interactions of cell surface integrins with adhesion ligands [13], adherent cells can sense the mechanical strength of implants [14], and therefore exert traction forces to influence cytoskeletal tension. Subtle variations in cytoskeletal tension render changes in cell shape and in associated signaling cascades, which ultimately regulate gene expression [15,16]. For instance, substrate stiffness could determine the differentiation lineage of the mesenchymal stem cell (MSC). Soft substrates are neurogenic, substrates with intermediate stiffness are myogenic, and stiff substrates are osteogenic [17]. Previous investigations also indicated that very different mechanical strengths facilitate stem cells to differentiate to chondrocytes *versus* osteoblasts [18]. Although cell proliferation and differentiation increase with increasing matrix stiffness, the optima of mechanical strength for different types of adherent cells varies widely [12]. Therefore, tunability of mechanical strength in a wide range for bone scaffolds is a potentially important mediator of behaviors and phenotypes of adherent cells.

For another thing, mechanical strength affected the mechanical stability of bone implants [19,20]. The mechanical strength of living bone varies between different bone types as well as within

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different regions of the same bone [21]. In addition, the mechanical strength of bone is influenced by porosity, mineralization level, and organization of the solid matrix [22]. The maturation of bone can also lead to changes in its mechanical properties [21]. Ideally, the mechanical strength of a bone scaffold should match the local biomechanical environment of specific bone defect site in case of mechanical mismatch which will cause stress shielding or collapse of implants [23–25]. Thus, if bone implants were to be transplanted into specific bone-defect sites, aligning the mechanical strength with the bone tissue of interest is of prime importance.

In order to tailor the mechanical properties of bone scaffolds, researchers had done much work including changing proportions of organic phase and inorganic phase [26,27], adjusting crosslinking degree [18], modulating the structures of inorganic fillers [28], optimizing scaffold design and fabrication technologies [29,30], *etc.* However, the composition, morphology, roughness, pore structure, pore density, thermal stability, and bioaffinity of scaffolds will change with varying organic/inorganic ratios and crosslinking degree. It has also been found that the morphology and size of nano-HAp particles could influence the mechanical strength of composite scaffolds [28,31], but the delicate control on the morphology and size of HAp particles has still been a big challenge. With the aid of 3 dimensional printing (3Dp) technology, the mechanical properties of scaffolds could be modulated, as well [32]. But the computer-aided structural design of scaffold is a complicated process, and extensive optimization is needed to process good quality parts [33].

In this work, we prepared a CS-TSF/HAp composite scaffold platform for cell culture or transplantation of bone reconstruction by using a novel *in situ* precipitation method. Through *in situ* inducing the conformational change of TSF in the CS-TSF/HAp hydrogel, the mechanical strength of the final CS-TSF/HAp composite became tunable in a wide range without changing its composition, morphology, roughness, and crystal structures.

TSF is produced and used by wild silkworms to construct cocoons [34]. The main molecular conformation of TSF is an extended antiparallel β-sheet structure caused by a hydrogen bond among the chains through —C═O and —NH groups, embedded in semi-amorphous matrices composed of α-helices and random coils [35]. Recent investigations reveal that *β*-sheet crystallites play a pivotal role in defining the stiffness and strength of TSF by providing stiff orderly crosslinking domains [36,37]. However, the molecular conformations of TSF regenerated from lithium thiocyanate are mainly α-helix and random coil in solution [38,39]. Fortunately, the molecular conformation of TSF can be changed from α-helices or random coils to β-sheet crystallites through treatment with heat or organic solvents (*e.g.,* methanol or ethanol) [40,41]. In this work, methanol was applied to *in situ* induce the conformation transition of TSF in the CS-TSF/HAp hydrogel [42]. From previous investigations, researchers also found that methanol concentration and immersion time could influence the transition rate [43]. Therefore, by adjusting the immersing time in methanol, the mechanical strength of the CS-TSF/HAp composite becomes tunable in a wide range.

Additionally, containing high contents of aspartic acid and arginine, TSF is abundant in Arg-Gly-Asp (RGD) sequences [41], which reside in the cell attachment region of fibronectin and are known to be the receptors of cell integrins. It was also reported that regenerated TSF supported the growth and functional maintenance of human bone marrow derived mesenchymal stem cell (hBMSCs) [44–46]. Thus, TSF was expected to improve the cytocompatibility of the CS-TSF/HAp composite

In order to have a better understanding of this new material, characterizations, including surface morphology, elementary distribution, crystal structures, and crystallinity, were carried out. The *in situ* conformation transition of TSF from random coil structure and α-helix structure to β-sheet structure in the CS-TSF/HAp hydrogel, which was induced by methanol treatment, was evidenced by X-ray diffraction (XRD), Fourier transform infrared spectrometer (FT-IR), thermogravimetric analysis (TGA), and differential thermal analysis (DTA). The influence of conformation transition on the mechanical strength of the CS-TSF/HAp composite was also investigated in detail. In addition, *in vitro* cytocompatibility of the CS-TSF/HAp composite (proliferation, differentiation, and fluorescence staining) was evaluated, as well.

2. Experimental section

2.1. Materials

Chitosan (*Mw* 1,000,000) was obtained from Golden-Shell Biochemical Co. (Zhejiang, China) with 95% degree of deacetylation. *Tussah* silkworm cocoons were bought from Nanyang, Henan, China. Genipin was bought from Chengdu ConBon Biotech Co., Ltd. (Chengdu, China). Polyethylene glycol (PEG, *Mw* 20,000) was purchased from Aladdin Industrial Co., Ltd. (Shanghai, China). Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), acetic acid, ammonia, ammonium thiocyanate (NH_4SCN), lithium hydroxide monohydrate ($LiOH{\cdot}H_2O$), glutaraldehyde, and methanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were all of analytical grade. Deionized ultrapure water was used throughout the experiment. For cytotoxicity assay, MC3T3-E1 cells were generously supplied by School of Stomatology, Wuhan University (Wuhan, China).

2.2. Preparation of regenerated TSF solution

In our previous work, the preparation process was depicted in detail [47]. Briefly, *tussah* silkworm cocoons were firstly degummed with 0.5% (w/v) Na_2CO_3 aqueous solution at 100 °C for 30 min and then washed vigorously with deionized water to remove the silk sericin. Afterwards, the degumming TSF was dissolved into 16 M LiSCN solution, prepared from NH_4SCN and $LiOH{\cdot}H_2O$. The as-prepared solution was then filtered and dialyzed to remove the undissolved parts and remaining chemicals. The resultant solution was then filtered by 0.8 μm millipore filter and concentrated against dry PEG-20,000 [48]. The final solid content of the solution was 3.3%, which was calculated by weighing method.

2.3. Preparation of the CS-TSF/HAp hydrogel

Firstly, 0.36 g CS was dissolved into 40 mL 2 vol% acetic acid solution at 45 °C with vigorous agitation for 30 min. Afterwards, 0.095 g $(NH_4)_2HPO_4$ and 0.282 g $Ca(NO_3)_2{\cdot}4H_2O$ (Ca/P = 1.67) were successively added into the CS solution. After the salts were totally dissolved, 3.63 g TSF solution (3.3 wt%) was dripped into the CS solution slowly. Next, 0.036 g genipin was added into the above solution. 10 min later, the resultant solution was then transferred into a constant temperature oven (25 °C)for 4 h. After that, ammonia was poured onto the surface of the hydrogel through the permeation from the top to the bottom. Simultaneously, *in situ* precipitation took place and the whole process could be depicted by the formula below: $10Ca^{2+} + 6HPO_4^{2-} + 8OH^- = Ca_{10}(PO_4)_6(OH)_2(\downarrow) + 6H_2O$ (pH > 10). 24 h later, the ultimate hydrogels were thoroughly rinsed with deionized water. For comparison, the above-mentioned process has been repeated in the absence of TSF to fabricate the CS/HAp hydrogel.