COMPOSITE HYDROGELS BASED ON SILK FIBROIN REINFORCED WITH CARBON NANOTUBES AND/OR GRAPHENES FOR TISSUE ENGINEERING APPLICATIONS

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Abstract

This paper focuses on the development of silkworm fibroin/polyacrylamide/carboxylated MWCNTs hydrogels as biomimetic materials to support the mineralization process. Hydrogels were obtained by free radical polymerization of acrylamide and methylenebisacrylamide in silk fibroin solutions containing dispersed carboxylated multiwall carbon nanotubes with a redox pair as initiator. The incorporation of fibroin and carbon nanotubes within the polyacrylamide matrix was proved bt ATR-FTIR spectroscopy. Swelling measurements in PBS solution were performed to evaluate the behvaiour of these hydrogels having various compositions. Hydrogels were then incubated in SBF1x in a biomineralization assay. SEM and EDX analysis revealed the presence of mineral nodules of apatite onto the surface of silk hydrogels, which explains the major role of the acidic groups upon mineralization process. Cytotoxicity tests on murine preosteoblastes cell line MC3T3-E1 revealed that these hydrogels have not developed cytotoxic effects.

1. Introduction

Silk fibroin is a natural fibrous protein produced by domesticated silkworm *Bombyx mori*, spiders or insects such as *Nephila clavipes* [1-2]. Silks are high molecular weight block copolymers consisting of a heavy (~370 kDa) and a light (~26 kDa) chain with varying amphiphilicity linked by a single disulfide bond [3-7]. These natural silkworm proteins have a highly repetitive amino acid sequence mainly composed of glycine (43%), alanine (31%), and serine (12%) residues [6, 9, 12-14]. The particular sequence of these amino acids with small side chains leads to the formation of hydrophobic domains, thus allowing the formation of thick packs of hydrogen bonded anti-parallel β-sheets [12-14]. Aqueous processing conditions, biocompatibility, good water vapour and oxygen permeability and biodegradability of silk fibroin, along with facile chemical modifications, are attractive features for its use as biomaterial.

Silk hydrogels have been thoroughly studied for potential biotechnological and biomedical applications [7]. To improve the properties of hydrogels, silk fibroin has been blended with various other polymers, which includes synthetic polymers such as polyvinyl

alcohol as well as natural macromolecules like gelatin, collagen, elastin, etc [7-9]. In a previous work [7] we have reported the synthesis and complex characterization of silk fibroin/polyacrylamide hydrogels for tissue engineering applications. The use of silk fibroin as a scaffold for bone reconstruction is a challenge for scientists all over the world. Therefore, the mechanical properties of silk fibroin should be improved to face the new application. The availability of molecular weight carbon nanotubes (MWCNTs), which have excellent mechanical properties make them suitable candidates for tailoring the mechanical properties of silk fibroin materials [10]. The functionalization of the nanotubes generates a novel, interesting class of materials, which combines the unique properties of nanotubes and the possibility of attaching an organic moiety for potential applications in tissue engineering and for biological purposes. Recent literature results showed that carboxylated carbon nanotubes could be capable of nucleating the HA crystals form simulated body fluid (SBF) resulting the formation of hierarchy assemblies.

This paper focuses on the development of silkworm fibroin/polyacrylamide/carboxylated MWCNTs composite hydrogels as biomimetic materials to support the biomineralization process. Composite hydrogels were incubated in SBF1x in a biomineralization assay [11]. SEM and EDX analysis revealed the presence of mineral nodules of apatite onto the surface of silk hydrogels, which explains the major role of the acidic groups upon mineralization process. The key role in mineralization process is the functionalization of MWCNTs together with the use of silk fibroin leading to the obtaining of very promising biomaterials for bone reconstruction. Cytotoxicity tests on murine preosteoblastes cell line MC3T3-E1 revealed that these hydrogels have not developed cytotoxic effects.

2. Materials and methods

2.1. Materials

The domesticated silkworm *(Bombyx mori* silk*),* cocoons were kindly supplied by S.C. SERICAROM SA (Bucharest, Romania). Acrylamide (AAm) was purified by recrystallization from water and dried in air. N,N'-methylenebisacrylamide (MBA), potassium persulphate (KPS) and triethanol amine (TEA) were purchase from Sigma-Aldrich. Lithium bromide (LiBr), sodium bicarbonate and sodium dodecyl sulphate (SDS) were provided by Alfa Aesar GmbH&Co KG, Germany, while dialysis tubing cellulose membrane by Sigma-Aldrich. Multiwall Carbon Nanotubes (MWCNTs) were purchased from Sigma-Aldrich having more than 90 % carbon basis (trace metal basis) and O.D. x I.D. x L 10-15 nm x 2-6 nm x 0.1-10 μm, produced by Catalytic Chemical Vapour Deposition (CCVD), Arkema Inc. The oxidation process was made using a mixture of 98 % sulphuric acid (Merck) and 70 % nitric acid (Merck). The functionalization process of MWCNT was performed using a standard protocol [12].

2.2. *Methods*

Composite hydrogels based on silk fibroin/polyacrylamide/MWCNT-COOH were obtained by polymerization reaction of acrylamide and methylenebisacrylamide in silk fibroin solution in which were dispersed the functionalized carbon nanotubes (MWCNT-COOH) using a constant concentration of carboxylated carbon nanotubes (1 wt. %) by a redox initiating system.

The FT-IR spectra were taken on a Jasco 4200 spectrometer equipped with a Specac Golden Gate attenuated total reflectance (ATR) accessory, using a resolution of 4 cm^{-1} and an accumulation of 60 spectra, in the 4000–400 cm^{-1} wavenumber region.

Morphological information including internal structure was obtained through the scanning electron microscopy (SEM) analysis of the gold-coated hydrogels. Hydrogel samples were frozen at –50 °C followed by lyophilization. The analysis has been performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG) with a resolution of 1.2 nm and with an X-ray energy dispersive spectrometer (EDS).

Evaluation of biomineralization potential of composite silk hydrogels

Hydrogel specimens with various compositions were immersed in synthetic body fluid (SBF1x) at pH=7.49, adjusted with tris(hydroxy-methyl) aminomethane (Tris) and hydrochloric acid (HCl), for 14 days, in containers with 45 mL of the incubation medium at 37 °C. After incubation, the hydrogels were rinsed with bi-distilled water to remove any traces of salts from the surface and dried at 40 °C for 24 h. The presence of mineral deposits onto the surface of the hydrogels was evaluated by SEM analysis. The Ca/P molar ratio was investigated by EDS spectroscopy.

Cell culture and cytotoxicity test on silk hydrogels

Murine preosteoblastes cell line MC3T3-E1 provided by American Type Culture Collection was cultured in monolayers, in DMEM (Sigma-Aldrich Co.) supplemented with 1% Antibiotic/Antimycotic (ABAM, Sigma-Aldrich Co.) and 10% Fetal Bovine Serum (FBS, Gibco). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, with the growth media changed every 3rd day.

The cytotoxic potential of the biomatrices on MC3T3-E1 cells was evaluated using "In vitro toxicology assay kit lactate dehydrogenase based" (TOX-7, Sigma Aldrich Co.), considering the spectrophotometric detection of LDH enzyme, released in the culture medium by the cells with damaged membrane.

3. Results and discussions

The compositions of the semi-interpenetrating networks were as follows: 0/100, 10/90, 20/80, 30/70, 40/60, 50/50, 70/30 in volumetric ratios. The polymerization and cross-linking reactions were almost complete after 4 days leading to three-dimensional polymeric networks where silk fibroin was physically entrapped. The last compositions were not furthermore studied due to low physical integrity.

The results of the FTIR-ATR spectra gave us the specific absorbance wavelengths of the specific bonds which appeared in the composite hydrogels, confirming the structure of the new materials. The presence of carbon nanotubes is hardly revealed by FTIR analysis (spectra not shown), even those functionalized with COOH groups (due to overlapping with the silk fibroin band). However we can see a slight peak shifting toward left of the signal from 3286 cm-1 in the hydrogel without nanotubes (silk fibroin-polyacrylamide 30/70, v/v) to 3385 cm-1 (SF/PAA/MWCNT-COOH, 30/70 v/v).

The biomineralization capacity of RSF/PAA/MWCNT-COOH hydrogels was assessed through SEM analysis. All the composite hydrogels were uniformly covered with a mineral layer whose morphology does not depended on hydrogels composition (fig. 1-2). Besides the effect of RSF, the COOH groups of functionalized carbon nanotubes potentiate the effect of mineralization. Due to the use of constant concentrations of carbon nanotubes, the

biomineralization process is similar; the mineral deposition is uniform, with minor differences.

EDS analysis clearly identified Ca and P onto the surfaces of RSF/PAAm/MWCNT-COOH hydrogels (nanostructured HA crystals). The Ca/P molar ratios ranged between 1.5-1.7 for all the hydrogels.

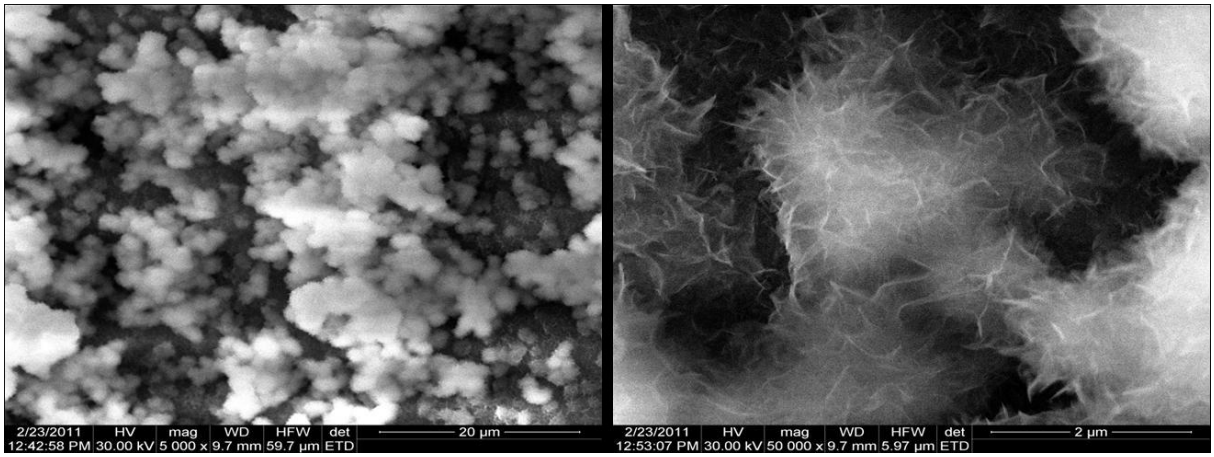

Fig.1. SEM microphotographs showing the mineral deposits onto the surface for SF/PAA.MWCNT-COOH hydrogels with SF/PAA 10/90 v/v, incubated in SBF 1x

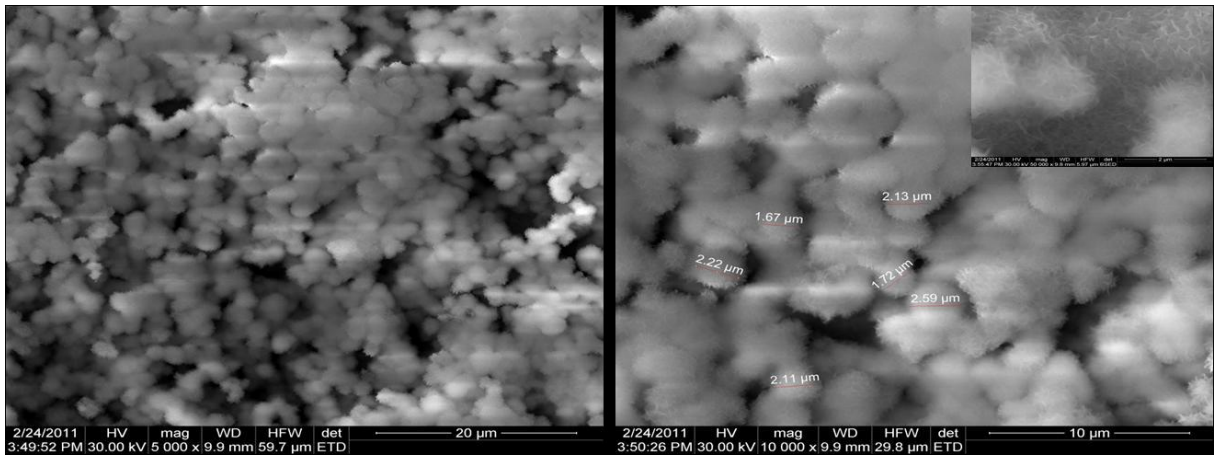

Fig.2. SEM microphotographs showing the mineral deposits onto the surface for SF/PAA.MWCNT-COOH hydrogels with SF/PAA 40/60 v/v, incubated in SBF 1x

In order to examine cell survival up to 9 days, the viability of MC3T3-E1 osteoblast-like cells in contact with P1 (SF10/90PAA/MWCNT-COOH), P2 (SF30/70PAA/MWCNT-COOH) and P3 (SF50/50PAA/MWCNT-COOH) scaffolds was evaluated at 2, 5 and 9 days post seeding using LIVE/DEAD assay. The ratio between the viable (green labeled) and the dead (red labeled) cells was found to be constantly positive, whereas a higher cellular density was revealed on P3 than on P2 and P1 respectively. Although all the matrices allowed cell proliferation, only in MC3T3-E1/P3 specimen, the fluorescent staining of the 3D culture revealed a full population of the scaffold.

Based on the evaluation of P1, P2 and P3 cytotoxic potential on MC3T3-E1 cells up to 9 days the activity of LDH enzyme in the culture media was found to be significantly increased at 2 days post seeding as compared to 5 and 9 days, in all composite hydrogels.

These findings suggest that the tested biomaterials displayed a lower cytotoxic effect on MC-3T3-E1 cells when the concentration of PAA decreased in their composition. In addition, the highest LDH activity was detected in all samples in during the first 2 days of culture and then the trend was constant, suggesting that MC3T3-E1 preosteoblastes require a short period of time to accommodate to the new microenvironment, rather that the biomaterial exert/produce/have a cytotoxic effect.

4. Conclusions

Composite hydrogels of SF/PAA/MWCNT-COOH with various compositions were developed and characterized by physico-chemical and biological means. Apatite-like crystals were found onto the surfaces of silk hydrogels incubated in SBF1x. The presence of functionalized carbon nanotubes significantly improved the biomineralization behaviour of hydrogels in SBF1x, which no longer depends on the hydrogels composition. Cytotoxicity tests on murine preosteoblastes cell line mc3t3-e1 revealed that these hydrogels have not developed significant cytotoxic effects. Results of this study lay down the fundament for the use of these silk fibroin biomaterials in bone tissue engineering applications.

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