Deposition of Bone-Like Hydroxyapatite on Grafted Fibroin Silk Fibers

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Abstract: Silk is naturally occurring protein polymer produced by a wide variety of insects and spiders. *Bombyx mori* silk, a member of Bombycidae family has been used as biomedical suture material for centuries. These types of proteins usually exhibit important mechanical properties. Because of these impressive mechanical properties, this family of proteins provides an important set of material options in the fields of controlled release of biomaterials and scaffolds for tissue engineering. The grafting of silk with acidic groups may lead to the formation of hydroxyapatite by incubation in medium that mimic blood plasma. The idea that the acidic functions stimulate the formation of hydroxyapatite gained attention in the last 15 years in the literature. Natural fibrous polymers were chemically modified to mimic the behaviour of bone proteins responsible for mineralization. In this respect, 2-acrylamido-2-methylpropane sulphonic acid (AMPSA), 2-hydroxyethyl methacrylate -2-acrylamido-2-methylpropane sulphonic acid (HEMA-AMPSA) and diethylamino ethyl methacrylate (DEAEMA) were grafted onto silk fibroin by cerium ammonium nitrate initiation. The resulted polymers were characterized by FTIR-ATR and XPS spectroscopy to prove the grafting reactions. The biomineralization capacity of the grafted fibroin was evaluated by incubation in simulated body fluid solutions (SBF1x). SEM analysis showed the presence of hydroxyapatite deposits onto the surface of the grafted fibroin samples and the value of Ca/P ratio was very close to 1.67 from bone hydroxyapatite. The new synthesized biomaterials prove to have real mineralization ability and could be a potential bone substitute in the future.

Keywords: silk, graft polymerization, hydroxyapatite, biomineralization, simulated body fluid (SBF1x)

1. Introduction

In the last years there has been an increasing interest in using silk fibroin in biomedical and biological applications. The reasons for using this kind of fibrous material are related to its high mechanical properties combined with flexibility, tissue biocompatibility and good oxygen permeability [1].

Natural silk fibers have excellent mechanical properties. For example, domesticated (*Bombyx mori, B. mori*) silk worm fibers possess a tensile modulus on the order of 5 GPa, strengths of 400 MPa, and tensile elongations of 15% or more and are able to undergo quite large deformations in compression without kinking [1-5]. The mechanical performance of silk is even more remarkable since the fibers are produced under ambient conditions from aqueous solutions. *B. mori* silk fibres consist primarily of two components, fibroin and sericin; fibroin is the structural protein of the silk fiber, and sericin is the water-soluble glue that serves to bond fibres together. The majority of the fibroin is highly periodic with simple repeating sections broken by more complex regions containing amino acids with bulkier side chains.

The basic, highly repetitive sections are composed of glycine (45%), alanine (30%), and serine (12%) in a roughly 3:2:1 ratio. These three residues contain short side chains and permit close packing of crystals through the stacking of hydrogen-bonded β-sheets. The structure is dominated by [Gly-Ala-Gly-Ala-Gly-Ser]n sequences, with corresponding side groups of H, CH_3, H, CH_3, H, CH_2OH.

The sericin proteins, which comprise approximately 25 wt % of the silkworm cocoon, contain glycine, serine, and aspartic acid totalling over 60%. Compositional details for the silks can be found in a number of references [4-6].

Hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] is the most commonly used calcium phosphate based biomaterial, which is the major mineral part of natural bones and teeth, due to its excellent biocompatibility, osteoconductivity and bioactivity [7]. To find new ways for the synthesis of improved bone implant materials, combination of HA with other biocompatible polymers or proteins was reported, such as recombinant collagen [7], chitosan [8], polylactic acid [8], and hyaluronic acid [10], etc. *Bombyx mori* silk

fibroin is of practical interest because of its excellent intrinsic properties utilizable in the biotechnological and biomedical fields, such as suture, artificial ligament and substrate for cell culture, as well as the importance of silkworm silks in the manufacture of highquality textiles.

The recent researches with biomineralized silk fibroin and silk fibroin/HA composite showed its potential application to be explored as hard tissue replacement materials [7, 11]. However, the mechanisms of silk fibroin mediated mineral initiation are far from understood.

In this paperwork, *B. mori* silk fibroin fiber grafted with different acidic and amino groups was used as a template for the HA crystal growth. After the different treatments, the silk fibroin fiber was immersed into a simulated body fluid (SBF) to induce the HA deposition. The results showed the growth of HA crystal after the mimicking biomineralization.

2. Experimental

2. 1. Materials

Silk cocoons were supplied by S.C. SERICAROM S.A Company (Bucharest, Romania). *Bombyx mori* silkworm cocoons were boiled for 30 min in an aqueous solution of 0.5% (w/v) Na_2CO_3 and sodium dodecyl sulphate and then rinsed thoroughly with distilled water to extract the sericin protein. This operation was repeated three times to get the pure silk fibroin. The degummed silk fibroin was dried at 40°C and atmospheric pressure.

2-acrylamido2-methylpropane sulphonic acid (AMPSA) was provided by Sigma Aldrich, St-Quentin Fallavier, France. Diethylamino ethyl methacrylate (DEAEMA) and 2-hydroxyethyl methacrylate (HEMA) were purified by distillation under reduced pressure. All other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich.

2.2. Methods

2.2.1. Grafting of specific monomers onto fibroin fiber

The grafting procedure was adapted from literature [12] and mainly consists in: the fibers are treated with ammonium cerium nitrate (Ce^{4+}) in sulphuric acid solution under inert atmosphere for 30 minutes. Then the monomer solutions of AMPSA, HEMA-AMPSA (10% molar composition of AMPSA) and DEAEMA were added in the reaction medium and the temperature was raised to 45°C. After 24 hours the grafting reactions were almost complete and the fibers were rinsed with demineralised water to remove the residual cerium salt and dried over night at 37°C. The recipes are presented in table 1.

TABLE 1. Recipes for fibroin grafting

System	Mass ratio
Initiator / Fibroin	1/5
AMPSA / Fibroin	4/1
AMPSA/ Fibroin	7/1
HEMA-AMPSA (molar ratio 90/10) / Fibroin	4/1
HEMA-AMPSA (molar ratio 90/10) / Fibroin	7/1
DEAEMA / Fibroin	4/1
DEAEMA / Fibroin	7/1

2.2.2. FTIR-ATR and XPS measurements on fibroin fibers

The FTIR Spectra were recorded using 32 scans with a resolution of 4 cm^{-1} in 600-4000 cm^{-1} on a VERTEX 70 BRUCKER instrument. XPS analysis was performed on a K-Alpha instrument from Thermo Scientific, using a monochromated Al Kα source (1486.6 eV), at a pressure of 2×10^{-9} mbar. Charging effects were compensated by a flood gun, and binding energy was calibrated by placing the C 1s peak at 285 eV as internal standard. The survey spectra were registred using a pass energy of 200 eV.

2.2.3. Biomineralization assays

The surface-modified fibroin fibers (preliminary immersed in $CaCl_2$ solution), prepared by the above method were soaked in 45 ml of SBF1x [3, 13] of pH 7.49 and ion concentrations (Na$^+$, 142.19; K$^+$, 4.85; Mg^{2+}, 1.5; Ca^{2+}, 2.49; Cl$^-$, 141.54; HCO$_3^-$, 4.2; HPO$_4^{2-}$, 0.9; SO$_4^{2-}$, 0.5 mM) approximately equal to those of human blood plasma at 37ºC for various periods up to 14 days. The SBF was prepared by dissolving reagent-grade chemicals of NaCl, NaHCO$_3$, KCl, K$_2$HPO$_4$, MgCl$_2$·6H$_2$O, CaCl$_2$ and Na$_2$SO$_4$ in distilled water, and buffering at pH 7.49 with tris(hydroxymethyl)aminomethane (TRIS) and 1.0 N HCl aqueous solution at 37°C. After the soaking period (14 days), the fibrous specimens were removed from the fluid, gently washed with distilled water, and then dried at room temperature for 24 h.

SEM analysis

The surface morphology of the samples incubated in synthetic body fluid was observed by using Zeiss Evo 50 XVP Scanning Electron Microscope (SEM). Prior to imaging the samples were sputtered with a thin layer of gold.

3. Results and Discussions

3. 1. Grafting yield

Gravimetric evaluation represents the "mass gain" compared to the initial weight of the unmodified fibres, offering quantitative information on the copolymer deposited on the fibres. The grafting yield (η) was calculated using the equation (1):

$$\eta = \frac{m_f - m_i}{m_i} \, x100 \qquad (1)$$

where: m_i – weight of the unmodified fibre before grafting,
$\qquad m_f$ – final mass.

The values for the grafting yields were between 40 and 60%: $\eta_{AMPSA} = 40\%$, $\eta_{HEMA-AMPSA} = 50\%$, and $\eta_{DEAEMA} = 45\%$.

3.2. FTIR-ATR and XPS measurements

FTIR spectra (spectra not presented) show the specific absorption band of fibroin sample and also the grafted fibroin: amide I at 1224.58 cm^{-1}, amide II 1555.78 cm^{-1}, amide III 1619.91 cm^{-1} and OH from fibroin grafted with HEMA-AMPSA at 3400 cm^{-1}.

XPS spectra of the grafted fibroin with HEMA-AMPSA and AMPSA reveal the presence of S peak in these samples (2.66, At., %, figure 1), which is not present within the blind fibroin sample.
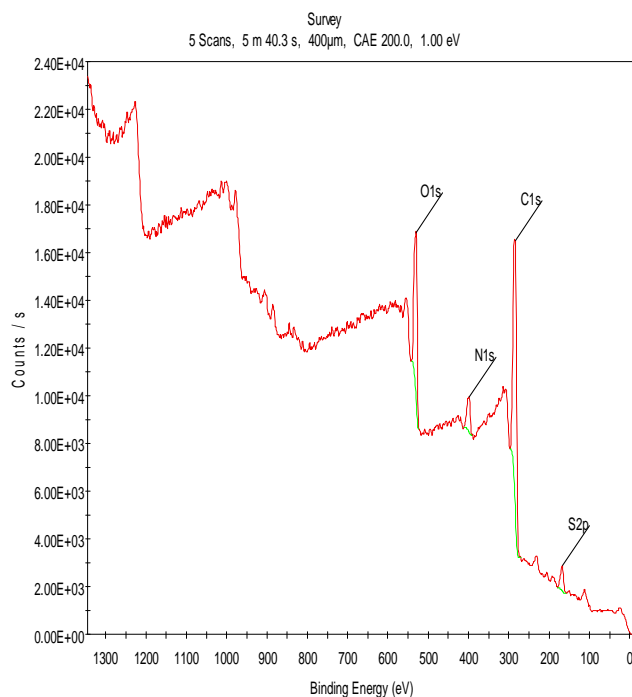

Figure 1. XPS spectrum of fibroin grafted with AMPSA (1/7, w/w)

3.3. Biomineralization assay and SEM analysis

Mineralization potential is a very important property of polymeric biomaterials, which greatly influences their application field. As we have already shown in the previous chapter Kokubo et al. (1990, 2006) method of *in vitro* evaluation was chosen. The procedure involves samples incubation in synthetic body fluid (SBF) in different concentrations.

Figures 2-5 show the SEM microphotographs of the crude fibroin and grafted with AMPSA, HEMA-AMPSA and DEAEMA.

As we could see from these figures, particles are observed on the grafted fibroin fibres soaked in 1xSBF, but they were not observed on the crude fibroin fibres. The morphology of the observed particles was that of an assembly consisting of finer particles. This particle morphology is similar to that of apatite crystals observed on bioactive glasses after having been soaked in a SBF.

The EDX results indicated that the particles contain predominantly calcium and phosphorus and Ca/P ratio is very close to natural bone hydroxyaapite (1.67).
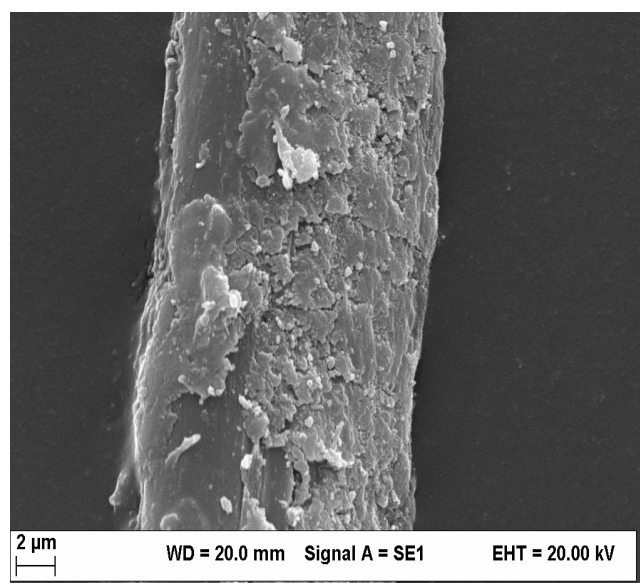

Figure 2. SEM microphotographs of the crude fibroin
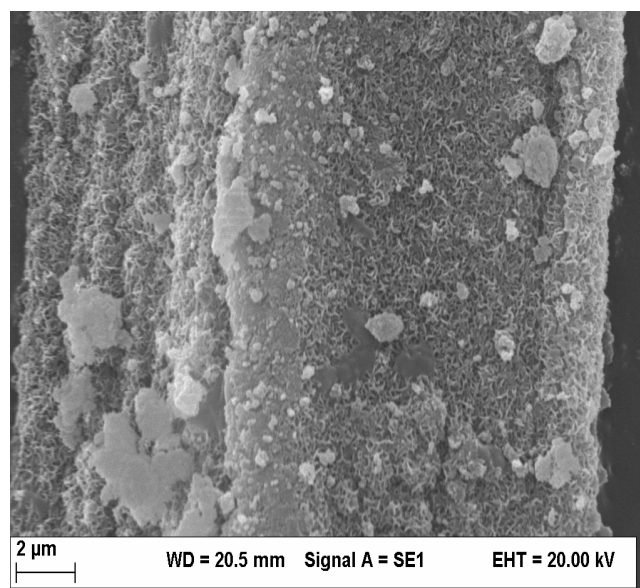

Figure 3. SEM microphotographs of the fibroin grafted with AMPSA
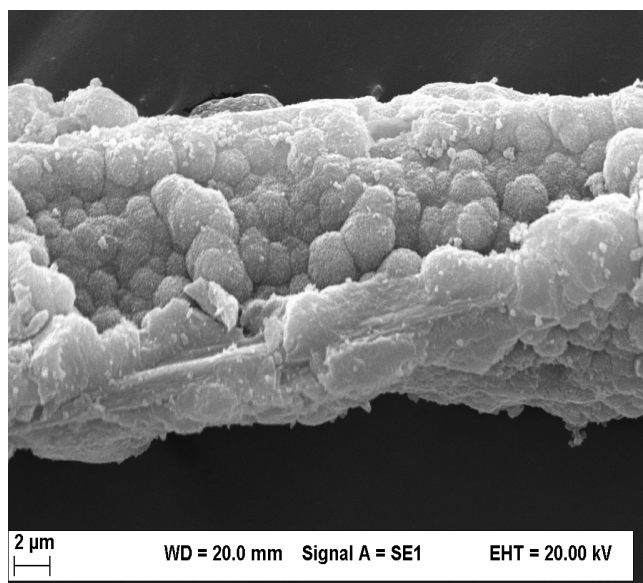
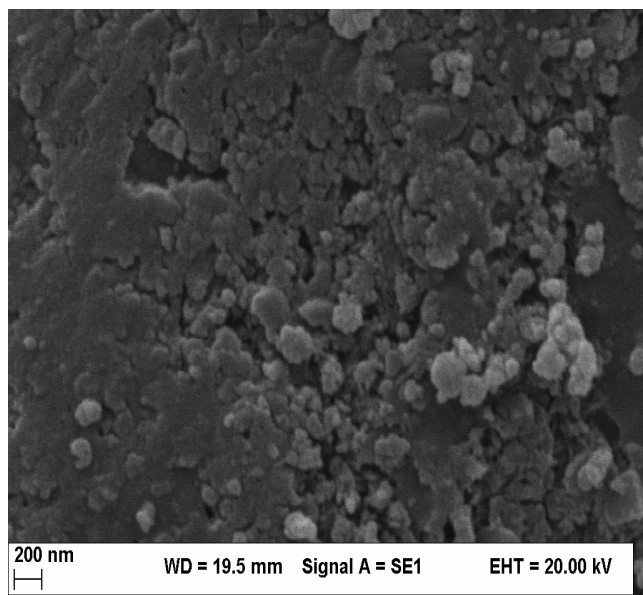Figure 4. SEM microphotographs of the fibroin grafted with
HEMA-AMPSA

Figure 5. SEM microphotographs of the fibroin grafted with
DEAEMA

It is clear from the results seen figures 2-5 that grafted fibers have the ability to deposit apatite in 1xSBF and the rate of apatite deposition is enough to cover the entire surface of the fibers after 14 days immersion.

In contrast crude fibroin fiber does not have the ability to deposit apatite under the same conditions. In this way the idea with the functional groups from the surface of the fibers is sustained. The acidic and amine groups present on the surface of the fibroin after the grafting procedure induce the mineralization phenomenon.

4. Conclusions

The results obtained in the present study indicate that grafted fibroin with acidic and amine groups has the potential to induce apatite deposition on its surface in a biomimicking solution, in this case 1xSBF. The presence of calcospherites and the apatite nucleation may be attributed to the existence of acidic and amine groups from the silk fibers.

Hydroxypatite formation on fibroin fiber can be accelerated by prior treatment with an aqueous solution containing calcium ions, such as a $CaCl_2$ solution, having a concentration of 1 kmol/m^3 or more. These findings support the use of these hybrid materials based on grafted silk fibers and HA as bone substitutes.

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