JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 16 (2005) 575–579

Physical properties and biocompatibility of chitosan/soy blended membranes

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Blends of polysaccharides and proteins are a source for the development of novel materials with interesting and tailorable properties, with potential to be used in a range of biomedical applications. In this work a series of blended membranes composed by chitosan and soy protein isolate was prepared by solvent casting methodology. In addition, cross-linking was performed *in situ* with glutaraldehyde solutions in the range $5 \times 10^{-3} - 0.1$ M. Furthermore, the influence of the composition and cross-linking on the degradation behaviour, water uptake and cell adhesion was investigated. The obtained results showed that the incorporation of chitosan, associated to network formation by cross linking, promoted a slight decrease of water absorption and a slower degradability of the membranes. Moreover, direct contact biocompatibility studies, with L929 cells, indicate that the cross-linking enhances the capability of the material to support cell growth.

1. Introduction

There is an increasing need to develop new biodegradable materials to be used in skin tissue engineering, wound cover or as dressings and barrier-membranes, since there is a high demand for skin replacements and skin repair treatments. For instance, an ideal material to be use as a wound dressing should associate availability with minimal storage requirements, long shelf-life, versatility and, biocompatible behaviour [1]. Many polymeric membranes have been investigated for the purpose of wound covering on account its importance in the treatment of burns, prevention of post surgical adhesions and cosmetic surgery. These materials include synthetic polymers like polyurethane, polyethylene, polylactides, polyglycolides, and polyacrylonitrile. However some of these polymers have disadvantages in such applications, i.e. poor biocompatibility and release of acidic degradation products [2, 3]. One alternative approach involves the use of biodegradable polymers from renewable resources, it including starch, collagen, gelatin, chitosan and proteins (soy protein, casein, silk fibroin and wheat), since these polymers are widely available in nature, and are biodegradable and non-toxic [1, 4–8]. Among these renewable polymers, soy protein, the major component of the soybean, has the advantages of being economically competitive and present good water resistance as well as storage stability. The combination of these properties with a similarity to tissue constituents and a reduced susceptibility to thermal degradation makes soy an ideal template to be used as a biomaterial for skin tissue engineering. Soy proteins are rich in polar groups, such as hydroxyl, amide and carboxyl groups among others, which enable soy protein to associate with many different types of compounds [9]. Some studies reported that the combination of soy protein with other proteins such as wheat gluten [10] or, casein [7], may promote physical and chemical interactions which improve some properties. Combination of polysaccharides such as carrageenan, xanthan [11], dialdehyde starch [12] with soy protein has also been investigated.

This work explored the combination of chitosan with a colloidal suspension formed from the soy protein, in the form of membranes, through chemical and/or physical means, which can allow to control the degradation rate, or the hydrolytic resistance, and to induce antibacterial properties in soy protein membranes. It is expected that the characteristics of a blend membrane system formed by these components can be tailored by controlling the particle charge of protein, which can be dependent, among other factors, on the pH medium and the composition of the blend. In addition, the blend membrane system can potentially be used for the delivery of water-soluble compounds

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that aid wound healing, such as antibiotics or anti-inflammatory agents. In a previous study, which also involved the combination of chitosan with soy protein [13], β-radiation was shown to be as a suitable sterilisation methodology to be used on chitosan/soy protein membranes aiming to be used in guide bone regeneration. The results showed that no substantial changes were detected in the studied properties, with the exception of the surface energy that was found to be slightly increased for higher applied doses.

The aim of this work was to evaluate the influence of chemical cross-linking in the water uptake, degradation rate and biocompatibility of the blend system composed by chitosan and soy protein. Cytotoxicity tests, both extract and direct contact tests were performed on these materials in order to evaluate the potential toxicity of the degradation products, and the ability of the membranes to promote cell attachment and growth.

2. Materials and methods

Chitosan-CHT (Sigma) with deacetylation degree about 85% was used. Soy protein isolate (SI) was provided by Loders Crocklaan (The Netherlands). All other reagents were analytical grade and used as received.

Chitosan/soy protein blended membranes (CS) (average thickness from 40 to 84 μm) were prepared by solvent casting. Chitosan flakes were dissolved in aqueous acetic acid 2% (v/v) solution at a concentration of 1% wt. A soy suspension (1% wt.) was prepared by slowly suspending the soy protein powders, under constant stirring, in distilled water with glycerol. After adjusting the pH to 8.0 ± 0.3 with 1 M sodium hydroxide, the dispersion was heated in a water bath at 50 °C for 30 min. Studies reported that the alkaline conditions favour soy film formation by aiding protein dispersion in film-forming solutions [14]. Then, the two solutions were mixed in different ratios, namely CS75, CS50 and CS25 corresponding to 75/25, 50/50, 25/75 wt% chitosan/soy. Glutaraldehyde (Ga) solutions in the range $5 \times 10^{-3} - 0.1$ M were prepared diluting 50 wt% Ga solution as it was provided by the manufacturer. After that, glutaraldehyde solutions were added to the mixture, to study the effect of crosslinking on the blend properties. After the chitosan/soy solutions had been homogenized, they were casted into Petri dishes and dried at room temperature for about 6 days. In order to neutralize acetic acid, the dried membranes were immersed in 0.1 M sodium hydroxide for about 10 min, and then washed with distilled water to remove all traces of alkali, followed by drying at room temperature. After that, the structural changes were assessed by FTIR-ATR spectroscopy (Perkin-Elmer 1600 Series).

Non-crosslinked and crosslinked blend membranes (1×2 cm) were submitted to swelling and *in vitro* degradation tests. Pre-weighed dried membranes were immersed for 0, 2, 7, 14, 30 and 60 days, at 37 °C, in a phosphate buffer saline solution, pH 7.4 (PBS). Sodium azide (0.02% w/v) was added to the buffer to prevent bacterial growth. After each ageing period, the samples were removed from the degradation solution, washed with distilled water and weighed. The water uptake was obtained by weighing the initial and swollen samples in various time intervals:

$$\text{Water uptake}\,(\%) = ((W_s(t) - W_i)/W_i) \times 100 \quad (1)$$

Where $W_s(t)$ and W_i represent the weight of the samples at time $\underline{t}$ and $\underline{0}$, respectively. After that, the samples were dried in an oven (60° C/24 h). The percentage weight loss of the soy materials was then calculated by:

$$\text{Weight loss}\,(t) = [(W_i - W_f(t))/W_i] \times 100 \quad (2)$$

where W_i is the initial dry weight of the sample. $W_f(t)$ denotes the weight loss of sample after a certain time $\underline{t}$ of immersion. Each experiment was repeated three times and the average value was taken as the weight loss.

In according to ISO standards [15] two categories of *in vitro* cytotoxicity evaluation were made: extract tests and direct contact tests. In extract tests the samples were extracted in culture medium for 24 h at 37 °C, 60 rpm. The filtered extracts were placed in contact with a monolayer of L929 cells (mouse fibroblasts) for 72 h. A control with cells grown in the presence of complete culture medium was included. Then, cell viability was evaluated by MTT assay and the results were expressed as percentage of cell viability. In direct contact test, L929 cells (8×10^4 cells/cm^2) were seeded on the biomaterials and incubated under standard culture conditions for 3 days.

3. Results and discussion

The FTIR-ATR spectra of chitosan and soy protein films were analysed (Fig. 1(a)) and compared with the spectra of the blended membranes (Fig. 1(b)). The ATR analysis of membranes was based on the identification of bands related to the functional groups present in chitosan and soybean, among others [16, 17].

As can be seen in Fig. 1(a), the main characteristic absorption bands of chitosan appear at 1650 cm^{-1} (C═O stretching), 1560 cm^{-1} (─NH angular deformation), 3450 cm^{-1} (OH hydroxyl group) and 1150–1040 cm^{-1} (─C─O─C─ in glycosidic linkage) [16]. The soy protein spectrum (Fig. 1(a)) showed an amide I band at 1632 cm^{-1} and a amide II band at 1536 cm^{-1} [17]. The amide I can be composed of several overlapping components due to various protein segments with different secondary structures [17]. Fig. 1(b) is one spectra representative of the crosslinked blended membranes, which showed the characteristic absorptions bands of both chitosan and soy being its proportional to ratio between the components of the blend. As a result, the absorbance of NH and CO deformation bands in the range 1580–1490 cm^{-1} and 1700–1630 cm^{-1} respectively, became gradually higher with the increase of soy content in the blend. Similar results were found for non-crosslinked membranes. Even though new peaks did not appear before and after the crosslinking reaction, we noted a displacement of these bands for lower wave-numbers (NH from 1584 to 1542 cm^{-1}and CO─from 1650 to 1634 cm^{-1}) with respect to pure chitosan. The above findings suggest that the chitosan and soy may have

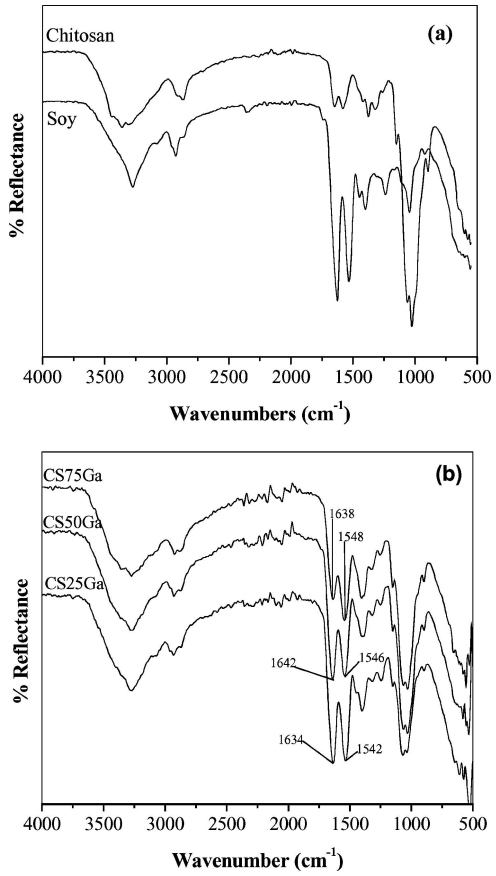

Figure 1 (a) FTIR-ATR spectra of chitosan and soy pure membranes; (b) CS75Ga, CS50Ga and CS25Ga blended membranes crosslinked with 5×10^{-2} M glutaraldehyde, after neutralization (CS75, CS50 and CS25 corresponding to 75/25, 50/50, 25/75 wt% chitosan/soy).

participated in a specific intermolecular interaction. In this case, more details about the protein-polysaccharide interactions and miscibility of this blend system, using measurements of proton spin-lattice relaxation times by solid state ^{13}C nuclear magnetic resonance (^{13}C NMR,) spectroscopy, are in progress.

On the other hand, water absorption ability of the blended membranes was evaluated through the monitoring of the water absorption ratio determined in phosphate buffer solution- PBS (pH 7.4). As expected, the samples showed high water uptake (ca.160–200%), as result of the hydrophilic character predominant of blend

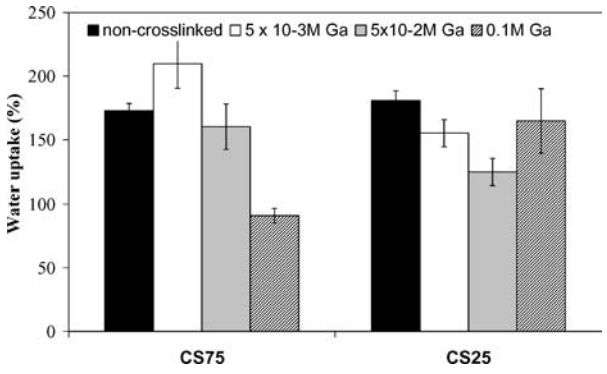

Figure 2 Water uptake of chitosan/soy blended membranes after 2 h in PBS: ■ CS75 and CS25 membranes non-crosslinked; □: CS75 and CS25 membranes crosslinked with 5×10^{-3} M Ga; ▩: CS75 and CS25 membranes crosslinked with 5×10^{-2} M Ga; ▨: CS75 and CS25 membranes crosslinked with 0.1 M Ga (CS75 and CS25 corresponding to 75/25, 25/75 wt% chitosan/soy).

components but, after cross-linking the samples present a decreasing trend. Some results can be associated to a preferential cross linking of chitosan instead of soy or vice-versa as well as the occurrence of a partial cross linking. In a previous work, Silva *et al.* [8] observed that for pure chitosan, slightly crosslinked films did not exhibit such decrease, mainly due to the stronger effect of crystallisation suppression during crosslinking. Also it was observed that both water sorption and degradation behaviour results obtained for CS50 blend composition presented an irregular behaviour (data not shown) compared to other blend compositions. This may be attributed to an irregularity found in the miscibility between soy protein and chitosan at this composition.

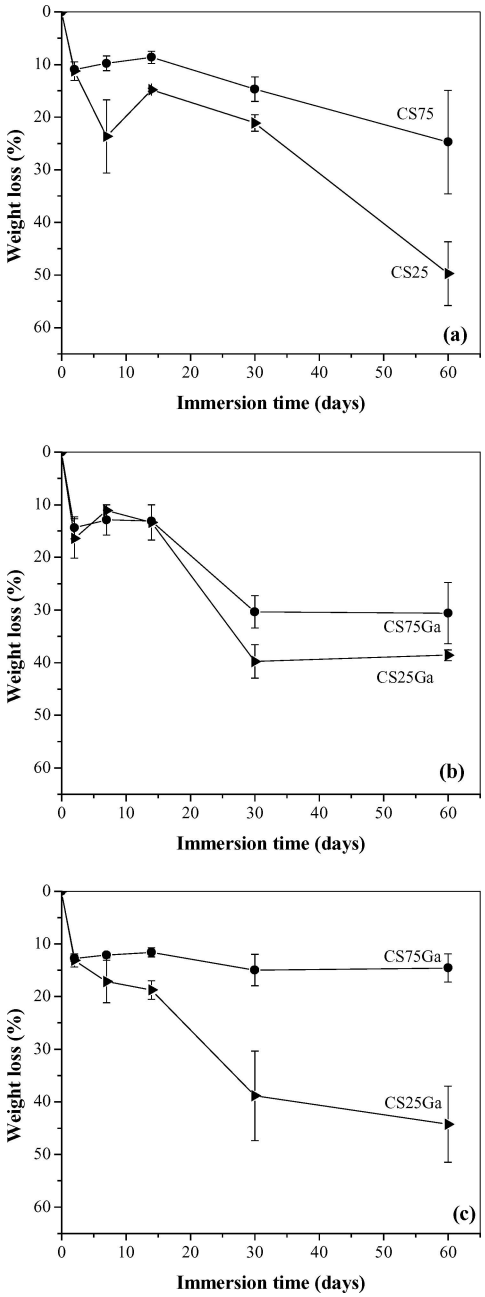

Figure 3 Weight loss of blended membranes as function of immersion time on a phosphate buffer solution at 37 °C: (a) CS75 and CS25 membranes non-crosslinked; (b) CS75 and CS25 membranes crosslinked with 5×10^{-3} M Ga; (c) CS75 and CS25 membranes crosslinked with 5×10^{-2} M Ga (CS75 and CS25 corresponding to 75/25, 25/75 wt% chitosan/soy).

All the samples revealed to be stable in the phosphate buffer solution (PBS), but with different degradation pattern in function of immersion time. In fact, it was observed that the degradation pattern of blended membranes occurs in three stages (Fig. 3(a)). The first one, between 0 and 14 days, can be related with the leaching of plasticizers (glycerol) and low molecular weight polymeric chains, as observed in composites based on casein and soy protein [7]. The second degradation stage, between 15 and 30 days, probably involves the degradation of a protein fraction, followed by third stage of degradation, correspondent to the final weight loss of the blend. Fig. 3(a), shows that the weight loss in the blended membranes increased with the percentage of soy in the blend. In the crosslinked samples (Fig. 3(b)–(c)), the weight loss tends to stabilize, mainly after 30 days in immersion. In particular, a positive influence of crosslinking on the degradation behaviour was more evident in the CS25 composition, since its weight loss along the time was reduced (Fig. 3(b)–(c)). On contrary, in the presence of the 0.1 M Ga, the weight loss of CS25 blend reached the highest value of 59% after 30 days of immersion (data not shown), probably due the brittleness of the samples. Furthermore, it is observed that the blended crosslinked membranes also present a degradation profile similar to the non-crosslinked membranes. In this case, the second stage degradation pattern can be related to degradation of the non-crosslinked protein fraction. The third stage can be associated to final weight loss (%) or stabilization due the presence of the chitosan/soy network, respectively. It is worth mentioning that the crosslinked membranes with higher chitosan content (CS75) maintained its physical integrity even after long immersion time (60 days).

The MTT studies with extracts demonstrated that for chitosan, CS75 and CS50 membranes crosslinked with 5×10^{-2} M Ga, the percentage of viable L929 cells was around 90%, thus comparable to the control. However, the viability of L929 cells slightly decreased in contact with the extracts of CS75 and CS50 blended crosslinked membranes prepared with higher Ga concentration (0.1 M) and CS75 (data not shown) but even in these cases the values were acceptable. On the other hand, cell adhesion studies, after 3 days of culture, show that L929 cells on the surface of chitosan membrane (CHT) were still spherical and with microvilli-like projections in appearance (Fig. 4(a)). It appears that cells on CHT were able to attach but unable to follow this attachment with spreading. Domard *et al.* [18] observed similar results in which chitosan is not cytotoxic towards fibroblasts but inhibits cell proliferation. In contrast, as can be observed (Fig. 4(b)–(d)) the C75 membranes show elongated and flattened cells, suggesting a tight cell adhesion to the membranes. This noteworthy improvement in cellular adhesion in comparison with chitosan membrane can be due the incorporation of the soy protein, which can provide more protein-binding sites on the membranes. In addition, the increased cellular adhesion in the crosslinked membranes CS75 (Fig. 4(c)–(d)) relative to the non-crosslinked membrane CS75 (Fig. 4(b)) suggests that the crosslinking with Ga has changed the surface membrane. Probably this is due to a better interaction between the blend components and, then improves the cellular adhesion of the blended membranes. The collective results from these experiments assure that chitosan/soy protein blended membranes and their extracts were non-cytotoxic and were able to support cell growth and proliferation.

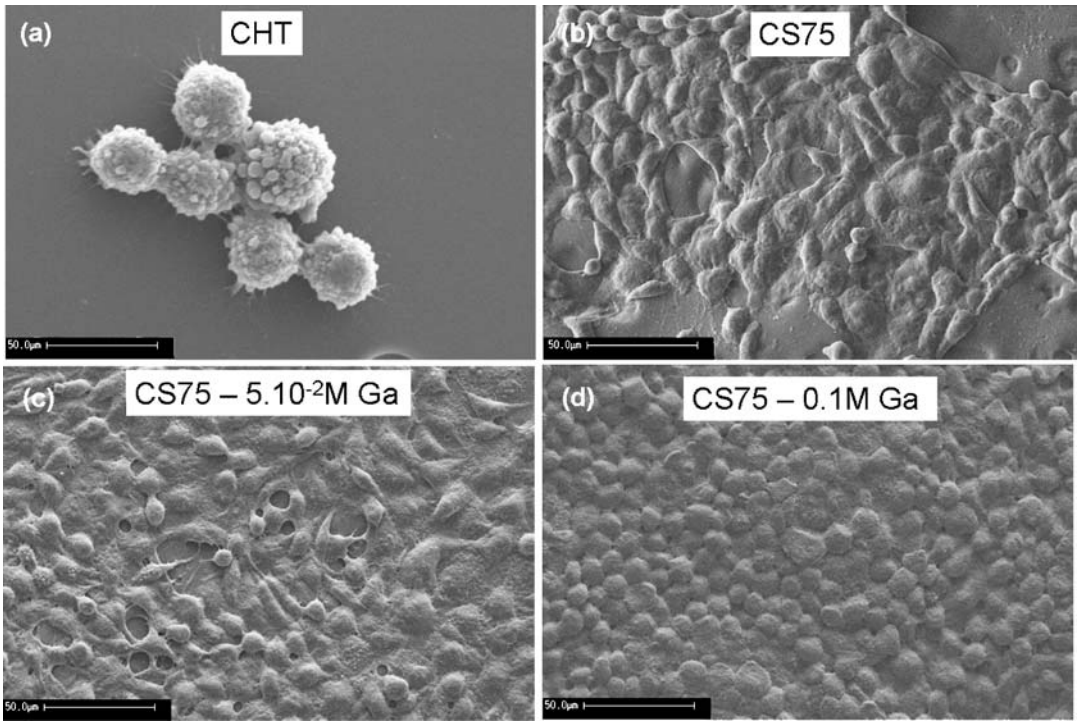

Figure 4 SEM micrographs of L929 cells cultured on chitosan/soy protein blended membranes after 3 days of culture: (a) chitosan membrane (CHT); (b) CS75 membrane non-crosslinked; (c) CS75 membrane crosslinked with 5×10^{-2} M Ga; (d) CS75 membrane crosslinked with 0.1 M Ga (CS75 corresponding to 75/25 wt% chitosan/soy).

4. Conclusions

Chitosan/soy protein isolate blended membranes were successfully prepared by means of a solvent casting methodology. The membranes exhibited different degradation pattern and, improved cell spreading with respect to pure chitosan. By the results, the incorporation of chitosan associated to network formation by cross linking promoted a slight decrease of water absorption and a slower degradability of the membranes. The biological studies performed suggest that the cross-linking with low glutaraldehyde concentration changed the membrane surfaces, promoting a better cell adhesion of the membranes.

Acknowledgements

S. S. Silva and M. I. Santos, thank the Portuguese Foundation for Science and Technology (FCT) for providing PhD scholarships (SFRH/BD/8658/2002 and, SFRH/BD/13428/2003, respectively). This work was partially supported by FCT, through funds from the POCTI and/or FEDER programmes.

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Received 15 August
and accepted 15 October 2004