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INTERACTION BETWEEN FIBROIN AND ALGINATE IN THE CORRESPONDING BLENDED FILMS

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ABSTRACT

Films blended with silk fibroin and sodium alginate of different ratios were synthesized and investigated. They were not treated with alcohols. They were subjected to FTIR, in vitro test and SEM analysis. The results showed that sodium alginate induced a conformation transition of the silk fibroin macromolecule and conversion of silk I to silk II. The fibroin's amido/amino groups and the carboxyl groups of the alginate interacted through hydrogen bonds. The ionic interaction between the alginate's COO^- groups and Ca^{2+} from 1.5 SBF was confirmed by FTIR analysis. The morphology of films was examined by SEM.

Keywords: biomaterials, silk fibroin, sodium alginate, FTIR, in vitro test, SEM.

INTRODUCTION

Natural biopolymer materials have good biocompatibility, biodegradability and bioactivity. Because of that they find wide range of applications in medicine and pharmacy. These properties make them irreplaceable in comparison to the variety of biomaterials based on synthetic polymers. Their application in the field of medicine requires a complex of properties, including physical, physico-chemical and chemical, as well as opportunities for their modification in view of the specifics of the corresponding application fields. Recent years of investigations have shown an increased interest to silk fibroin (SF), which is used in various fields of medicine [1 - 8] in the form of films, gels, membranes, sponges and fibers. Alginic acid and its various salts are used to treat wounds and burns because of their homeostatic properties. Their first application was in the form of a gel, but sodium alginate (SA) in the form of a porous material [9, 10] was found to be more effective.

Preparation and application of blends in various fields of medicine can be described as a rapid and growing research area involved in achieving optimal healing conditions. There are investigations [11 - 14] concerning the preparation of blends based on SF and a second component comprising gelatin, alginate, chitosan, etc., that achieve a desired change in SF properties.

There is no unanimous view in the literature about the interaction between SF and SA in their composites. Some authors believe that there is no interaction between SF and SA [13]. Others [14] interpret the results achieved on the ground of the interaction between SF's amide groups and the carboxyl group of alginate [15], or the connection between the carbonyl groups of the SF and the hydroxyl groups of alginate [16].

The present study aims to support the thesis that there is an interaction between SF's amino/amido groups and SA's carboxyl groups based on intermolecular hydrogen bonds. The results obtained can provide useful information regarding preparation and implementation of biomedical materials. This work is focused to the preparation of blended films not treated with alcohols and the investigation of the interaction between fibroin and alginate.

EXPERIMENTAL

Materials

Silk fibroin used for these experiments was in form of degummed textile fabrics. SF without sericin was dissolved in the system $CaCl_2$: C_2H_5OH: H_2O (in a molar ratio of 1:2:8) [17] for 60 min at a temperature of 75°C. It was subjected to dialysis with distilled water for 3 days. Cellulose membranes were used (Slide-A-Lyzer). The

insoluble materials were removed through centrifuging for 20 min at 3500 rpm/min. It was applied twice. The final concentration of SF aqueous solution was 1.5 %. It was determined by drying at room temperature to constant mass.

SA used in the experiment was also in the form of 1 % aqueous solution purchased from Sigma-Aldrich. The blends were prepared by mixing using different mass % ratios - 75:25; 50:50; 25:75 (Table 1). It proceeded for 30 min under stirring. The solutions were poured into polystyrene dishes and dried at room temperature.The SF/SA ratio of 25 to75 resulted in a layered non-homogeneous structure with fibroin fibrils and which is why it was not further investigated. Most authors studying SF/SA composites observed a homogeneous structure in case of ratios applied, whereas Moraes M., Silva M., Weska R., Beppu M. [18] reported the formation of SF fibrils in composites containing more than 25 % of SF [18].

The films were obtained by drying at room temperature to exclude the solvent. Water was used as a solvent in this study.

The formation usually proceeds through three stages. The last one provides conformational changes in the polymer's macromolecules, which determine the film's good physical and mechanical properties [19]. Unlike almost all studies no post-treatment of the films with methanol was applied. This was done aiming greater clarity in respect to the transformational structural changes in the blends.

Methods

Fourier Transform Infrared Spectroscopy (FTIR) was applied to investigate the polymer's structure, the conformational transformation and other changes resulting from the interaction. The characteristic absorption bands of SF are known as amide I, amide II, amide III, amide IV and amide V. The initial SF fibers show wave numbers indicative for amide I (1625 cm^{-1} and 1701 cm^{-1}), amide II (1518 cm^{-1}), amide III (1229 cm^{-1}), amide IV (1064 cm^{-1}), amide V (725 cm^{-1}) and β-sheets regions [20]. SA is the second natural polymer used in the present study. It is characterized by intensive absorption bands of ionized carboxyl groups at 1610 cm^{-1} and 1400 cm^{-1}, non-ionized carboxyl groups positioned at 1420 cm^{-1} and hydroxyl groups at 1110 cm^{-1} and 1031cm^{-1} [21, 22].

Table 1. Composition of the blended films prepared.

Sample numbers	Blends composition , %	
	Fibroin	Sodium Alginate
1	100	0
2	75	25
3	50	50
4	25	75

The FTIR spectra were recorded using *Bruker Tensor 27* spectrometer in the range of 4000 cm^{-1} - 400 cm^{-1} with the samples macerated in KBr matrixes.

The *in vitro* test is widely used to determine the bioactivity, as it is easily applicable and reproducible. It was applied using a solution of 1.5 SBF of pH of 7.4. The films were soaked in the solution under static conditions for 3 days, 14 days and 25 days. The aqueous 1.5 SBF aqueous solution contained NaCl (11.9925 g), $NaHCO_3$ (0.5295 g), KCl (0.3360 g), K_2HPO_4.3H2O (0.3420 g), $MgCl_2.6H_2O$ (0.4575 g), $CaCl_2.2H_2O$ (0.5520 g) and Na_2SO_4 (0.1065 g) in 1L distilled water with buffering to reach pH of 7.4; TRIS = 9.0075 g and 1M HCl [23].

The *in vitro* behavior was defined by the change in the samples mass. The calculation was done on the ground of the following equation:

$$\text{Change in mass} = \frac{W_0 - W_t}{W_0}.100, \%$$

where W_0 is the mass of the initial sample, while W_t is the mass of the sample after soaking in 1.5 SBF.

Samples of a certain mass were dried at 55°C for 1 hour and then tempered in a desiccator and weighed (the weighing was read to the fourth digit). The procedure was repeated until a constant weight (W_0). Then the sample was soaked in 1.5 SBF solution at 37°C under static conditions. The soaking time was varied. After that the samples were washed out with distilled water. This step was followed by additional drying at 37°C until constant weight. All samples were weighed to the fourth decimal digit.

Scanning electron microscopy (SEM) was applied to investigate the micro structure and surface morphology of the materials prepared. Philips SEM 515 microscope, model WEDAX 3A and Zeiss model EVO MA-15 were used.

RESULTS AND DISCUSSION

Interaction between silk fibroin and sodium alginate

Natural SF is considered a stereo-regular copolymer containing mainly alanine (30 %) and glycine (44 %). It is characterized by the absence of bulky side residues.

A composition of the kind determines the maximum approximation of the macromolecular chains, which in turn provide a dense network of intermolecular interaction through hydrogen bonds. Thus a structure of a high level of organization is achieved.

It is well known that the fiber-polymers are heterogeneous two-phase systems containing crystalline and amorphous regions. The geometry of the polypeptide macromolecular chain of SF is β-conformational allowing a high level of organization. It includes crystal areas /β–sheets/.

SF dissolution is associated with destruction of the compact network of hydrogen bonds and the orderliness of its structure. Thus the structural elements are transformed in a random coil, while α-helix conformation of some parts of the macromolecular chains proceeds.

The FTIR analysis can verify the transition of the macromolecular chains from α-helix to β-conformation. It can also outline the interaction and formation of new bonds between the two components of the blends.

The process of film formation is related to reorganization of the SF structure from a random coil and α-helix as a part of the macromolecular chains to β-conformation and the resulting higher level of organization (β-sheets). The protein of SF has the capacity to self-assemble and transit from silk I to silk II [6, 24].

Fig. 1 shows independent participation of SF (curve a) characterized by intense absorption bands. The latter cover large areas and refer to the typical values for amide I from 1621 cm^{-1} to 1701 cm^{-1}, for amide II from 1523 cm^{-1} to 1558 cm^{-1} and for amide III 1241 cm^{-1}. This result indicates that the film structure contains both α-helix and β-conformation of the polypeptide macromolecule chains. This means that partial conformational changes occurred during the drying process. The results [25] referring to the "curve-fitting" of amide I and amide II are in agreement with the results reported so far.

The incorporation of the SA as a second component of the composite affects the spectrum range. It is evident by curve b in Fig. 1 referring to a blend of SF/SA ratio of 75 to 25. The broad absorption band areas of amide I and amide II are divided into separate bands. Three well defined peaks at 1621 cm^{-1}, 1678 cm^{-1} and 1701 cm^{-1} appear in the field of amide I. It is worth adding that a high level of organization (β-sheets) is observed [26] in the area of amide I. Bands at 1697 cm^{-1}-1703 cm^{-1} and 1617 cm^{-1}-1637 cm^{-1} are reported [26]. Two well defined

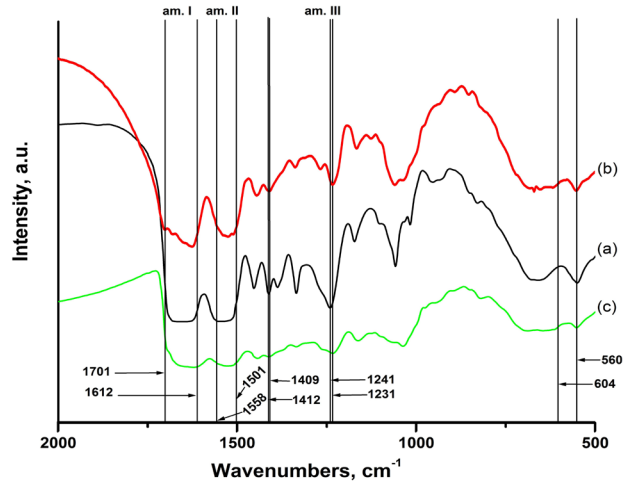

Fig. 1. FTIR spectra of the films obtained: (a) 100% SF; (b) blended SF/SA (75:25); (c) blended SF/SA (50:50).

and intense absorption bands at 1542 cm^{-1} and 1501 cm^{-1} are also formed in the area of amide II. The absorption maximum of amide III is shifted to shorter lengths. It is recorded at 1231 cm^{-1}. The carboxyl groups of SA show a strong absorption at 1420 cm^{-1} and 1400 cm^{-1}. The blend of SF/SA ratio of 75 to 25 shows a value of 1409 cm^{-1}.

Spectral curve c in Fig. 1 refers to the material of SF/SA ratio of 50:50. It is different in terms of intensity when compared to curve b. It has wide absorption bands with no separate peaks formation. There is a shift towards shorter wave lengths for amide I band recorded at 1612 cm^{-1} and amide II band at 1501 cm^{-1}. The absorption maximum of amide III appears at 1231 cm^{-1}. It can be assumed that this is due to the increased amount of SA in the blend. The carboxyl groups present show absorption at 1412 cm^{-1}. The explanation is similar to that pointed above, i.e. it refers to the macromolecular interactions between SA and SF based on hydrogen bonds formation. The change in the blended materials structure shows that there is a reorganization process of SF in presence of SA. It includes a conformational change of SF macromolecule from α-helix to β-conformation as well as a process of recrystallization (β-sheets). The pure SF film and the SF/SA blended films have co-existing random coil and β-sheets in their structure.

SA has a polyelectrolyte nature. It has macromolecules containing a large number of carboxyl groups. Some of the latter are non-ionized (-COOH), while others are ionized in the form of $–COO^- Na^+$. That is why the different acidic conditions applied for the preparation of SF/SA (75:25) blend (pH of 4 and 6) affect the amount of -COOH – it is increased and hence the capacity of

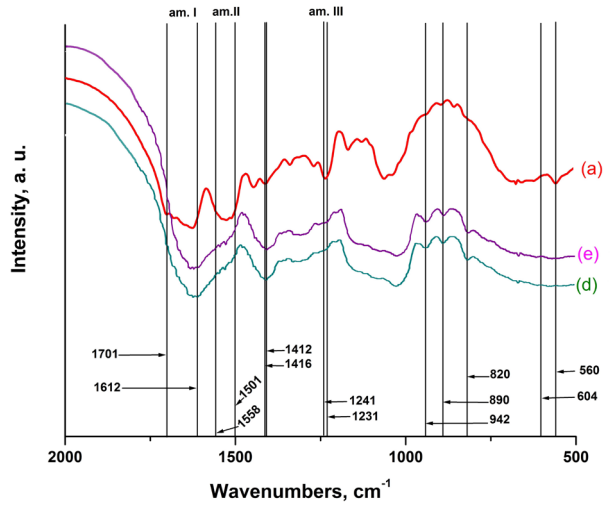

Fig. 2. FTIR spectra of films obtained at different pH: (a) blended SF/SA (75:25); (e) under acidic conditions at pH of 6; (d) under acidic conditions at pH of 4.

their interaction with the amide/amino groups of SF through hydrogen bonds formation increases also. This process is illustrated in Fig. 2, where the significant differences in the spectrum of the SF (curve a) and the composite materials under different acidic conditions (curves e and d) are clearly outlined. The absorption bands of the latter blends are similar, but different when compared to curve a. The absorption band of amide I shifts and reaches a value of 1612 cm^{-1}, while those of amide II (1518 cm^{-1}), amide III (1229 cm^{-1}) and amide IV (650 cm^{-1}) are not recorded. The outlining of an intense band of absorbance at 1416 cm^{-1} refers to -COOH of SA. Amide II and amide III, participating in the interaction of the amide groups (N-H) through hydrogen bonds formation, shift the absorption bands. On the other hand, three well defined and intense absorption bands are visualized at 820 cm^{-1}, 890 cm^{-1} and 942 cm^{-1} , i.e. in the area [20] of primary aliphatic amines (-N_2H). Furthermore, an electrostatic interaction between ionized -COO^- of SA and ionized -NH_3^+ of SF is possible under the acidic conditions applied. Thus the carboxyl groups' absorption band is seen at 1416 cm^{-1}. The consideration just presented is an evidence of intermolecular interactions during the treatment.

The results presented so far demonstrate that a macromolecular interaction between -COOH of SA and amide groups of SF proceeds leading to hydrogen bonds formation (see Fig. 3). This conclusion is in an agreement with Liang and Hirabayashi [15].

Similar results, concerning interaction between SF and other natural polysaccharides such as chitosan, cellulose, are observed by other authors [27 - 31]. The interaction between the two polymers is realized through hydrogen bonds formation. The structural changes in SF are induced by the polysaccharides.

***In Vitro* behavior**

Structure change and ionic interaction

The results illustrating the structural changes in the films after their stay in 1.5 SBF are shown in Fig. 4.

The spectrum of pure SF (curve a) is characterized by intense absorption bands. The structural changes in the films obtained refer to both α-helix and β-conformation of the polypeptide macromolecular chains. The 25 days-stay in the physiological medium results in reorganization processes in the areas of amide I, amide II and amide III. The large areas are divided into well-defined separate bands (curve b) with outlined peaks. In the area of amide I, Two new peaks appear in the area of amide I (1678cm^{-1} - 1701cm^{-1}). This evidences the existence of a fraction of a high level of organization - β-sheets. Two well-defined peaks at 1540 cm^{-1}, at 1508 cm^{-1} refer to amide II. A shift to the right of amide III peak – from 1241 cm^{-1} to 1233 cm^{-1}, is observed. The solution of 1.5 SBF induces processes of reorganization and recrystallization in SF structure.

The incorporation of SA in the process of preparation of SF/SA (75:25) blend results in reorganization

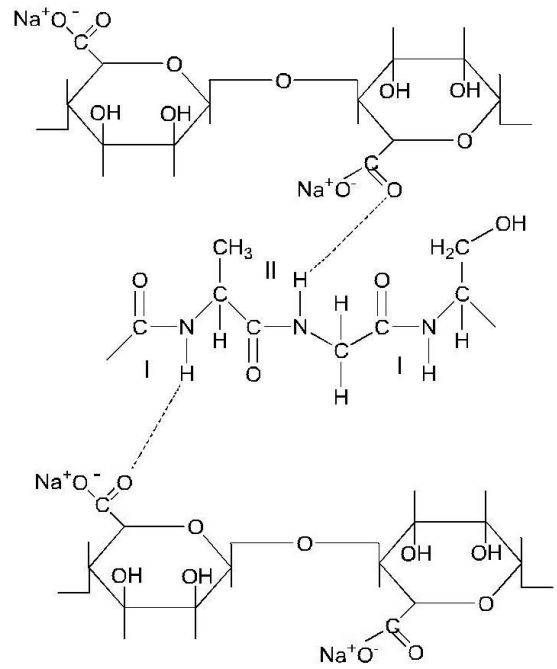

Fig. 3. Hydrogen bonds formation between fibroin and sodium alginate.

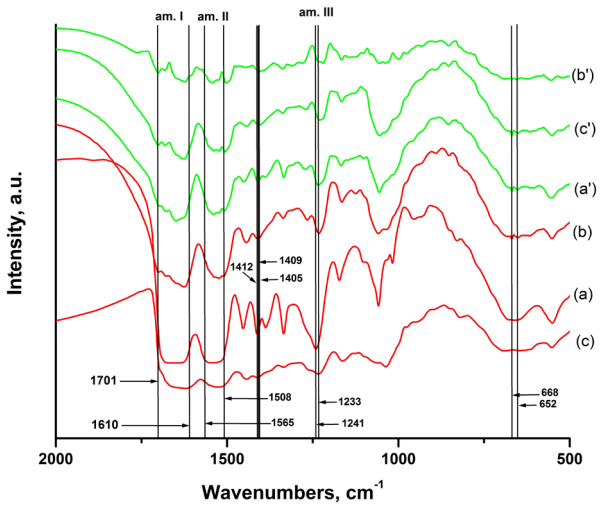

Fig. 4. (a) FTIR spectra of films obtained for 100 % SF prior to (a) and after soaking (a') in 1.5 SBF for 25 days; (b) and (b') refer to SF/SA (75:25), while (c) and (c') refer to SF/ SA(50:50).

and recrystallization of SF as visualized in Fig. 1. The process complicates when the blends are immersed in 1.5 SBF. Intense, characteristic peaks of β-conformation and crystal structures appear in the area of amide I (at 1681 cm^{-1}, 1701 cm^{-1} and 1623 cm^{-1}) and in the area of amide II (1538 cm^{-1} and 1501 cm^{-1}). The ionized carboxyl groups of SA (absorption at 1610 cm^{-1} and 1400 cm^{-1}) may be involved in the formation of an ionic bond with Ca^{2+} provided by 1.5 SBF. The mechanism of this interaction is investigated and in fact verified [32 - 35]. The peak at 1610 cm^{-1} is not seen because of the overlap with that of amide I. It may also contribute to the peak at 1623 cm^{-1}. The peak of amide II appears at 1409 cm^{-1}. It changes to 1405 cm^{-1} after 25-day stay in 1.5 SBF solution.

The spectrum of SF/SA (50:50) blended film shows wide bands of amide I and amide II, α-helix and β-conformation. Fragmentation connected with separation of the broad bands is observed - narrow bands with well and clearly defined maxima are seen. This means that 1.5 SBF solution facilitates the transformation of SF from α-helix and random coil (amide I at 1650 cm^{-1}, amide II at 1540 cm^{-1} and amide III at 1233 cm^{-1}) to β- conformation (amide I at1624 cm^{-1}, amide II at 1523 cm^{-1}) and the formation of crystalline structures in the field of amide I (at 1681 cm^{-1} and at 1701 cm^{-1}) and amide II (1508 cm^{-1}). It is plausible to accept that the two biopolymers interact through hydrogen bonds formation. The behavior of SA carboxyl groups is similar to that observed in 75:25 blend - the film's spectrum shows an absorption band at 1412 cm^{-1} prior to the immersion, while after 25 days stay in 1.5 SBF solution this band is shifts to 1405 cm^{-1}.

All samples (100, 75:25, 50:50) tested show an identical trend of their properties variation as a result of 1.5 SBF solution effect – the latter induces a conformation transition of SF macromolecule from α-helix to β-conformation and β-sheets formation.

An ionic bond between $-COO^-$ of the SA and the Ca^{2+} contained in 1.5 SBF can be formed under the conditions examined. It is expected to be of $-(COO^-)_2$ Ca^{2+} type. The most common method to prepare alginate hydrogels from an aqueous solution is to combine alginate with divalent cations acting as ionic crosslinking agents [32, 33]. The divalent cations cooperatively interact blocking G monomers to form ionic bridges as shown in Fig. 5.

One of the most commonly used divalent cations to ionically cross-link alginate and calcium chloride ($CaCl_2$) is one of the best choices [34, 35].

Mass changes

The study reported includes four test samples of different blends in the form of films. They are immersed in 1.5 SBF solution for 3 days, 14 days and 25 days. The obtained results are shown in Fig. 6. The experiment monitors and analyzes the effect of the blended component ratio, the soaking time in 1.5 SBF solution and the morphological structure.

The change in the samples' mass indicates a process running in biomaterials blends under the effect of 1.5 SBF.

Fig. 6 indicates that the test samples show different increase in weight after soaking in 1.5 SBF depending on their composition. The general trend observed refers to mass increase with soaking time increase. A higher mass increase of the samples is achieved in blends containing SA when compared to those of pure SF. The greater SA amount in the blends leads to a higher increase in the samples mass. These significant differences are due to the fact that pure silk SF has a very compact structure, which hinders the penetration of SBF components (Sample 1). The combination of SF with SA gives blends of "a loose structure" which is more hydrophilic because of the hydrophilic nature of SA (large amount of -COOH groups and natural porosity). An ionic bond of the type $(COO^-)_2$ Ca^{2+} can be formed under these conditions - the carboxyl groups of SA react with Ca^{2+} contained in 1.5

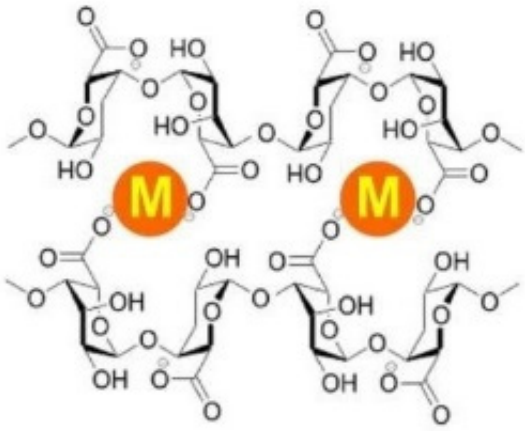

Fig. 5. An illustrative presentation of the mechanism of ionic interaction between alginate and divalent cations.

SBF solution. There are studies [32 - 35] demonstrating the formation of such an ionic bond. Some of them implement crosslinking of SA. It is known that the polymer crosslinking leads to a stabilization of their structure. The reported increase of the film weight resulting from increase of the residence time in the physiological medium may be associated with of a stable structure formation. The comparison of the growth mass of pure SF films with that of the blends confirms in fact the positive role of SA in obtaining materials of greater accessibility in physiological media.

The observed increase of the films mass is proportional to the increase of SA amount in the blends and the soaking time in 1.5 SBF solution.

Morphological structure of blended films

The films studied can be visually distinguished depending on their composition. The pure SF film is smooth, transparent, and homogeneous. It has a good strength.

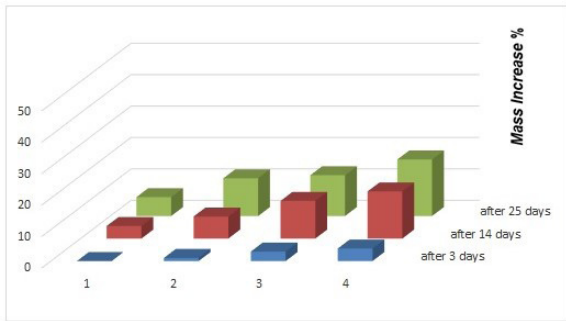

Fig. 6. Mass increase of the films depending on blended ratio* and soaking time in 1.5 SBF solution. (*the samples numbers are listed in Table 1).

The presence of SA in the blends changes the type of the film when the ratio of the blend is of 50:50. The film becomes cloudy and non-homogeneous to a certain extent. The SF/SA 25:75 blended film is not studied as it has a non-homogeneous structure with SF fibrils.

SEM analysis of the tested samples is performed at a magnification of 5000 aiming to observe a more detailed topography of the surface.

The films obtained can be visually distinguished on the ground of their blend. The SEM micrograph (Fig. 7, image a) of a pure SF film exhibits beads and irregular morphology. Analogical results are reported [1]. They explain the topography as a result of the amorphous structure conversion to the β–pleated structure (silk II). The presence of SA in the blends changes the type of the film. This change depends on the ratio of the blends. Adding SA to SF in the ratio of 75:25 results in a film of a flatter surface when compared to that of pure SF film (Fig. 7a and 7b). The film of SF/SA ratio of 50:50 has also a flat surface, but it is cloudy, opaque and non-uniform to a certain extent (Fig. 7c). SF/SA (25:75) blend yields a non-homogeneous structure with SF fibrils. SF/SA (25:75) blend has a layered non-homogeneous structure. Most authors studying composites based on SF/SA observe a homogeneous structure in case of all ratios investigated On the other hand Moraes M., Silva M., Weska R., Beppu M. [18] find formation of SF fibrils in composites containing 25% of SF. In fact the same happens during the process of our blends preparation.

It is worth adding that no monitored pores are monitored for all test samples investigated.

CONCLUSIONS

The obtained results show interaction between fibroin's amido groups and alginate's carboxyl groups based on an intermolecular interaction through hydrogen bonds formation. 1.5 SBF induces structural changes in the materials. An ionic bonds of $(COO^-)_2Ca^{2+}$ type are formed between $–COO^-$ groups of SA and Ca^{2+} supplied from 1.5 SBF solution.

The films produced contain both α-helix and β-conformation of the polypeptide macromolecule chains. This means that conformational changes are proceed during the drying process. But they are partial in case the films are not treated with an alcohol. SA in the blended materials induces conformational changes of fibroin's macromolecules from α-helix to β-conformation and β-sheets formation.

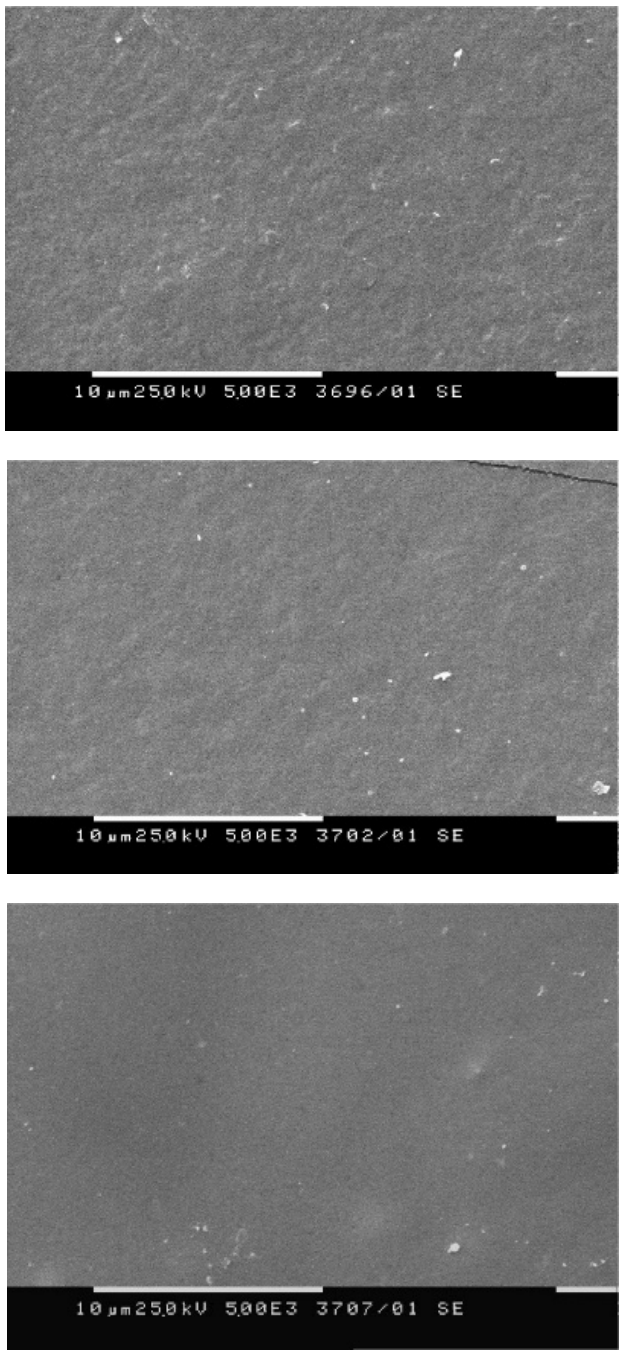

Fig. 7. SEM images of the prepared blended films: (a) 100 % SF film; (b) SF/SA (75:25); (c) SF/SA (50:50).

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