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Elucidation of differential mineralisation on native and regenerated silk matrices

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ABSTRACT

Bone mineralisation is a well-orchestrated procedure triggered by a protein-based template inducing the nucleation of hydroxyapatite (HA) nanocrystals on the matrix. In an attempt to fabricate superior nanocomposites from silk fibroin, textile braided structures made of natively spun fibres of *Bombyx mori* silkworm were compared against regenerated fibroin (lyophilized and films) underpinning the influence of intrinsic properties of fibroin matrices on HA nucleation. We found that native braids could bind Ca^{2+} ions through electrostatic attraction, which initiated the nucleation and deposition of HA, as evidenced by discrete shift in amide peaks via ATR-FTIR. This phenomenon also suggests the involvement of amide linkages in promoting HA nucleation on fibroin. Moreover, $CaCl_2$-SBF immersion of native braids resulted in preferential growth of HA along the c-axis, forming needle-like nanocrystals and possessing Ca/P ratio comparable to commercial HA. Though regenerated lyophilized matrix also witnessed prominent peak shift in amide linkages, HA growth was restricted to (211) plane only, albeit at a significantly lower intensity than braids. Regenerated films, on the other hand, provided no crystallographic evidence of HA deposition within 7 days of SBF immersion. The present work sheds light on the primary fibroin structure of *B. mori* which probably plays a crucial role in regulating template-induced biomineralisation on the matrix. We also found that intrinsic material properties such as surface roughness, geometry, specific surface area, tortuosity and secondary conformation exert influence in modulating the extent of mineralisation. Thus our work generates useful insights and warrants future studies to further investigate the potential of bone mimetic, silk/mineral nanocomposite matrices for orthopaedic applications.

1. Introduction

Bone is a complex nanocomposite consisting of organic–inorganic phases of collagen-hydroxyapatite (HA) crystals, where HA [$Ca_{10}(PO4)_6(OH)_2$] measures 20–30 nm in width, 100 nm in length with 3–6 nm thickness [1], precisely embedded between longitudinally arranged collagen fibres such that HA is synthesized on the c-axis plane along the longitudinal axis of collagen [2]. In addition, HA exhibits intimate chemical bonding between the organic-inorganic elements with an overall poor crystallinity [2]. Growing interest in the development of biomimetic synthetic HA-based composites is primarily due to; (i) developing synthetic "analogs" of HA to be able to precisely replicate the morphology and chemistry of bone tissue [3], (ii) HA coating on constructs for improving osteoconductivity and (iii) evaluating the bioactivity of novel materials by studying the nucleation and growth of "bone-like" apatite upon immersion in supersaturated fluids such as simulated body fluid (SBF) [4]. However, there are still gaps in understanding the nature of such depositions; surface features and crystallisation phase especially with respect to the morphology and chemistry of the base material.

Silk fibroin, another fibrous protein that closely mimics collagen type I of bone has gained popularity in tissue engineering due to interesting intrinsic properties such as resorbability [5], toughness [6], minimal immunogenicity [7,8], cytocompatibility with abundant polar, hydrophilic groups [9], strong chemical bonding with HA [10–13] and versatility in processing depending upon the target application [14]. Silk fibroin template fabricated in the form of nanofibres [12], films [15], porous 3D matrices [16] and composites [17] exposed to supersaturated ions, SBF [4,10] or fetal bovine serum [11], have been tested before for HA deposition. Silk fibroin plays a crucial role in regulating the synthesis and growth of HA nanocrystals [18], as HA combines with fibroin through chemical interactions which have been identified by FTIR analyses whereby the strong chemical bonding between the two caused peak shifts in the amide bonds of fibroin, commonly referred to as the 'blue shift' [2]. However, majority of these studies have largely ignored the role of template chemistry of native fibroin in directing the nucleation and growth of HA. The primary reason could be that most of

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the studies used regenerated silk fibroin materials formed by the subsequent dissolution and re-assembly of native silkworm cocoons which tends to disrupt the original protein conformation [19]. Moreover, the role of different morphologies and other physical characteristics as a potential cause of large variations in the resultant mineralisation attained have not been addressed. In this study, we hypothesize that native silk fibroin will experience superior mineralisation in the presence of supersaturated solution, credited to the intact organization of the primary amino acid composition and structure. To test this hypothesis and understand the underlying chemical interactions governing the process of HA nucleation on fibroin matrix, we examined the change in the intrinsic properties of fibroin and thoroughly characterized HA crystallisation on the material surface initiated by incubating silk structures in 1.5× SBF.

Furthermore, there are theories which attribute the mineralisation potential of fibroin to the amorphous anionic bridges present between the β-sheets present within the structure [20]. In this study, Marelli et al. compared the hydrophobic chains in fibroin against the hydrophilic, electronegative chains and found apatite formation only in the hydrophilic fragments. They concluded that the differences in the mineralisation potential of silk fibroin were due to its constituent anionic chains which typically resembled the non-collagenous, anionic fragments of collagen type I. In another study, Kino et al. prepared regenerated silk fibroin films with varied calcium chloride content and reported deposition of HA post-immersion in 1.5× SBF, but only in films possessing >3 wt% of calcium chloride relative to silk fibroin [15]. The reason was attributed to the presence of Ca^{2+} ions at a particular concentration which reportedly induced more β-sheets in the fibroin structure [21] and such modified silk fibroin films demonstrated improved HA coating [15]. However, quantitative analysis of this varying β-sheet content was not done. Also, the reason as to why mineralisation could not be observed on methanol treated pure fibroin films (without calcium chloride) which contained at least some amount of β-sheet content remained unexplained. Nevertheless, most studies have reported an additional requirement of either pre-exposure of fibroin or its association with calcium- and phosphate-based solutions to promote nucleation and growth of HA nanocrystals [12,13,16,22]. Whether it is the processing parameters during dissolution and reconstitution that disrupt the native structure of the fibroin chain; firstly by dissociating the specific amino acid chains and secondly by hampering their tendency to re-assemble the necessary β-sheet conformation, or if it is the inherent inert nature of fibroin chain that modulates the extent of mineralisation is still under wraps. These studies further highlighted the fact that our current understanding of the primary structure of fibroin is insufficient to elucidate the mineralisation capacity of silk fibroin in order to use it as a potential bone graft substitute [9].

Apart from the secondary conformation, there are other contributing factors that determine apatite formation on biomaterials including molecular size, surface chemistry, topography, surface charge, stiffness, target site and rate of *in situ* degradation which needs to be taken into account. For instance, hydrophobic crystalline fractions of silk fibroin with a 40 kDa MW are weak templates for mineralisation, whereas 2–10 kDa MW hydrophilic chains are strong templates [20]. Moreover, the specific surface area of the biomaterial plays a crucial role in determining the amount of apatite formation, as a larger hydrated layer of organic template with strong tendency for ion exchange facilitates higher precipitation of nanocrystalline HA [3]. Therefore, in the present study, we aim to address the following three specific questions with respect to *in vitro* mineralisation potential of silk fibroin; (i) the role of fibroin as an osteogenic substrate (in regenerated versus native form) in regulating nanocrystalline apatite synthesis, (ii) whether change in 3D morphology (lyophilized versus braid) influences HA deposition on fibroin and (iii) whether the modified 3D morphology and resultant variation in the intrinsic material properties modulate the extent of mineralisation. To the best of our knowledge, this is the first study to provide an indepth analysis of the difference in mineralisation in silk fibroin matrices between the native and regenerated fibres. We hypothesized that by inducing morphological changes in the fibroin structure, we could modulate the material properties which will eventually enhance control over the nucleation and growth of the HA phase. In order to characterize the mineralisation quotient, we used a combination of several sophisticated analytical techniques combining high end imaging and compositional analysis of the fabricated matrices to generate correlation between the material properties and the resultant mineralisation. We believe that understanding the process of mineralisation on the hierarchical assembly of fibroin will provide some innovative ideas for fabricating functional silk-based materials with customized morphology and chemistry best suited to promote biomineralisation *in situ*.

2. Materials and methods

2.1. Isolation of silk fibroin solution

12 tex 2-ply *Bombyx mori* silk yarns were procured from Starling Mills Pvt. Ltd., Malda district, West Bengal, India. Silk solution was prepared directly from the fibres according to a protocol used routinely [23,24]. Briefly, 5 g of *B. mori* fibres were weighed and cut into fine pieces, followed by boiling in 0.02 M Na_2CO_3 for 30 min to remove sericin. The fibres were then thoroughly rinsed in deionized (DI) water to isolate fibroin protein. Once completely dried, fibres were solubilized in 9.3 M LiBr solution at 60 °C for 4 h. Fibroin-LiBr solution was dialysed using Slide-A-Lyzer cassette (Thermo, molecular weight cut off 3500) yielding 6.1 wt% solution of silk fibroin, which was stored at 4 °C until subsequent usage.

2.2. Preparation of regenerated silk fibroin matrices

Aqueous silk fibroin solution of the same concentration (i.e. 6.1 wt%) was used for the fabrication of both lyophilized matrices and 2D planar films.

2.2.1. Silk films

To generate silk solution in the form of planar films, 2 mL of silk fibroin solution was pipetted over 10 × 10 mm^2 teflon plates and dried at RT overnight. Post-drying, the films were immersed in sufficient volume of 80% ethanol for 2 h at RT in order to induce β-sheet crystallisation.

2.2.2. Lyophilized

A randomly porous three-dimensional (3D) morphology was obtained by lyophilising fibroin solution. Approximately 10 mL of the solution was poured into a 30 cm^2 glass petri dish and frozen at −20 °C in a refrigerator for 24 h. The resulting silk solution was then lyophilized to generate a 3D porous matrix, which was subsequently immersed in ethanol for 2 h to induce β-sheet crystallisation.

2.2.3. Silk braids

Fibres of *B. mori* silk were fabricated into 3D braids using a 17 spindles flat braiding machine type NG1/16-120 (August Herzog Maschinenfabrik GmbH & Co. KG, Germany). Briefly, yarn bundles (17 multi-filament yarns per yarn bundle) were produced from multi-filament yarns comprising of 30 filaments/yarn of silk. The resulting structure was made such that yarns were oriented at 32° angle to the long axis of the braid using a production rate of 1 m/min to produce braided structures. The 3D braids were manually cut into smaller structures approximately 4 × 2 × 1 mm^3 dimensions.

2.3. SBF immersion

Post-fabrication, silk fibroin 3D matrices and films were immersed in 1.5× SBF solution for 7 days. SBF (1.5×) solution was prepared as