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Dehydro-thermally cross-linked collagen–poly(vinyl alcohol) blends: mechanical, biological and surface properties

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The mechanical, biological and surface properties of films, based on blends of soluble collagen and poly(vinyl alcohol), were investigated. Films prepared by casting were cross-linked by a dehydro-thermal treatment. The mechanical behaviour of the materials was studied by dynamic-mechanical thermal analysis. The biological properties of the films were evaluated by performing cytotoxicity and cytocompatibility *in vitro* tests. Surface characterization was carried out by X-ray electron spectroscopy. The results obtained indicate that these materials behave as two-phase systems and a remarkable enrichment of collagen in the surface with respect to the bulk was observed. Cytocompatibility tests show that the blending of the two polymers creates a better substrate for cell growth in comparison with pure components.

1. Introduction

Blends of synthetic and natural polymers have been used in the last few years to develop new materials called "bioartificial polymeric materials" [1–3]. Their capability of combining good physical and mechanical properties with good biocompatibility characteristics was studied with the purpose of making new materials for biomedical applications.

On dealing with collagen-based materials, it is typical practice to cross-link collagen in order to reduce its degradation rate and to minimize the immuno response of the host. In addition, cross-linking is necessary to avoid rapid dissolution of the material when in contact with biological fluids.

In previous works, materials based on collagen-poly(vinyl alcohol) blends were cross-linked by means of a dehydro-thermal procedure consisting of dehydration and heating under vacuum [4]. It was observed that this treatment performed for 24 or 72 h induces high degrees of cross-linking in collagen and high crystallinity in poly(vinyl alcohol), thus reducing the solubility and increasing the biological stability of the films. The biological stability in term of resistance to enzymatic digestion, and the thermal properties studied by calorimetric analysis suggest that the two components of the blends tend to form independent cross-linked structures, and an interpenetrating network-like behaviour could be inferred.

Because of the greater reliability of the dehydro-thermal treatment with respect to other cross-linking procedures, a deeper investigation of collagen-poly (vinyl alcohol) based materials has been performed in this work. Blends of soluble collagen and poly(vinyl alcohol) have been obtained by solution casting in the form of films with different composition. Dynamic-mechanical thermal analysis of the cross-linked films has been performed, and the biological properties in term of cytotoxicity and cytocompatibility have been studied through *in vitro* tests based on the cell culture method. In addition, X-ray electron spectroscopy has been performed to investigate the surface characteristics of the films [5].

2. Materials and methods

Materials used in this study were commercially available poly(vinyl alcohol) (PVA) (Aldrich Chemie, Steinheim, Germany), with average molecular weight of 114 000 and hydrolysis degree 100%; acid soluble collagen (SC) (type I from calf skin, Sigma, St Louis, MO, USA).

A 1% w/v PVA aqueous solution was prepared by adding the powdered PVA to distilled water and raising the temperature to 60 °C. A 1% w/v SC solution, in 0.5 M acetic acid, was prepared at 0 °C under mild stirring. From the two formulated solutions, SC\PVA mixtures with 0/100, 20/80, 50/50, 80/20 and 100/0 w/w polymer ratios, were obtained. The mixtures were poured into Petri dishes at room conditions and films were obtained by casting.

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The dehydro-thermal treatment (DHT) of the films was carried out through two dehydration steps and a final cross-linking step [4]. First, the films were placed in a vacuum over at 50 °C and subjected to a vacuum of less than 13.3 Pa for 3 h. Second, the temperature within the vacuum oven was increased to 90 °C and this value maintained for 30 min. Finally, cross-linking was achieved by raising the temperature to 120 °C. The SC/PVA films were then removed from the oven after 72 h.

Dynamic mechanical thermal analysis (DMTA) was performed by a Polymer Laboratories DMTA analyser at a frequency of 1 Hz and over a temperature range from room temperature to about 250 °C at a rate of 4 °C/min.

The biological properties were evaluated by both cytotoxicity and cytocompatibility *in vitro* tests. Samples (45 mg) of the cross-linked films were added to 10 ml phosphate-buffered saline solution (PBS) and then kept at 37 °C for 5 days. Extracts were filtered with 0.4 μm cellulose acetate syringe filters (Nalgene, USA) and tested for cytotoxicity on 3T3 cells, from a mouse fibroblast cell line, cultured in complete culture medium (Dulbecco's Minimal Essential Medium with 10% foetal bovine serum). The cells were seeded onto 96-well plates at a density of 6×10^3 cells/cm^2. Three different colorimetric methods were used: Neutral Red Uptake assay (NR), 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl Tetrazolium Bromide assay (MTT), and Kenacid Blue R-Binding method (KB) from modified FRAME protocols [6].

NR was used to asses cell lysosomal damage, MTT to verify mitochondrial functionality, and KB for total protein determination.

The extracts were added to the wells (100 μl/well) 24 h after seeding. PBS was added to the negative control wells. The plates were then incubated for 72 h at 37 °C in a CO_2 atmosphere. After the incubation period, the procedures were different for the three assays, the details being described elsewhere [7].

In cytocompatibility tests, cross-linked films were cut to obtain 5 cm^2 area samples and dry heat sterilized in a vacuum oven (3 h at 50 °C, 30 min at 90 °C and 2 h at 110 °C). Sterilized films were rinsed twice with complete culture medium and then seeded with 3T3 fibroblast cells. Tissue culture polystyrene (TCPS) was used as a positive control. Seeding density was 4.5×10^4 cells/cm^2. The samples were incubated at 37 °C in an air atmosphere containing 5% CO_2. Seven days after seeding the cells were tripsinized (0.05% trypsin/0.02% EDTA w/v) from the wells and counted with a Neubauer haemocytomer. Cell proliferation on the films was also evaluated by optical microscope analysis. X-ray electron spectra (XPS) were recorded on a VG Instrument electron spectrometer using a $MgK_{\alpha 1,2}$ X-ray source (1253.6 eV). The X-ray source in the standard conditions was working at 300 W, 15 kV and 20 mA. The base pressure of the instrument was 6.5×10^{-8} Pa and the operating pressure 2.7×10^{-6} Pa typically. A pass energy of 100 eV, 50 eV and 20 eV was used for widescans, and narrowscans for area ratio calculation and curve fitting, respectively. The take-off angle of the electrons was 60° with respect to the surface of the sample, corresponding to a sampling depth of approximately 75 nm. All data analysis (linear background subtraction, peak integration and curve fitting) were accomplished using a VGX900x (version 5) software. Binding energies were referenced to the C-H level at 285.0 eV.

3. Results

3.1. DMTA analysis

The analysis of the mechanical properties of the SC/PVA films suggests that they behave, in the temperature and composition range analysed, as two-phase systems, according to the previous results obtained by calorimetric measurements [4].

The trend of the storage modulus E' and the loss factor tan δ, as a function of the temperature are shown in Figs 1 and 2, respectively, for different compositions of film. The curves of the storage modulus of pure SC and pure PVA each show a single slope change related to SC denaturation (at about 210–220 °C) and to the PVA glass transition (T_g, at about 120 °C), respectively. The modulus curves of SC/PVA blends show both the phenomena with little changes.

3.2. Biocompatibility tests

The results of NR, MTT and KB tests suggest that no cytotoxic effects (one factor Anova, $p > 0.05$) were exerted on the cell lysosomal, mitochondral or proliferation activity by SC/PVA = 0/100 and 20/80 samples extracts after 72 h incubation. The extracts from 50/50 and pure SC performed worse (one factor Anova, $p < 0.05$) than the negative control (Fig. 3).

Cellular proliferation, measured as a percentage of the seeded cells, in comparison with the positive control (64.10%) was very good on 50/50 (82.4%), good on 20/80 (63.33%) and 80/20 (50.43%), and poor on pure PVA (34.82%) and pure SC (5.26%) (Fig. 4). Fig. 5 shows optical microscope images of 3T3

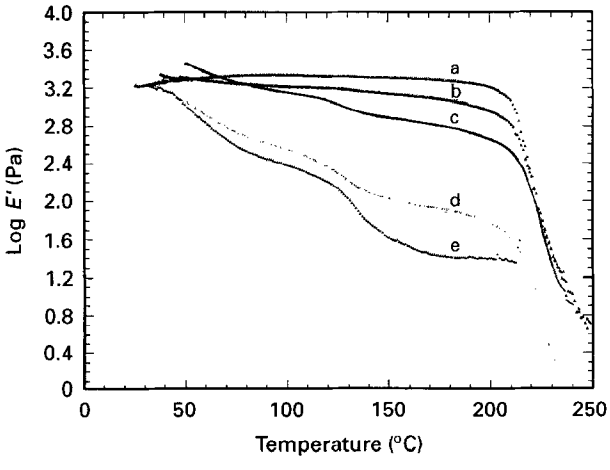

Figure 1 Storage modulus, E', of dehydrothermally cross-linked collagen/poly(vinyl alcohol) (SC/PVA) films, with the following compositions: (a) SC/PVA = 100/0; (b) SC/PVA = 80/20; (c) SC/PVA = 50/50; (d) SC/PVA = 20/80; (e) SC/PVA = 0/100, by dynamic mechanical thermal analysis (scanning rate: 4 °C min^{-1}; 1 Hz).

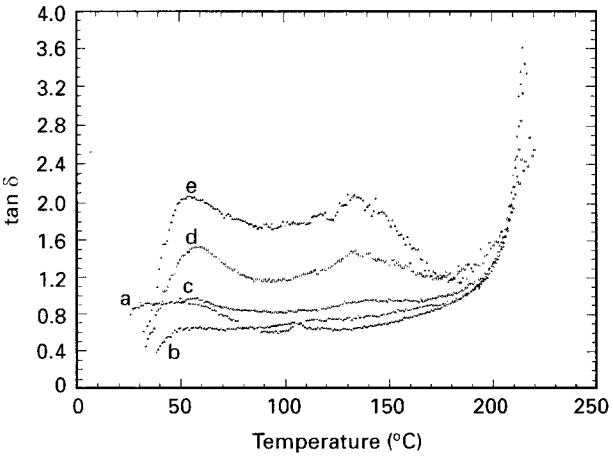

Figure 2 Loss factor, tan δ of dehydrothermally cross-linked collagen/poly(vinyl alcohol) (SC/PVA) films, with the following compositions: (a) SC/PVA = 100/0; (b) SC/PVA = 80/20; (c) SC/PVA = 50/50; (d) SC/PVA = 20/80; (e) SC/PVA = 0/100, by dynamic mechanical thermal analysis (scanning rate: 4 °C min^{-1}; 1 Hz).

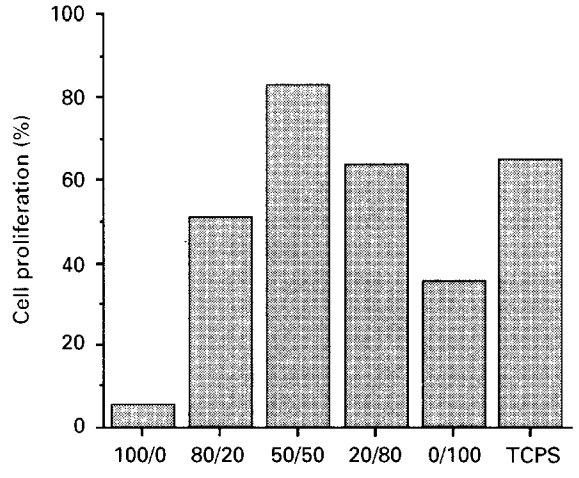

Figure 3 Viability of 3T3 mouse fibroblasts measured as a function, respectively, of Neutral Red (NR) uptake into the lysosomes, mitochondrial integrity (MTT) and total protein content (KB) after 72 h incubation of the cells with the SC/PVA film extracts. The statistical significance of the absorbance values is referred to the PBS values (negative control) (Fisher PLSD test, $P < 0.05$). The results are the mean (±SE) of five measurements.

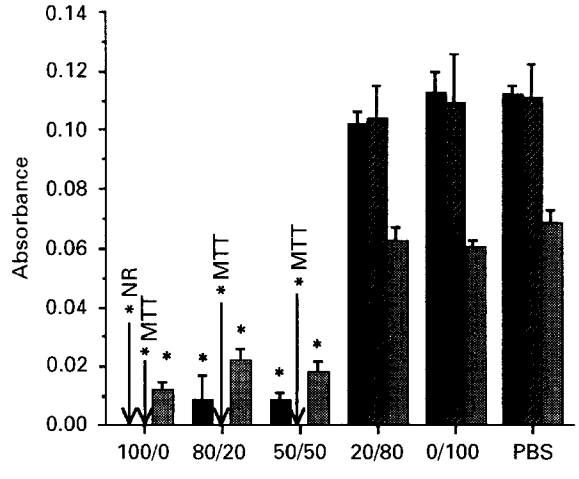

Figure 4 Proliferation of 3T3 mouse fibroblasts on SC/PVA films and TCPS, measured as percentage of the seeded cells, 7 days after seeding. The values are the mean of two measurements: ■ NR; ▨ MT; ■ KB.

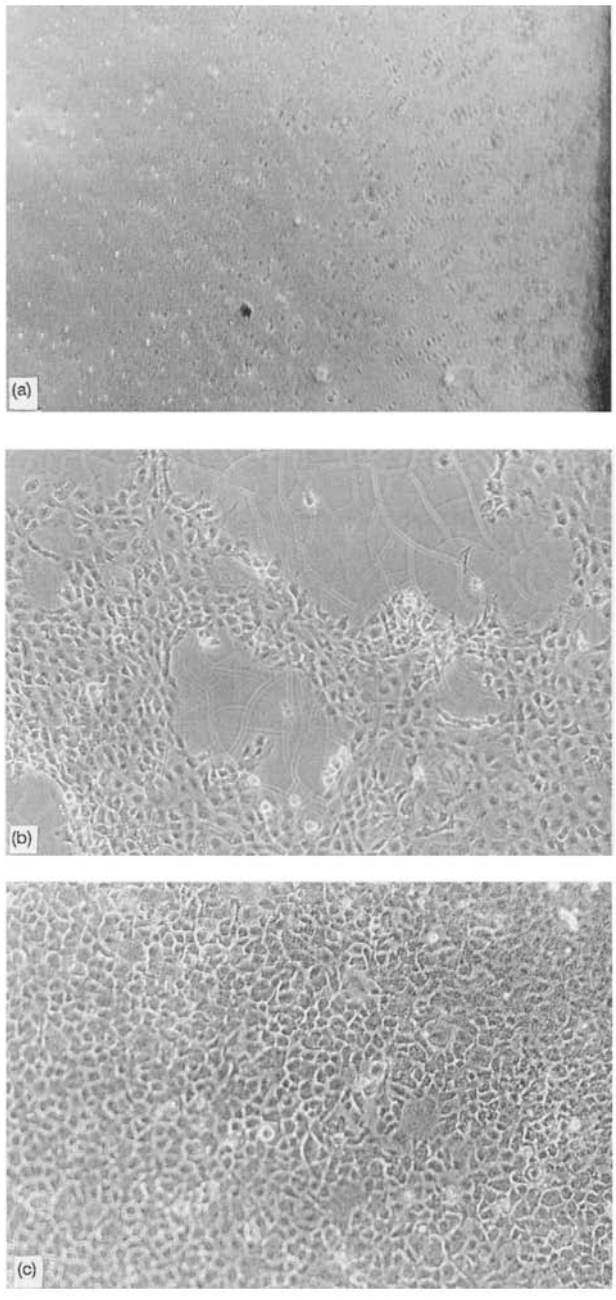

Figure 5 Optical microscope images of 3T3 murine fibroblasts growth on (a) pure SC (×40), (b) pure PVA (×100) and (c) SC/PVA = 50/50 (×100) films 7, days after seeding.

fibro-blasts growth on pure SC, pure PVA, and SC/PVA = 50/50 films.

3.3. XPS analysis

Quantitative XPS analysis of the SC/PVA blends was accomplished in two ways. The first, by utilizing an empirical "factor" derived from the nitrogen to carbon ratio (N/C) of pure cross-linked SC and, the second, by curve fitting of the C_{1s} envelope. The N/C ratio used in this analysis is derived from peak areas of the N_{1s} and C_{1s} regions corrected with the appropriate instrument sensitivity factors for the stoichiometries.

The calculation of percentage collagen present in the surface using the empirical "factor" has been done assuming that the N/C ratio for a composition of 100% of collagen would be the experimental N/C ratio of cross-linked pure collagen.

TABLE I Surface composition of SC/PVA samples from XPS analysis

Sample (SC/PVA)	N_{1s}/C_{1s} ratio	CO–NH/C_{1s} ratio	Experimental percentage of SC from N_{1s}/C_{1s} ratio
100/0	0.25	0.19	100
80/20	0.22	0.17	88
50/50	0.21	0.17	84
20/80	0.14	0.11	56
0/100	0	0	0

The results (Table I) show a surface excess of collagen, more evident for samples 20/80 and 50/50, while for samples 80/20 the excess is less pronounced.

Surface weight percentages were also determined by curve fitting the C_{1s} spectrum. The parameters used to fit the SC/PVA blends were determined from the curve fits of the homopolymers. Since in the C_{1s} feature the component centred at 288.2 eV (CO–NH) is characteristic of the collagen due to the presence of peptidic bonds, it has been possible to determine the CO–NH/C_{1s} area ratios for the samples.

4. Discussion

Evaluation of the mechanical properties by DMTA analysis has clearly shown a biphasic behaviour of SC/PVA films dehydro-thermally cross-linked for 72 h: the variations of the modulus and loss factor curves observed for the various SC/PVA blends correspond to the transitions of the single pure components. This result is in agreement with previous data obtained by calorimetric analysis [4] which indicated that the two components of the blend tend to create independent structures.

From the results of the cytotoxicity tests, it seems that a higher collagen content induces a toxic effect on the cells after 72 h incubation with the material extracts. The cytocompatibility tests show a different trend; in this case cell adhesion and proliferation onto the blends is as good as on TCPS and better than on pure components.

The results of these biocompatibility tests seem not to agree. However, the apparent cytotoxicity of the samples having higher SC content could be attributed to acetic acid traces released by the samples during the extraction procedure. The presence of acetic acid is instead negligible in the case of cytocompatibility tests because the samples were rinsed with culture medium before cell seeding.

The most interesting result of the XPS analysis is the surface enrichment of collagen with respect to the bulk. The calculation of the N/C ratio indicates that this enrichment is more pronounced on the samples with lower collagen content. This is confirmed by the calculation of the CO–NH/C_{1s} ratio, although it is less precise due to the overlap of the peak observed for pure cross-linked PVA at 288.4 eV with the peptidic peak of collagen. Nevertheless the contribution of this carbonyl peak to the C_{1s} envelope of pure cross-linked PVA is only 4% of the total area and therefore the error introduced by not considering its presence could be accepted.

The surface enrichment of collagen could be due to a different value of the interfacial energy of the pairs SC–water and PVA–water: as water evaporates from the SC/PVA solutions, the concentrations increase and the polymer with the highest polymer–water interfacial energy is preferentially forced towards the surface where water molecules can be easily transferred to the air. This hypotheses is in agreement with the qualitative observation that SC is less hydrophilic than PVA, that is the interfacial energy of SC–water pair is higher than that of PVA–water.

5. Conclusions

Films based on blends of collagen and poly(vinyl alcohol) can be dehydro-thermally cross-linked to increase biological stability and reduce water solubility. This cross-linking method is a good alternative to the use of chemical agents, i.e. glutaraldehyde, that could affect the biocompatibility of the materials owing to the release of cytotoxic residuals.

The films behave as two-phase systems and the surface collagen content is higher than in the bulk. The high surface content of collagen cannot by itself be responsible for cell adhesion and proliferation, since very poor cytocompatibility has been observed for the pure collagen sample. The cytocompatibility tests indicate that it is the mixing of the two polymers which enables a better substrate for cell growth. Therefore, bioartificial polymeric materials obtained from the blending of synthetic and natural polymers can provide new materials with enhanced properties.

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