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Multiscale design and synthesis of biomimetic gradient protein/biosilica composites for interfacial tissue engineering

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ABSTRACT

Continuous gradients present at tissue interfaces such as osteochondral systems, reflect complex tissue functions and involve changes in extracellular matrix compositions, cell types and mechanical properties. New and versatile biomaterial strategies are needed to create suitable biomimetic engineered grafts for interfacial tissue engineering. Silk protein-based composites, coupled with selective peptides with mineralization domains, were utilized to mimic the soft-to-hard transition in osteochondral interfaces. The gradient composites supported tunable mineralization and mechanical properties corresponding to the spatial concentration gradient of the mineralization domains (R5 peptide). The composite system exhibited continuous transitions in terms of composition, structure and mechanical properties, as well as cytocompatibility and biodegradability. The gradient silicified silk/R5 composites promoted and regulated osteogenic differentiation of human mesenchymal stem cells in an osteoinductive environment *in vitro*. The cells differentiated along the composites in a manner consistent with the R5-gradient profile. This novel biomimetic gradient biomaterial design offers a useful approach to meet a broad range of needs in regenerative medicine.

1. Introduction

An attractive feature of natural tissue interfaces is their unique gradient structure [1,2]. Osteochondral interfaces, for example, consist of spatial variation in the extracellular matrix (ECM) composition and orientation from subchondral bone to cartilage [3–5]. Hydroxyapatite (HA) also gradually diminishes from subchondral bone to calcified cartilage and completely disappears in hyaline cartilage [6]. These gradients of minerals and ECM lead to a continuous change of mechanics along the trend [4,7], and also play an important role in connecting mechanically mismatched tissues, facilitating load transmission as well as reducing or eliminating delamination at the junctions [2,4,8,9].

To mimic these natural gradient structures, a variety of biphasic [10–14], multiphasic [15–18], and gradient [4,19–21] composites have been developed. However, unlike natural mineralization during which non-collagenous proteins initiate and regulate mineral formation on the collagenous matrix [22], most of these materials were prepared through mixing inorganic bioactive phase (e.g., chemosynthetic HA crystallites) with additional substrate materials (e.g., collagen) [12,15,18,23–25]. These approaches generally result in weakened structural uniformity and mechanical performance of the resultant composite materials [26,27]. To address these problems, several *in vitro* biomineralization approaches have been reported [9,28] where the nucleation and growth of inorganic mineral phase was regulated directly on well-organized polymer templates. For instance, a three-layer scaffold composed of HA nanocrystals nucleating on self-assembled collagen fibers was developed to support cartilage and bone formation [28]. The gradient mineralization was achieved via changing the ratio of HA/collagen in different layers. However, HA crystallization on self-assembled collagen fibers lacks non-collagenous proteins as mediators of the mineralization process, thus well-controlled mineral deposition on the polymer template is difficult to achieve for the synthesis of advanced materials that mimics the complicated hierarchical natural tissues [29]. Moreover, the use of caustic chemicals during scaffold preparation limits the

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incorporation of bioactive molecules during material fabrication [9]. Therefore, new strategies are needed to design and synthesize continuous gradient materials using biomimetic mineralization [30].

In this paper, inspired by the growth of natural sponge spicules, we designed a novel route to fabricate gradient protein/biosilica composites via site-specific *in vitro* biomineralization. The biosilica selective peptide-R5, a bioinspired analog derived from the silaffin peptides that are used for silica synthesis in *Cylindrotheca fusiformis* [31], was introduced into the silk fibroin (thereafter referred to silk) hydrogel composites through enzymatic crosslinking. A well-controlled gradient distribution of the R5 peptide was achieved by regulating the peptide concentration along the longitudinal direction of the composites. The final gradient silicified silk/R5 (GSSR5) composites presented a structure with varied pore sizes and a gradient distribution of biosilica particles, similar to the structural features of natural osteochondral interfaces. More remarkably, these continuous gradient structures endowed the composites with gradient mechanical properties and distinct osteogenesis capacity. In addition, unlike chemical synthetic methods which use caustic chemicals [32–35], this simple and versatile approach allows for surface modification of biosilica particles to encapsulate biomolecules and drugs [36]. This novel gradient biomaterial design offers a useful approach to meet a broad range of needs in regenerative medicine involving osteochondral tissue engineering.

2. Materials and methods

2.1. Materials

All chemical reagents used for making the GSSR5 composites were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. All materials and reagents used for cell culture and analysis were purchased from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise specified.

2.2. Preparation of aqueous silk solution

Silk solutions were prepared using our previously established procedures [37]. Briefly, ten grams of silk cocoons were cut into pieces and boiled in 4 L of 0.02 M sodium carbonate solution for 30 min to remove the coating of sericin protein. Degummed fibers were rinsed with deionized water three times and dried in air overnight. Five grams of dried fibers were dissolved in 20 ml of 9.3 M lithium bromide solution at 60 °C for 4 h. The silk fibroin solution was then dialyzed against deionized water using a dialysis cassette (Pierce 3.5 kDa MWCO; Fisher Scientific, PA) for 2 days. The solubilized silk solution was then centrifuged twice at 9000 RPM, 4 °C for 20 min to remove insoluble particulates. Protein concentration was determined by drying a known mass of the silk solution at 60 °C for 12 h and measuring the mass of the remaining solid.

2.3. Preparation of the gradient silk/R5 hydrogels

The biosilica selective peptide R5 (H-SSKKSGSYSGSKGSKRRIL-OH) with a purity of 95% was synthesized by GenScript (Piscataway, NJ, USA). Lyophilized horseradish peroxidase (HRP, type VI) powder and the R5 peptide powder were dissolved in deionized water to form a stock solution of 1000 U/mL and 0.7 mg/μL, respectively. To generate the gradient silk/R5 hydrogels with high, medium and low loadings of the R5 peptide (thereafter referred to high, medium and low regions, respectively), the R5 peptide solution was first mixed with the silk solution in a R5/silk molar ratio of 250/1, 125/1 and 62.5/1, respectively. The mixture was then loaded sequentially into a cylindrical container layer by layer. First, the high region was loaded and gelation were initiated by adding the HRP and hydrogen peroxide (H_2O_2, 165 mM) solution at a ratio of 40 units and 40 μL per 1 mL of silk solution, respectively. Before the complete gelation in high region, the medium region was added on top of the high region followed by the addition of HRP and H_2O_2 with the same concentration as in the high region. Then the low region was added on top of the previous medium region followed by the addition of HRP and H_2O_2. The mixture was then incubated at 37 °C overnight to ensure complete gelation. Unsilicified plain silk control hydrogels were prepared by crosslinking silk solution using HRP and H_2O_2 with the same ratio as in the gradient silk/R5 hydrogels.

2.4. Silicification

Biosilica deposition was introduced into the gradient silk/R5 hydrogels post gelation. Pre-hydrolyzed tetraethoxysilane (TEOS) solution was prepared by mixing 2.23 ml of TEOS solution with 7.76 ml of 50% ethanol/water solution, 10 μl of 1 M hydrogen chloride (HCl) and left at room temperature for 15 min. A 50 μl aliquot of the silk/R5 hydrogel was immersed in 200 μl silicifying medium consisting of 40 μl prehydrolyzed TEOS solution and 160 μl buffer solution (10.4 μl 1 M bis-tris propane, 5.6 μl 1 M citric acid solution and 144 μl deionized water) to allow for silicification at room temperature overnight. The silicifying medium was changed twice every 4 h during the silicification process.

2.5. Fluorescence imaging of the FITC-labelled GSSR5 composites

The fluorescein isothiocyanate (FITC)-labelled R5 peptide (FITC-SSKKSGSYSGSKGSKRRIL-OH, GenScript, NJ, USA) was added into the R5 peptide solution in a molar ratio of 1–4000. The resultant peptide mixture was used to create the FITC-labelled GSSR5 composites following the same gelation and silicification process as described earlier. The FITC-labelled composites were imaged using with the Olympus mvx10 macroscope and captured by cellSens Dimension (ver 1.8.1) program (Olympus, Tokyo, Japan).

2.6. Quantification of encapsulation efficiency

Quantification using FITC-labelled R5 peptide was performed to determine the encapsulation efficiencies of the R5 peptide in the GSSR5 composites. The FITC-R5 peptide was added into the R5 peptide solution in a molar ratio of 1–4000 following the preparation process as described earlier. The silicifying medium was collected after silicification of the silk/R5 hydrogels. The amount of the FITC-R5 peptide in the supernatants was quantified by recording the emission signal using a SpectraMax M2e multimode microplate reader (Molecular Devices, CA, USA). All conjugation reactions were repeated in triplicate. The reaction efficiency was calculated as follows:

$$\text{Encapsulation efficiency} = \frac{\text{Peptide amount added} - \text{Peptide amount in supernatant}}{\text{Peptide amount added}} \times 100\%$$