NATURAL FIBERS MODIFIED BY CHEMICAL METHODS FOR APPLICATION IN BONE PATHOLOGY

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Polimerii naturali sub formă de fibre au fost modificați chimic pentru a obține proprietăți de mineralizare asemănătoare cu proteinele din os. Astfel acidul itaconic (AI) şi 2-hidroxietilmetacrilatul (HEMA) au fost grefați pe celuloză şi fibroină din mătase naturală cu două sisteme de inițiere, materialele rezultate fiind incubate în plasmă sintetică şi analizate prin microscopie electronică de baleiaj şi dozare de calciu şi fosfor. Prin grefarea acidului itaconic pe fibroină se obține un material care conduce la formarea unor depozite minerale foarte asemănătoare cu hidroxiapatita.

Natural fibrous polymers were chemically modified to mimic the behaviour of bone proteins responsible for mineralization. In this respect, itaconic acid (IA) and 2-hydroxyethyl methacrylate (HEMA) were grafted onto cellulose and fibroin from natural silk by two initiating systems. The resulted materials were incubated in synthetic body fluid and analyzed by scanning electronic microscopy and dosage of calcium and phosphorus. The new material resulted from the grafting of itaconic acid onto the fibroin led to the formation of mineral deposits very similar to hydroxyapatite.

Keywords: fibroin, cellulose, itaconic acid, hydroxyapatite

1. Introduction

The development of osteoconductive biomaterials for bone repair represents a major challenge in the biomedical research. A wide range of materials were used as synthetic bone grafts. The design of osteoconductive behaviour is considered one of the most important characteristics of natural grafts, consisting in apatite-type content and porous interconnected microarchitecture. Combining these two attributes in a

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new orthopaedic formulation could ensure the success of the implant via osteoconduction and osteoinduction. The use of polymers for bone repair allows the synthesis of numerous biomaterials with specific structure and properties. Polymers are used in osseous applications due to their wide chemical composition, to their different mechanical behaviour and because the organic bone matrix are mainly formed by macromolecules. In order to form apatite deposits in/on the implant, after implantation, polymer must contain negative chemical groups [1-6], in order to mimic the activity of bone proteins responsible for mineralization [7-9]. The respect of the bone porous microarchitecture can be obtained by an innovative solution, using fibrous polymers, forming micro- and macropores by plying. The present research focuses on the design of porous materials based on natural fibrous polymers (silk fibroin and cellulose for comparison), modified by grafting with itaconic acid (IA), responsible for the presence of negative groups. IA was chosen because its efficacy, one molecule introducing two acid groups on the fibrous support, this diminishing the level of the grafting agent in the reaction. IA was used in mixture with 2-hydroxyethyl methacrylate (HEMA), a very biocompatible monomer. Fibroin and cellulose were selected as natural fibres due to their wide use in different biomedical applications [10-19]. Two types of fibroins were used, secreted by *Bombyx mori* and Phylosamia ricini silk worms. The present work reports on the synthesis and on the morphology of these fibrous biomaterials for bone application.

2. Experimental

2.1. Materials

Commercial 2-hydroxyethyl methacrylate (HEMA) was purchased from Aldrich and purified by extraction with 3% (w/v) solution of sodium bicarbonate ($NaHCO_3$) in distilled water, extracted with chloroform ($CHCl_3$) and the solvent was removed by rotary evaporation under vacuum. HEMA was made free of $CHCl_3$ by distillation (b.p. 75°C at 3 mmHg).

Itaconic acids (IA) (Aldrich), potassium persulphate (PSK) (Fluka) and ammonium cerium nitrate (ACN) (Merck) were used without any further purification.

Cellulose and fibroin fibres were provided by S.C.Sericarom S.A. Bucharest and other chemicals by Chimopar Bucharest.

2.2. Methods

IA-HEMA grafting onto cellulose and fibroin

Cellulose was washed three times for 1 hour with boiling demineralised water, followed by drying at 40°C (up to constant mass).

The grafting reaction developed in heterogeneous medium because the reaction mixture was liquid and the fibrous support solid. Cellulose was weighted and introduced in an acid solution of ceric salt, with nitrogen bubbling for 10 minutes.

The monomers mixture and sulphuric acid 0.1N was added, followed by nitrogen bubbling for 15 minute. The reaction temperature was 45°C, in inert atmosphere, for 90 minutes. At the end of the reaction, the fibres were washed with demineralised water and extracted in order to completely eliminate the residual monomers.

Several compositions were synthesized, as shown in table 1, using constant IA:HEMA ratios (30:70) and different monomer/substrate ratios.

Table. 1

Reaction mixture for grafting of IA-HEMA onto cellulose

Reactive	Role	Quantity		
		1	2	3
Cellulose	Substrate	0.5 g	0.5 g	0.5 g
ACN	Initiator	5.45 mmole	5.45 mmole	5.45. mmole
HEMA	Monomer	1.95 ml	2.54 ml	3.9 ml
IA solution 5%	Monomer	17.94 ml	23.4 ml	35.88 ml
Sulphuric acid 0.1N	Catalyst	25 ml	25 ml	25 ml

The solution of ACN was used in 0.1N sulphuric acid. All samples were washed with distilled water, extracted to remove residual monomers and dried (up to constant mass).

Two types of fibroin were used as fibrous substrates: *Bombyx mori* (FBM) and *Phylosamia ricini (*FPR).

Fibroin samples were extensively washed with boiling water solution of Na_2CO3 0.5M and $NaHCO_3$ 0.5M for 30 minutes in order to eliminate the sericine and then dried at 40°C. Two initiators were used for grafting of IA-HEMA: PSK and ACN. Two copolymers series were synthesized using both FPR and FBM fibres. The ratios initiator fibroin were 1/10 and 1/5 (w/w), table 2.

Table 2

Reaction mixture for IA grafting onto fibroin

Reactive	Role	Quantity			
		1/10		1/5	
		FPR	FBM	FPR	FBM
FPR or FBM	Substrate	0.5 g	0.5 g	0.5 g	0.5 g
ACN	Initiator	0.31 g	0.31 g	0.61 g	061 g
HEMA	Monomer	1.7 ml	1.67 ml	3.4 ml	3.4 ml
Aqueous solution of IA 5%	Monomer	15.6 ml	15 ml	11.2 ml	11.2 ml
Sulphuric acid 0.1N	Catalyst	25 ml	25 ml	25 ml	25 ml
FPR or FBM	Substrate	0.5 g	0.5 g	0.5 g	0.5 g
PSK	Initiator	0.05 g	0.05 g	0.05 g	0.05 g
HEMA	Monomer	1.7 ml	1.7 ml	3.4 ml	3.4 ml
Aqueous solution of IA 5%	Monomer	15 ml	15 ml	11 ml	11 ml

Fibroin was initially treated with acidic solution of cerium (IV) salt at 45°C under nitrogen bubbling for 10 minutes. The mixture of monomers was added under nitrogen atmosphere for 15 minutes. The reaction developed at 45°C under inert atmosphere for 90 minutes.

The grafting initiated by PSK (1/10 w/w PSK/fibroin) was performed at 70°C, for 90 minutes. All samples were washed with distilled water, extracted to remove residual monomers and dried up to constant mass.

The results of the grafting reactions were gravimetrically evaluated and also by scanning electronic microscopy (SEM).

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

$$GR = \frac{(m_f - m_i)}{m_i} \cdot 100 \quad (1)$$

where: m_i - weight of the fibre before grafting; m_f - finall mass of the fibre after grafting

SEM analysis

SEM analysis of the morphology of the fibrous materials was achieved with an electronic microscope SMPE XL 30 Philips.

In vitro test

In vitro tests were performed by two methods:

- The classical incubation method (biomimetic) [2, 17] in 1x and 1.25x synthetic body fluid (SBF) having the composition similar to that of human plasma, for 14 days at *p*H=7.42 and 37°C, see table 3.
- The incubation method based on alternating cycles in two solutions: $CaCl_2$ 200mM/Tris-HCl at *p*H=7.42 (Ca solution) and Na_2HPO_4 150mM (P solution) [15].

The samples were weighed and then immersed in 25 ml Ca solution at 37°C for 2 hours. After this procedure the samples were washed with distilled water and immersed in P solution at 37°C for 2 hours. This procedure was repeated three times.

Table 3

Synthetic body fluid (SBF) and human blood plasma compositions

Ion	SBF 1x (mM)	Human plasma (mM)
Na^+	142.19	142.0
K^+	4.85	5.0
Mg^{2+}	1.5	1.50
Ca^{2+}	2.49	2.5
Cl^-	141.54	103.0
HCO_3^-	4.2	27.0
HPO_4^{2-}	0.9	1.0
SO_4^{2-}	0.5	0.5

Ca and P dosage

Calcium and phosphorus dosage was performed by the colorimetric method. For calcium deposits orthocresolphtaleine was used at 570 and 660 nm, and for phosphorus deposits the complex ammonium phosphomolibdate was used at 340 and 375 nm.

3. Results and discussions

The results of the gravimetric analysis are presented in table 4.

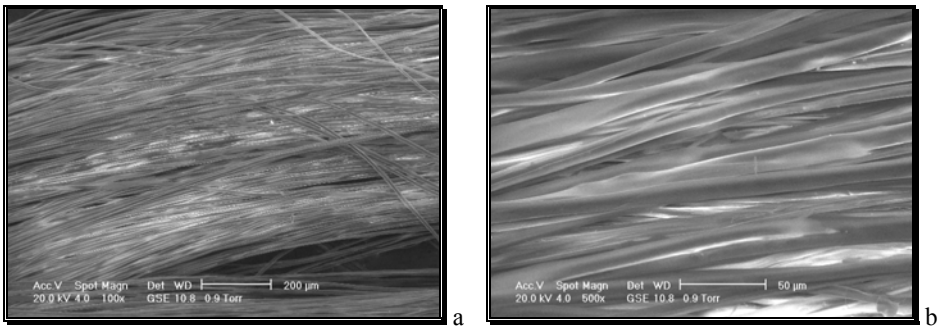
Table 4

Grafting ratio values

No.	Fibrous support	Grafting with IA-HEMA		GR (%)
1	FBM	Initiator Ce^{4+}		37
2	FBM	Initiator PSK		122
3	FPR	Initiator Ce^{4+}		42
4	FPR	Initiator PSK		302
5	Cellulose	Initiator Ce^{4+}	0.023 moles (IA-HEMA)	79
6	Cellulose	Initiator Ce^{4+}	0.03 moles (IA-HEMA)	60
7	Cellulose	Initiator Ce^{4+}	0.046 moles (IA-HEMA)	61

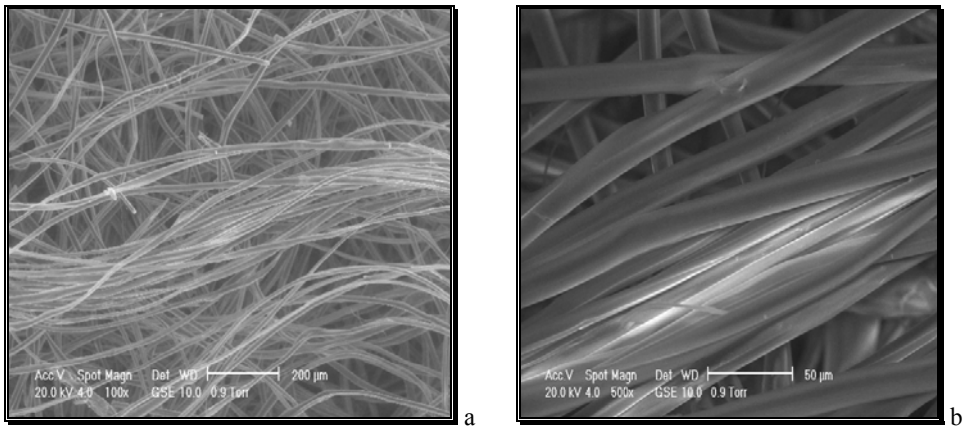
All reactions have significant values of the GR with values ranging from 37% and 302%., these results proving the grafting reactions. When PSK is used as initiator, the values of GR are higher than 100%, but these values show also the homopolymerisation and copolymerisation reactions that accompany the grafting ones. The presence of the homopolymer PHEMA and copolymer IA-HEMA covering the fibres was also noticed by the SEM analysis.

The results of the SEM examination of the fibrous samples are selectively presented in Figs. 1-4. The grafted samples were analysed as compared to the original fibres, at different magnification levels (100X, 500X, 2500X, 5000X).



a b

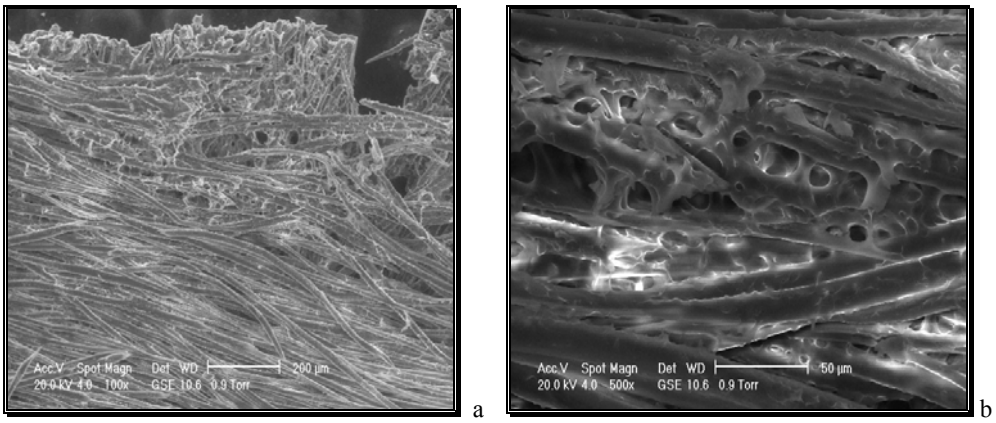
Fig. 1. SEM morphology of the original *Bombyx mori* silk fibroin general view (a - 100X; b - 500X)



a b

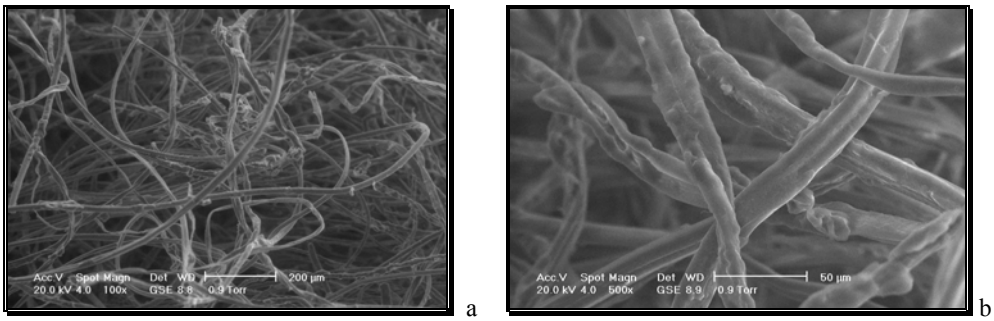
Fig. 2. SEM morphology of *Bombyx mori* silk fibroin grafted with IA-HEMA (initiator Ce^{4+}) general view (a - 100X; b - 500X)

SEM allows the analysis of the morphology of the fibres following the grafting reaction. This technique offers the possibility to establish the formation of copolymer grafts or/and copolymer deposition on fibrous substrate, without offering specifications about the chemical nature. Some modifications, even if proved by the gravimetric evaluation are not obvious from the point of view of morphological changes. *Bombxy mori* silk fibroin has the same appearance when grafted with IA-HEMA monomers and cerium salt initiator (fig. 1 and 2). The results obtained for the PSK initiation of silk fibroin grafting are very interesting (fig. 3).



a b

Fig.3. SEM morphology of *Bombyx mori* silk fibroin grafted with IA-HEMA (PSK initiator) general view (a - 100X; b - 500X)

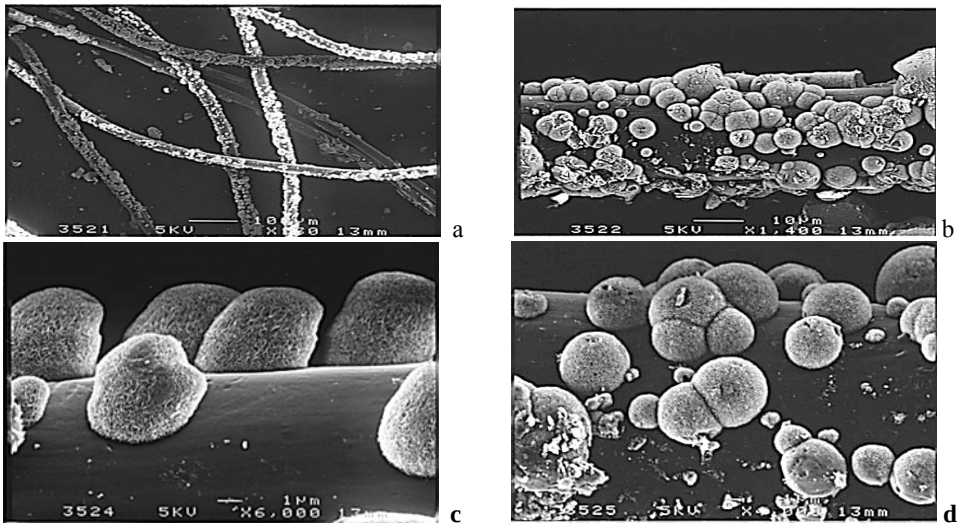


a b

Fig.4. SEM morphology of cellulose grafted with 0.03 moles of IA-HEMA general view (a - 100X; b - 500X)

The morphology of the grafted fibres corresponds to interconnected pores, mimicking the trabecular bone architecture. The interconnection is evident (Fig. 3b), and the copolymer IA-HEMA connection to the fibroin fibres. This behaviour is very important by combining the interconnected porosity with the presence of numerous negative groups in the material. The morphology of cellulose fibers after modification is selectively presented in Fig. 4. Copolymer globular deposits appeared on the cellulose fibres.

For the fibroin modified with ammonium cerium nitrate and monomer mixture HEMA / IA = 90 / 10 (mole / mole), the mineral deposits resulted after incubation were analysed (Fig. 5 a, b, c, d). The Ca/P ratio was estimated at 1.6, value that is very close to that of hydroxyapatite (1.67).



a b

c d

Fig.5. SEM of the globular mineral deposits on grafted silk fibroin using $(NH_4)_2Ce(NO_3)_6$ as initiator, incubated in SBF at 37°C: a, b – grafts based on HEMA-IA 10%, monomers/fibroin=2/1; c, d – grafts based on HEMA-IA 10%, monomers/fibroin=4/1

4. Conclusions

1. The presence of calcospherites was observed on silk samples grafted with HEMA / IA after incubation in synthetic body fluid, respectively Ca and P solutions.
2. The best results were obtained for the modification of silk fibroin with IA-HEMA mixture following a PSK initiation. Following research will be done in order to completely elucidate the chemical nature of the formed copolymers, the grafting mechanism and the biological behaviour.

R E F E R E N C E S

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