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Silk fibroin films for tissue bioengineering applications

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Silks are fibrous proteins with remarkable mechanical properties produced in fiber form by silkworms and spiders. Silk fibers in the form of sutures have been used for centuries. The unique mechanical properties, controlled biodegradability, haemostatic properties of these fibres provided important clinical repair options for many applications. During the past 20 years, some biocompatibility problems have been reported for silkworm silk. Recently regenerated silk solutions have been used to form a variety of biomaterials, such as gels, sponges and films, for biomedical applications. The aim of this study consists in the obtaining of regenerated fibroin films and their modification with different groups for some specific applications. In this respect there were obtained fibroin-polyvinyl alcohol films, fibroin-poly(itaconic acid) films, fibroin-poly(2-acrylamido-2-methylpropane sulphonic acid) films, fibroin-poly(2-hydroxyethyl methacrylate-co-itaconic acid) and fibroin-poly(2-hydroxyethyl methacrylate-co-2-acrylamido-2-methylpropane sulphonic acid) films. The structure of the new biomaterials was evaluated by RAMAN spectroscopy. Contact angle measurements were also performed. Cytotoxicity assays show the good *in vitro* biocompatibility of the fibroin-based biomaterials. Mineralization assays in SBF1x solution revealed the presence of apatite-like crystals onto the surface of the films of silk fibroin modified with acidic groups. The positive results may lead to the potential use of apatite-coated grafted silk fibroin scaffolds as bone-repairing material (bone tissue bioengineering). The silk fibroin-polyvinyl alcohol films are simply to be obtained and could be used for blood-vessel engineering.

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1. Introduction

Silk, popularly known in the textile industry for its luster and mechanical properties, is produced by cultured silkworms [1-4]. Silks are produced by members of the class Arachnida (over 30,000 species of spiders) and by several worms of the order Lepidoptera, which includes mites, butterflies and moths. Silks are fibrous proteins synthesized in specialized epithelial cells that line glands in these organisms [2, 4-5].

Silk fibroin polymers consist of repetitive protein sequences and provide structural roles in cocoon formation, nest building, traps, web formation, safety lines and egg protection [1-3]. Silks are generally composted of β-sheet structures due to the dominance of hydrophobic domains consisting of short side chain amino acids in the primary sequence. These structures permit tight packing of stacked sheets of hydrogen bonded anti-parallel chains of the protein. Large hydrophobic domains interspaced with smaller hydrophilic domains foster the assembly of silk and the strength and resiliency of silk fibres [4, 6-10] .

Silks from silkworms (e.g., *Bombyx mor*i) and orb-weaving spiders (e.g., *Nephila clavipes*) have been explored to understand the processing mechanisms and to exploit the properties of these proteins for use as biomaterials [5-8].

Biomaterial design is an important element of tissue engineering, incorporating physical, chemical and biological cues to guide cells into functional tissues via cell migration, adhesion and differentiation. Biomaterials may need to include provisions for mechanical support appropriate to the level of functional tissue development. In general, biomaterials must be biocompatible and elicit little to no host immune response [1, 11-12].

Nowadays, silk fibroin has found diverse applications in the biomedical field which can be attributed to its high tensile strength, controllable biodegradability, haemostatic properties, non-cytotoxicity, low antigenicity, and non-inflammatory characteristics [13-22].

Thus, silks have been investigated as biomaterials due to the successful use of silk fibers from *B. mori* as suture material for centuries. Adverse effects experimented in the historical use of silk as suture thread have been attributed to inflammatory reactions promoted by sericins residues in the material. A number of research groups have investigated fibroin (after sericin extraction) for new biomedical applications [20-25]. While native fibres have been investigated, more commonly cocoon fibres are dissolved to obtain a fibroin solution, which can then be processed into a variety of states, including films, hydrogels and membranes, or blended with other polymers.

Fibroin films have been used to coat surfaces, resulting in improved cell adhesion for anchorage-dependent cells comparable to collagen substrates [26-27]. Fibroin films have also been shown to induce bone tissue growth *in vitro* when seeded with osteoblasts [28-30].

Aiming at preparing scaffolds suitable for tissue-engineering applications or even implant biomaterials, we have investigated the preparation of fibroin films regenerated from lithium bromide solutions. Fibroin-polyvinyl alcohol matrixes were obtained and then characterised by contact angle measurements. Next, we have functionalized the fibroin fibres with different carboxylic and sulphonic groups and then we have dissolved the grafted fibres into lithium bromide solution resulting various films. The films were subjected to contact angle measurements and cytotoxicity assays against macrophage cells. Mineralization assays in SBF1x solution revealed the presence of apatite-like crystals onto the surface of the films of silk fibroin modified with acidic groups.

2. Materials And Methods

2.1. Materials

Silk cocoons were kindly supplied by S.C. SERICAROM S.A Company (Bucharest, Romania). Itaconic acid (IA) and 2-acrylamido2-methylpropane sulphonic acid (AMPSA) were provided by Sigma Aldrich, St-Quentin Fallavier, France and used without any further purification. 2-hydroxyethyl methacrylate (HEMA) was purified by distillation under reduced pressure. Polyvinyl alcohol (PVA 88%) was supplied from Fluka and used as such. Lithium bromide (LiBr), sodium bicarbonate and sodium dodecyl sulphate (SDS) were provided by Alfa Aesar GmbH&Co KG, Germany and dialysis tubing cellulose membrane from Sigma All other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich.

2.2. Methods

2.2.1. Functionalization of fibroin fibre with acidic groups

Bombyx mori silkworm cocoons were boiled for 30 min in an aqueous solution of 0.5 % (w/v) $NaHCO_3$ and SDS 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. The grafting procedure was adapted from literature [16] and mainly consists in: the fibres are treated with ammonium cerium nitrate (Ce^{4+}) in sulphuric acid solution under inert atmosphere for 20 minutes. Then the monomer solutions of IA, AMPSA, HEMA-IA (10% molar composition of IA) and HEMA-AMPSA (10% molar composition of AMPSA) were added over the reaction medium and the temperature was raised to 45 °C. After 4 hours the grafting reaction was almost complete and the fibres 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 silk fibroin grafting.

System	Mass ratio
Initiator / Fibroin	1/5
IA / Fibroin	2/1
IA / Fibroin	3/1
IA / Fibroin	4/1
HEMA-IA (molar ratio 90/10) / Fibroin	2/1
HEMA-IA (molar ratio 90/10) / Fibroin	3/1
HEMA-IA (molar ratio 90/10) / Fibroin	4/1
AMPSA / Fibroin	2/1
AMPSA / Fibroin	3/1
AMPSA / Fibroin	4/1
HEMA-AMPSA (molar ratio 90/10) / Fibroin	2/1
HEMA-AMPSA (molar ratio 90/10) / Fibroin	3/1
HEMA-AMPSA (molar ratio 90/10) / Fibroin	4/1

2.2.2. Preparation of silk fibroin films

1g of purified *Bombyx mori* fibroin was solubilized in 9.5 M aqueous LiBr solution and dialyzed in cellophane bags for 3 days. The clear solution was kept into the refrigerator. Polyvinyl alcohol solution was obtained by dissolving PVA in demineralised water at 50°C for 2 hours.

The fibroin and PVA solutions were mixed together at different weight ratios (100/0, 50/0 and 0/100). 10 ml of this mixture was cast onto Petri dishes to obtain films of fibroin-PVA films. Then they were dried in vacuum oven at room temperature over night.

The functionalized fibroin fibres were obtained as films by the same procedure described below. The fibroin-IA, fibroin-AMPSA, fibroin-HEMA-AMPSA and fibroin-HEMA-IA films were dried under vacuum at room temperature over night and subjected to further analyses.

2.2.3. RAMAN analysis

A Bruker spectrophotometer with ionised argon laser COHERENT Innova 90-4 (4W) device was used to perform the RAMAN analysis of the grafted fibres.

2.2.4. Contact angle measurements

KSV CAM 200 apparatus was used for static contact angle measurements performed on dried films. Ultrapure water droplets were used with a drop volume of 20 µl. The measurement of each contact angle was made within 10 s after each drop to ensure that the droplet did not soak into

the compact. The contact angles reported were the mean of 10 determinations. Smaller contact angles correspond to increased wettability.

2.2.5. Cytotoxicity assays

Biomaterials biocompatibility can be verified by testing *in vitro* cytotoxicity. It was used cell line L929 of murine fibroblasts, which was cultivated in culture medium (DMEM or RPMI 1640), supplemented with calf foetal serum and antibiotics. The cells were microscopically examined for detecting cytotoxicity visible signs, cellular lysis or cellular components dimensions and conformation. SEM analysis has been performed using a QUANTA INSPECT F SEM device equiped with a field emission gun (FEG) with a resolution of 1.2 nm and with an X-ray energy dispersive spectrometer (EDS)

2.2.6. Mineralization tests

For mineralization assay, three samples of each grafted silk fibroin films were incubated in synthetic body fluid (SBF1x) at pH=7.45, adjusted with tris(hydroxy-methyl) aminomethane (Tris) and hydrochloric acid (HCl), for 14 days, under sterile conditions, in containers with 45 mL of the incubation medium at 37 °C. The incubation medium was changed every 48 h. After incubation, the hydrogels were rinsed with distilled water to remove any traces of salts from the surface and dried at 40 °C for 24 h. The composition of SBF1x is presented below: Na^+: 142.19 mM, Ca^{2+}: 2.49 mM, Mg^{2+}: 1.5 mM, HCO_3^-: 4.2 mM, Cl^-: 141.54 mM, HPO_4^{2-}, 0.9 mM, SO_4^{2-}: 0.5 mM, K^+: 4.85 mM.

The presence of mineral crystals onto the surface of the hydrogels was evaluated by SEM analysis. The Ca/P molar ratio was investigated by EDS spectroscopy.

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):

$$\eta = (m_f - m_i)/m_i \times 100 \quad (1)$$

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

The values for the grafting yields were between 37 şi 40%: η_{AI}=37%, $\eta_{HEMA\text{-}AI}$=40%, η_{AMPSA}=39%, $\eta_{HEMA\text{-}AMPSA}$=40%.

3.2. RAMAN analysis

The RAMAN analysis of the modified fibroin fibres are presented in figure 1 by comparison with the virgin silk fibroin. The specific bands of the fibroin are presented in table 2.

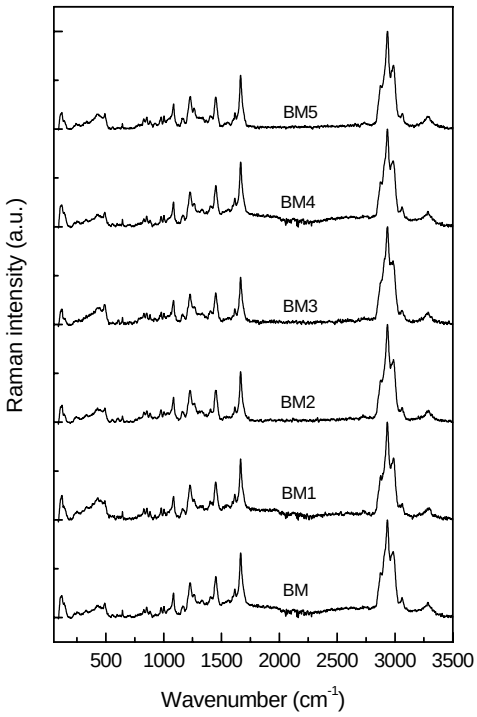

Fig.1. RAMAN spectra of the non-grafted (BM) and grafted fibroin with HEMA-AMPSA 10% and 10% molar (BM1 and BM2), HEMA-IA (BM3), IA (BM4) and AMPSA (BM5)

Table 2. RAMAN spectrum interpretation of the Bomby mori silk fibroin

Absorption band (cm^{-1})	Intensity	Assignment
645	Medium	Tirosine
830-855	Medium	Tirosine
1004	High	Phenyl-alanine and triptophan
1232-1269	Medium	III amide group
1449	Very high	CH_2 and CH_3 groups
1605	Medium	Phenyl-alanine
1668	Very high	I amide
3286	Medium	N-H

RAMAN spectrum of *Bomby mori* fibroin is very complex as it contains 17 natural aminoacids in which the major sequences are of Gly-Ala-Gly-Ala-Gly-Ser type. It is possible that the absorption peaks of the carboxylic and sulphonic groups to overlap the peaks of the fibroin functions. Due to this aspect it is very difficult to exactly compare the spectra of the non-grafted and grafted fibroin fibre. Nevertheless, we could observe differences in the 1000-1500 cm^{-1} region, fact that could confirm the

grafting of the carboxylic and sulphonic at some extent onto the fibroin surface.

3.3. Contact angle measurements

The contact angles measured on the different silk fibroin films modified with PVA or carboxylic and sulphonic groups are listed in table 3. Smaller contact angle usually indicates that the material surface is more hydrophilic, enhancing for example the cell adhesion and proliferation.

Table 3. Contact angle values for fibroin-PVA matrix film and fibroin grafted with acidic groups.

Material	Mean contact angle (°)
Fibroin film	54
PVA film	48
Fibroin-PVA matrix film	45
Fibroin-HEMA-AMPSA film	47
Fibroin-HEMA-IA film	55
Fibroin-IA film	54
Fibroin-AMPSA film	43

Data are shown as average ± standard deviation (n = 60).

3.4. Cytotoxicity using cell cultures

Numerous cells were encountered at the surface of the fibroin films after a three day period. The L929 murine fibroblast cells were mainly found in a round shape, but sometimes, they exhibited an elongated shape (figures 2 and 3). They exhibited numerous thin filopodia, and sometimes, thicker extensions were encountered, allowing communication with cells. No images of necrotic cell could be evidenced at the surface of polymeric films. The conclusion is that the fibroin-based biomaterials show no cytotoxicity against the cells so. From this point of view, the films could have potential use in medicine and biology as scaffolds or bone substitutes.

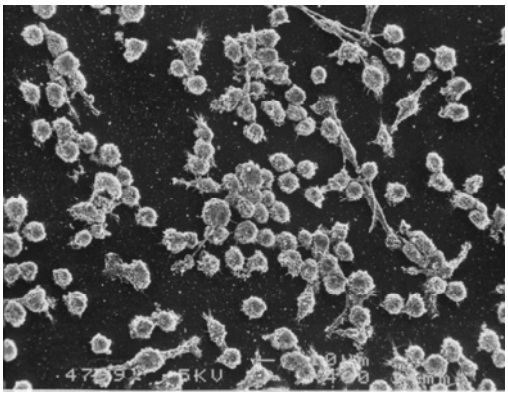

Fig.2. SEM microphotgraphs for fibroin-HEMA-AMPSA biomaterials

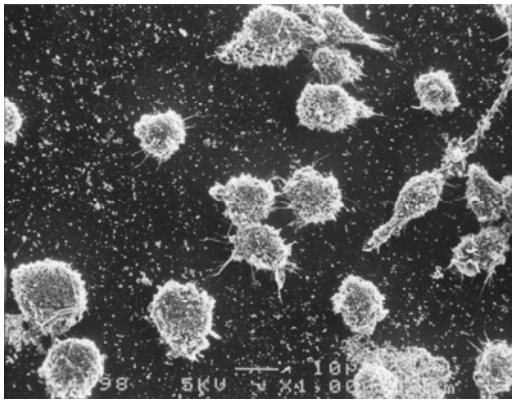

Fig.3. SEM microphotgraphs for fibroin-HEMA-IA biomaterials

3.5. Mineralization results

The mineralization capacity of the silk fibroin films grafted with carboxyl and sulphonic groups was assessed through SEM analysis. Most of the silk fibroin grafted films were uniformly covered with a mineral layer whose morphology resembles very much that of apatite crystals. The best results were achieved with silk fibroin films grafted with AMPSA and HEMA-AMPSA copolymer. As shown in figures 5 and 6, the mineral phase emerged from silk fibroin film surface. EDS analysis clearly identified Ca and P onto the surfaces of grafted silk fibroin films. The Ca/P molar ratios ranged between 1.5-1.7 for all the samples except pure silk fibroin film (fig. 4).

Apatite deposition is known to be initiated by some functional group that exists on the surface of a material [31-33]. In this case, the presence of specific acidic groups (carboxylic from itaconic acid and sulphonic from 2-acrylamido-2-methylpropane suphonic acid) is responsible for the formation of apatite-like deposits onto the surface silk fibroin films. This means that the silk protein rich in carboxyl and sulphonic groups may act as template substances to induce the deposition of apatite crystals.

Designed film materials can be useful for application not only as bone substitutes but also as scaffolds for tissue engineering.

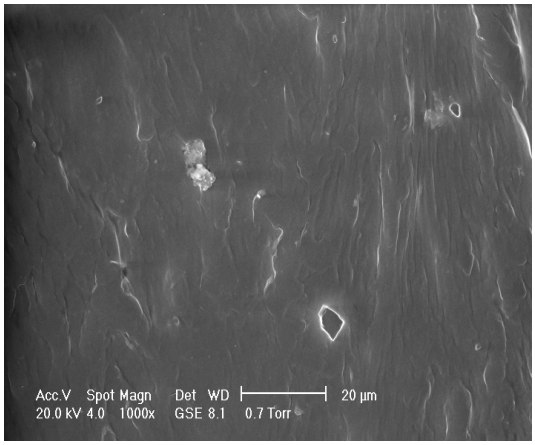

Fig.4. SEM microphotographs for silk fibroin crude film incubated in SBF 1x

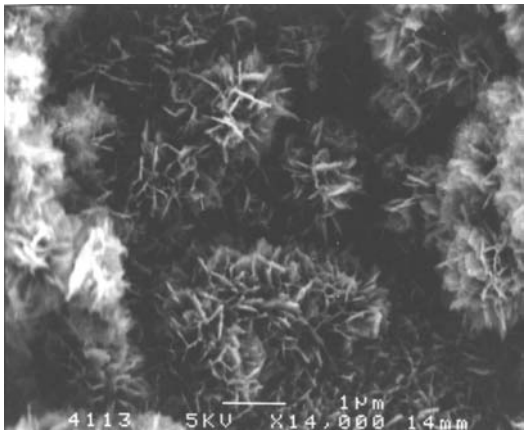

Fig.5. SEM microphotographs for fibroin-HEMA-AMPSA biomaterials incubated in SBF 1x.

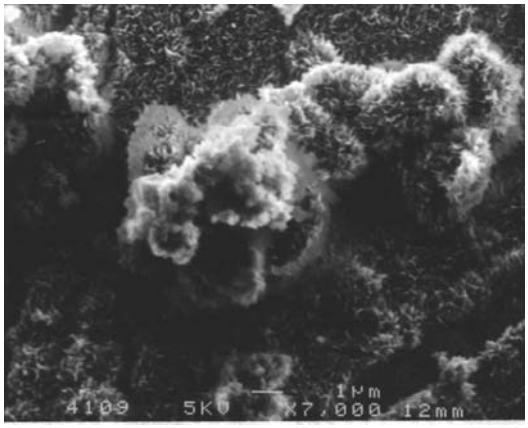

Fig.6. SEM microphotographs for fibroin-HEMA-IA biomaterials incubated in SBF 1x

4. Conclusions

We have reported in this article the preparation of fibroin-PVA matrixes and films of fibroin grafted with acidic groups. The idea of using fibroin films proved to be very efficient in medical applications as the biocompatibility of the materials could be improved. The contact angles revealed the hydrophilic character of the fibroin films, in this way enhancing for example the cell adhesion and proliferation. Silk fibroin-polyvinyl alcohol film could be potentially used in blood-vessel engineering applications. The cytocompatibility of the fibroin films was very good. The results obtained in the present study indicate that silk fibroin films modified with carboxylic and sulphonic groups have the potential to induce apatite deposition on its surface in a biomimicking solution, in this case SBF1x.

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