JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 882–886

Physical and biological stability of dehydro-thermally crosslinked collagen–poly(vinyl alcohol) blends

N. BARBANI, L. LAZZERI, L. LELLI, A. BONARETTI, M. SEGGIANI, P. NARDUCCI, G. PIZZIRANI, P. GIUSTI
Dipartimento di Ingegneria Chimica, Universita di Pisa, via Diotisalvi 2, 56126 Pisa, Italy

Dehydro-thermal treatments for 3, 24 and 72 h were used to crosslink blends of collagen and poly(vinyl alcohol) with various compositions. This crosslinking method increases the biological stability *in vitro* of collagen, as was established by an enzymatic test. When the poly(vinyl alcohol) content is not more than 20% the resistance of collagen to enzymatic digestion is not affected by the presence of the synthetic component. A higher content of poly(vinyl alcohol) produces a steric hindrance screening that enhances the resistance of collagen to the collagenase. Dehydro-thermal treatment performed for 24 and 72 h increases the crystallinity of poly(vinyl alcohol), thus reducing the solubility of this component of the blend. Calorimetric analysis was carried out by differential scanning calorimetry to investigate the structure and the thermal stability of the blends. Dehydro-thermal treatments carried out for 24 and 72 h induce high degrees of crosslinking in collagen and high crystallinity in poly(vinyl alcohol). The two components of the blend seem to create independent structures and the blend can show interpenetrating-network-like behaviour.

1. Introduction

Collagen-based materials have been used in a wide variety of biomedical applications. Because of its low antigenicity, reconstituted collagen (especially type I collagen) has been used as the structural component of many biopolymer-based prostheses such as vascular prostheses, heart valves, vitreous body and corneal replacement, artificial skin, wound dressing and dialysis membranes [1–5]. Although collagen-based biomaterials stimulate only a mild inflammatory response, they are still recognized as "foreign" by the tissue. Consequently, a sequence of cellular events starts, leading to the degradation of the implant by collagenase and other host enzymes. Crosslinking is an effective mean in reducing the biodegradation rate of collagen-based biomaterials. In contrast, crosslinking is less effective in rendering the reconstituted collagen as stiff as the native collagen, so that the mechanical properties of the crosslinked collagen-based materials are often poor. In searching for new collagen-based biomaterials that are both biocompatible and mechanically satisfactory, blends of collagen and synthetic polymers, such as poly(vinyl alcohol) (PVA), have been developed by us in the last few years [6, 7]. These materials, belonging to the class of the so-called "bioartificial polymeric materials" [7], could in fact possess good biocompatibility due to the biological nature of collagen, and adequate mechanical properties due to the synthetic component. PVA is a synthetic polymer already used in several biomedical applications [8]. Previous works [9] were dedicated to collagen–poly(vinyl alcohol) blends crosslinked by glutaraldehyde (GTA), which is a crosslinking agent widely used to increase the biological stability of collagen. However, the cytotoxicity due to the glutaraldehyde residuals is often relevant in the failure of the implant. Crosslinking methods, such as UV irradiation and dehydro-thermal treatment, have been described [10, 11] as an alternative to using chemical crosslinkers. The dehydro-thermal treatment was used in this work to crosslink collagen–poly(vinyl alcohol) blends. The crosslinking of collagen depends upon the duration of the treatment. Thus the biological stability of collagen in the blends was evaluated, by an enzymatic test *in vitro*, for different dehydro-thermal treatment lengths. It is also known that heat treatments modify the crystalline structure of poly(vinyl alcohol) [12]. Therefore calorimetric analysis was also carried out to study structure modifications that could affect the properties of the blends.

2. Materials and methods

All the materials used in this study were commercially available: (i) poly(vinyl alcohol) (PVA) (Aldrich Chemie, Steinheim, Germany), with average molecular weight of 114 000 and hydrolysis degree of 100%; (ii) acid soluble collagen (TC) (type I from calf skin, Sigma Chemical Co, St Louis, MO, USA); (iii) collagenase (Sigma Chemical Co, St Louis, MO, USA)

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TABLE I TC digested by collagenase[a], measured as percentage of the initial TC content, for both uncrosslinked and DHT-crosslinked samples, versus percentage weight of PVA in the blend

TC/PVA (w/w)	Uncrosslinked samples (%)	D1-samples[b] (%)	D2-samples[b] (%)	D3-samples[b] (%)
100/0	100	53	0,2	0,2
80/20	100	53	0,2	0,2
60/40	78	43	0,3	0,1
50/50	80	31	0,6	0,3
40/60	74	22	0,6	0,3
20/80	55	24	1,3	0,9

[a] 100 units of collagenase per mg of collagen at 37 °C; incubation time 90 min
[b] D1: 3 h DHT treatment; D2: 24 h DHT treatment; D3: 72 h DHT treatment

from *clostridium histoliticum*, with an activity of 1700 U/mg.

A 1% w/v PVA aqueous solution was prepared by adding the solid PVA to distilled water and raising the temperature to 60 °C. A 1% w/v TC solution, in 0.5 M acetic acid, was prepared at 0 °C under mild stirring. From the two formulated solutions, TC/PVA mixtures with 0/100, 20/80, 40/60, 50/50, 60/40, 80/20 and 100/0 w/w polymer ratios, were prepared. The mixtures were poured into Petri dishes at room conditions and films were obtained by casting. For each composition, films were obtained in quadruplicate: one was used as an uncrosslinked reference, while the others were crosslinked according to the procedures described below.

The dehydro-thermal treatment (DHT) of the films was carried out through two dehydration steps and a final crosslinking step [10]. First, the films were placed in a vacuum oven at 50 °C and subjected to a vacuum of less than 13.5 Pa for 3 h. Second, the temperature within the vacuum oven was increased to 90 °C and this value maintained for 30 min. Finally, crosslinking was achieved by raising the temperature to 120 °C. The TC/PVA films were then removed from the oven after 3 h (D1-samples), 24 h (D2-samples) and 72 h (D3-samples).

Calorimetric analysis of the films was carried out by a differential scanning calorimeter (DSC) Perkin-Elmer DSC-7, in the temperature range 50 to 250 °C, at a scan rate of 10 °C/min.

The biological stability of collagen was estimated by evaluating the enzymatic digestion due to collagenase. Samples of each film were cut in pieces all containing the same TC amount (4 mg). They were soaked into a buffer solution (2 ml, pH 7.4, 0.1 M Tris HCl 0.005 M $CaCl_2$) containing collagenase. In each experiment the collagenase/TC ratio was 100 U/(mg of TC). After incubation at 37 °C for 90 min the samples were centrifuged at 3000 rpm for 10 min. Subsequently, 0.5 ml of the supernatants were collected from each sample, and hydrolysed with 1.5 ml of HCl/water 2/1 at 105 °C for 16 h. The concentration of the digested protein was evaluated using the standard hydroxyprolyne method [13].

Samples of both uncrosslinked and DHT-crosslinked films were placed in distilled water at 37 °C for 3 days and the amount of released PVA was evaluated spectrophotometrically according to the method of Buganda and Rudin [14].

Morphological analysis was carried out by a scanning electron microscope (SEM) Jeol T 300 on samples fractured in liquid nitrogen and sputter-coated with gold.

3. Results

The results obtained by the collagenase assay are shown in Table I. The amount of digested TC is reported as percentage of the initial TC content of the blend. In D1-samples the amount of TC digested after 90 min incubation is about half of that measured for the corresponding uncrosslinked blends. The digested TC in both D2- and D3-samples is practically negligible for all compositions.

Results obtained by DSC analysis carried out on uncrosslinked samples are reported in Fig. 1a. Pure uncrosslinked TC shows an endothermic peak at about 150 °C, related to the denaturation of TC, and a degradation phenomenon at about 200 °C. The pure untreated PVA gives a melting endotherm with a peak temperature, T_m, at about 222 °C. The thermograms of uncrosslinked TC/PVA films showed two endothermic peaks, the first corresponding to TC denaturation and the second to PVA melting. The intensity of the denaturation peak decreases as the PVA content increases and is not detectable when PVA content is higher than 40%.

The thermograms of D1-samples are reported in Fig. 1b. Pure TC denatures at about 180 °C. The DSC curve of pure PVA does not show any relevant variation in comparison with the untreated PVA. The thermograms of the blends show only one endothermic event in which the TC denaturation is present as a shoulder superimposed on the sharper melting peak of PVA.

In Fig. 1c the DSC curves of D2-samples are illustrated. The denaturation of pure TC is revealed by a rather sharp peak at about 200 °C. The pure PVA shows a melting peak quite similar to the previous ones, and a secondary, small endothermic event at about 125 °C. The denaturation of TC and the two peaks of PVA are detectable for all compositions of D2-samples.

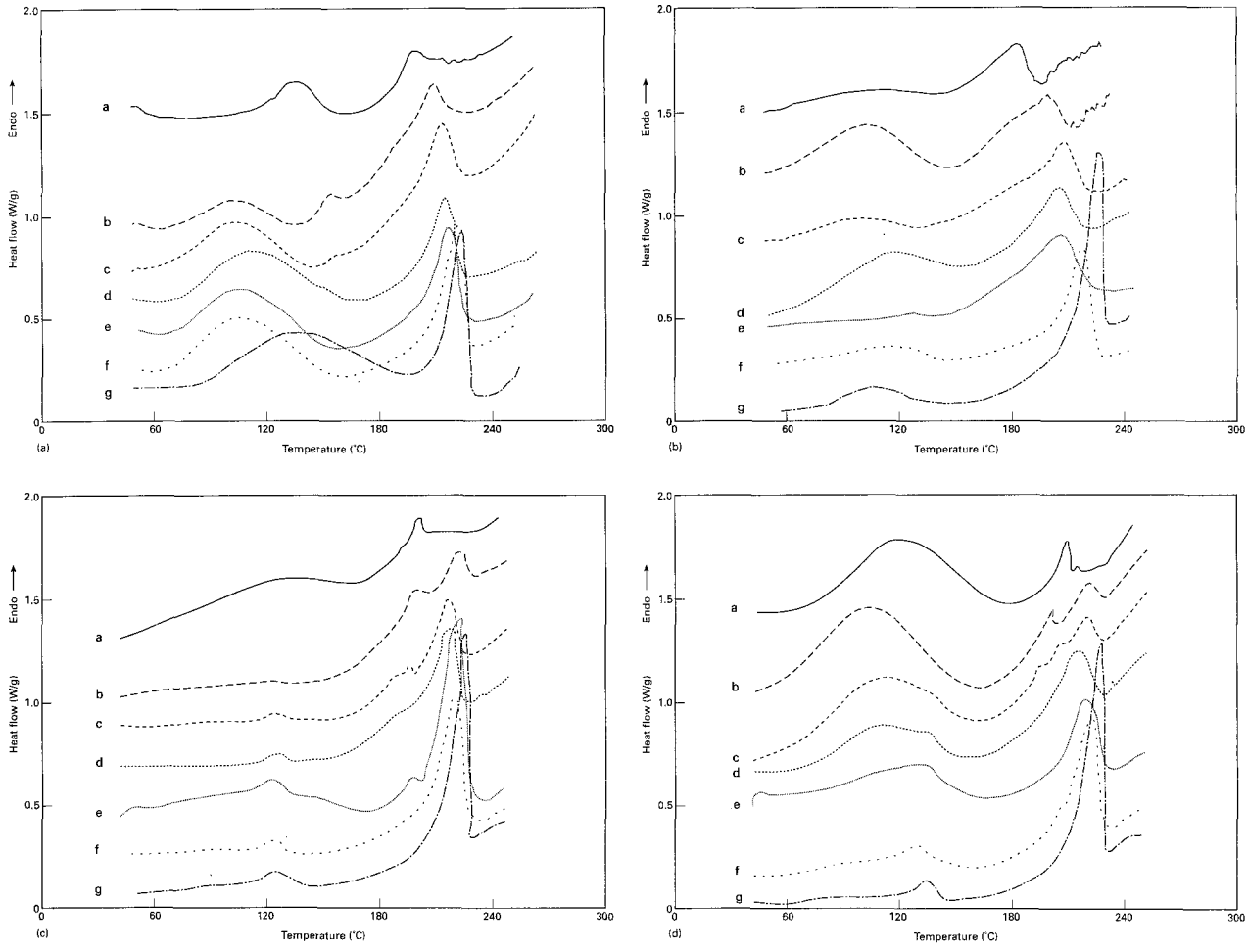


Figure 1 Normalized DSC thermograms of TC/PVA blends: (a) uncrosslinked samples, (b) D1-samples, (c) D2-samples, (d) D3-samples (TC/PVA 100/0a; 80/20b; 60/40c; 50/50d; 40/60e; 20/80f; 0/100g).

The DSC curves of D3-samples are quite similar to those of D2-samples (Fig. 1d). The denaturation of pure TC occurs at 207 °C, and the secondary peak of PVA at about 130 °C.

Most of the DSC curves show a spread endothermic event related to the evaporation of water contained in the samples.

For each sample series the melting point depression of PVA, $\Delta T_m = T_m^\circ - T_{blend}$, is reported as a function of TC content (Fig. 2). T_m° is the melting point of pure PVA and T_{blend} is the melting point of PVA in the blend. The highest values of ΔT_m were observed for D1-samples.

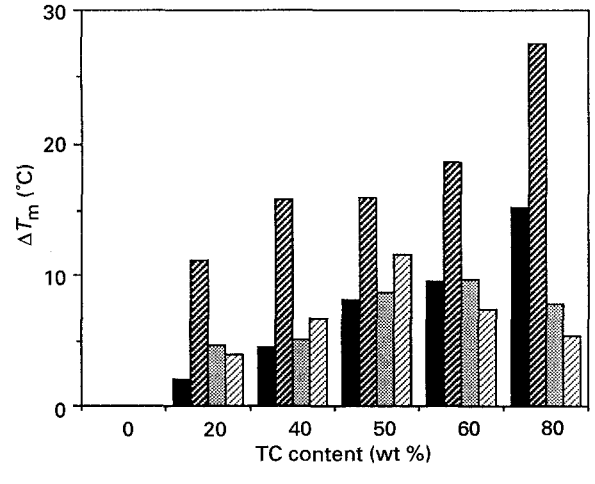


Figure 2 Melting point depression of PVA as a function of TC content (■ uncrosslinked; (▨ D1; ▩ D2; ▨ D3).

In Fig. 3a–d the values of the enthalpies, ΔH_t, are reported as functions of the TC content. Because of the superimposition of peaks in the DSC curves, the TC denaturation enthalpy and the PVA melting enthalpy cannot be separately evaluated. Thus, the values of ΔH_t have been obtained by measuring the total area below the two superimposing peaks. The continuous lines are obtained assuming that the total enthalpy follows the additivity rule with the blend composition. Deviations from linear behaviour of ΔH_t indicate the presence of interactions between the two components of the blend. Slight deviations are observed for uncrosslinked samples, while D1-samples show the most marked positive deviations. No relevant interaction is observed for D2- and D3-samples.

The amounts of released PVA are reported in Table II as percentages of the initial PVA content of the blend. A decreasing trend of released PVA is observed as the time of DHT treatment increases.

SEM analysis does not show any relevant differences between uncrosslinked and crosslinked samples (Fig. 4a–c). Generally, all the observed structures were dense and relatively homogeneous. SEM images at higher magnification revealed fibrillar-like structures in all crosslinked films (Fig. 5a,b).

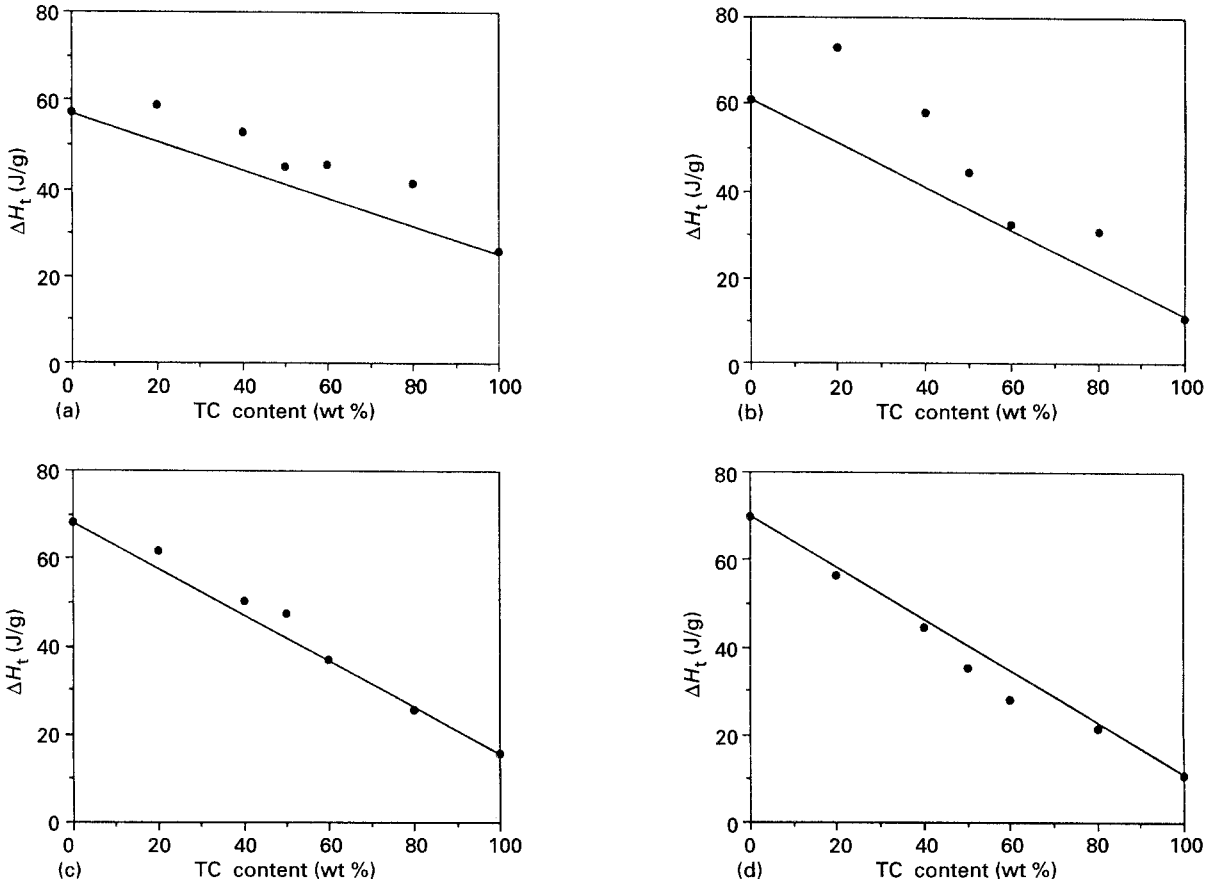


Figure 3 Enthalpies of TC/PVA blends as function of TC content for: (a) uncrosslinked samples, (b) D1-samples, (c) D2-samples, (d) D3-samples. The continuous lines are obtained assuming that ΔH_t follows the additivity rule with the blend composition.

TABLE II PVA released by TC/PVA films as percentage of the initial PVA content, for both uncrosslinked and DHT-crosslinked samples

TC/PVA (w/w)	Uncrosslinked samples (%)	D1-samples[a] (%)	D2-samples[a] (%)	D3-samples[a] (%)
80/20	78,50	10,76	2,27	0
50/50	28,04	10,67	2,80	0,15
20/80	7,00	2,24	6,25	1,70
0/100	25,50	4,18	4,14	1,04

[a] D1: 3 h DHT treatment; D2: 24 h DHT treatment; D3: 72 h DHT treatment

4. Discussion

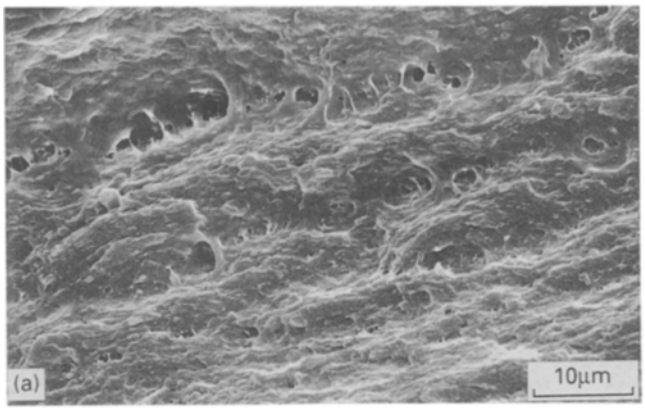
As expected, DHT treatment increases the resistance of TC to enzymatic digestion. The enzyme action is not affected by the presence of PVA when the PVA content in the blend is not greater than 20%. At higher PVA contents, collagenase is less effective on uncrosslinked and D1-samples. This is quite similar to previous results obtained with TC/PVA blends crosslinked with glutaraldehyde [6]. This could be due to a steric hindrance screening around the protein molecules produced by high PVA contents. The very low values of digested TC measured for D2- and D3-samples indicate that a very high degree of crosslinking between TC molecules was achieved. In this case variations in the PVA content are irrelevant to the TC response to the enzyme.

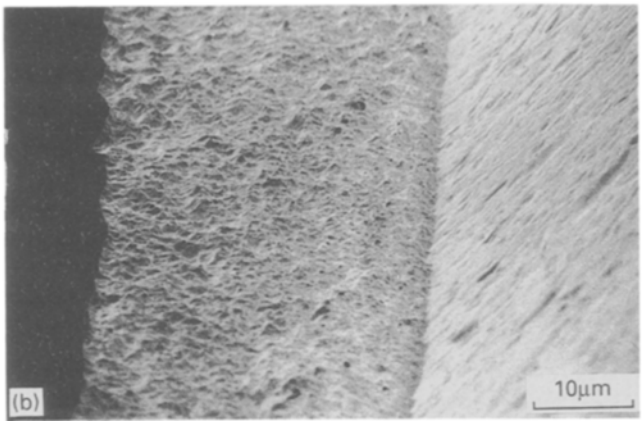
The thermal stability of TC is also enhanced by DHT treatment, as is revealed by the shifting of the denaturation peak towards higher temperatures.

The melting point depression of PVA, in TC/PVA uncrosslinked samples, is probably due to hydrogen-bond interactions between hydroxyl groups of PVA and functional groups of aminoacidic residues of TC. These calorimetric data are in agreement with those obtained by us in previous work [6, 7]. The enhancement of the PVA melting point depression observed for D1-samples could be related to the increase of hydrogen bonding due to the higher mobility of PVA chains at the DHT temperature (120 °C).

DHT treatments of 24 and 72 h increase the crystallinity of PVA [12] as revealed by the appearance of the small secondary peaks. The increase of crystallinity, in turn, can explain the lower solubility of PVA found by releasing tests on these sample series.

The trend of ΔH_t with blend composition confirms that interactions between TC and PVA occur to a greater extent in uncrosslinked and D1-samples. The values of ΔH_t very close to linear behaviour for D2-





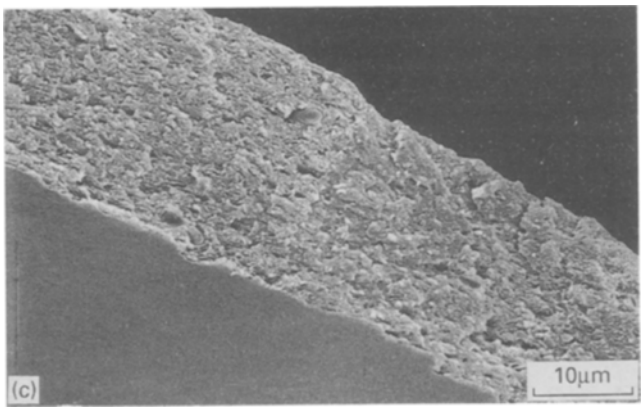
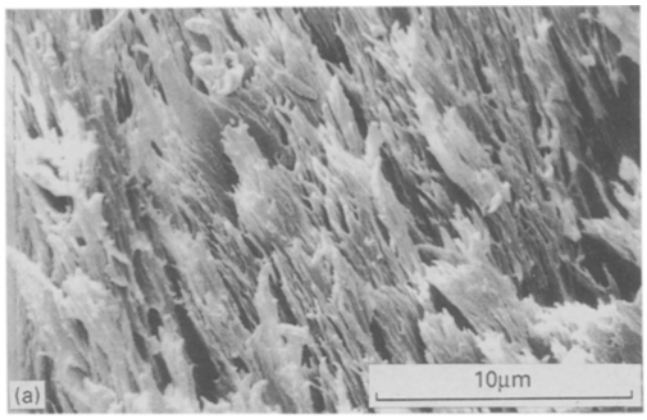


Figure 4 SEM images of TC/PVA = 50/50: (a) uncrosslinked; (b) D1-sample; (c) D3-sample.



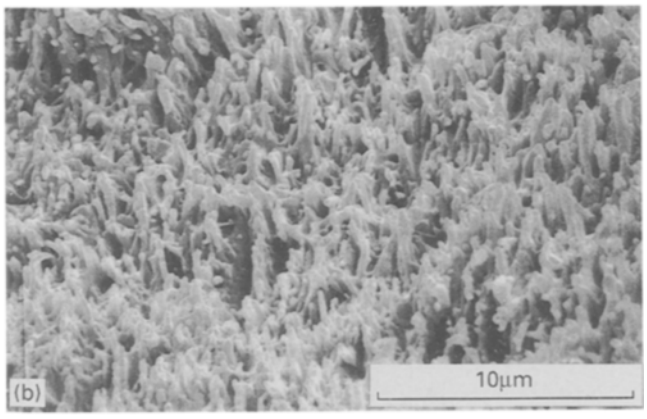


Figure 5 SEM images of TC/PVA = 20/80: (a) D1-sample; (b) D3-sample.

and D3-samples suggest that the highly crosslinked TC and the highly crystallized PVA form independent structures and that these blends could behave like interpenetrating polymer networks.

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