Gellan–Adipic Acid Blends Crosslinked by Means of a Dehydrothermal Treatment

Niccoletta Barbani,[1] Maria Laura Coluccio,[2] Caterina Cristallini,[3] Giulio D. Guerra,[3] Elisabetta Rosellini[1]

[1]*Department of Chemical Engineering, Industrial Chemistry and Materials Science, University of Pisa, Via Diotisalvi 2, I-56122 Pisa, Italy*
[2]*Department of Agro-Forestal and Environmental Sciences and Technologies, University of Reggio Calabria, Piazza S. Francesco di Sales 4, I-89061 Gallina Reggio Calabria (RC), Italy*
[3]*Institute for Composite and Biomedical Materials, Research Unit of Pisa, Italian National Research Council, Via Diotisalvi 2, I-56122 Pisa, Italy*

Received 15 September 2009; accepted 18 February 2010
DOI 10.1002/app.32277
Published online 9 July 2010 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Blends of gellan gum (GE) and adipic acid (ADA), at various ratios, were manufactured in the form of films by casting from aqueous solutions and crosslinked by a dehydrothermal treatment (DHT). The materials, before and after DHT, were characterized by both physicochemical tests and cellular adhesion and growth on the film surfaces. The total reflection and spotlight Fourier transform infrared (FTIR) spectroscopy and optical and scanning electron microscopy showed the presence of both GE-rich and ADA-rich regions and the formation of ester groups after DHT. Differential scanning calorimetry, thermogravimetric analysis, and dynamic mechanical analysis (DMA) showed that the crosslinking by DHT made the materials more thermally stable. The swelling in water, which diminished in the films subjected to DHT, confirmed that the crosslinking enhanced the whole stability of the material. DMA also showed that the behavior of the GE–ADA blends was quite similar to that of some living tissues, such as the skin. The cell cultures indicated that the materials, especially that with a 6 : 10 ADA-to-GE ratio, were very able to promote cellular adhesion and proliferation. In conclusion, the GE–ADA crosslinked blends appeared very suitable for a use as biomaterials; in particular, the cell cultures indicated that they might be useful as scaffolds for tissue reconstruction. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 3131–3140, 2010

Key words: biomaterials; crosslinking; FTIR; polysaccharides; thermogravimetric analysis (TGA)

INTRODUCTION

Natural polysaccharides are polymers that are widely used as biomaterials because they mimic the features of the extracellular matrix and, thus, have the potential to direct the migration, growth, and organization of cells.[1] Many of them also demonstrate adequate biocompatibility and biodegradability, but, often, they must be chemically modified or crosslinked to improve their mechanical properties and to regulate their degradation rates in a physiological environment.[2]

Particularly interesting are those polysaccharides that can form gels with water or biological fluids, such as alginate, carrageenan, and gellan gum (GE).[3] GE is a linear polysaccharide, produced by the bacterium *Sphingomonas paucimobilis*, which has the tetrasaccharide repeating unit [(→3)-β-D-Glc-(1→4)-β-D-GlcA-(1→4)-β-D-Glc-(1→4)-α-L-Rha-(1→)].[4] The polysaccharide in the native state contains *O*-acetyl groups, approximately one for every repeating unit;[5] these groups, however, are completely removed in the commercial products.[6] As a result, the deacetylated GE contains, in the salt form, two $-CH_2OH$ groups and one $-COO^-$ group per repeating unit. The very versatile properties of GE make it an excellent multifunctional agent.[1]

This macromolecule forms with water a gel with a double-helix structure,[7,8] which has a reversible gel–sol transition at about 30°C in the salt form.[9] The mechanical properties of the gel form depend mainly on the starting solution concentration, the counterion content, and the deacetylation degree. To obtain a homogeneous mixture of GE with other components, for example, hydroxyapatite,[4] its concentration must not exceed 1% w/v. Because a gel made with a GE solution having this concentration generally shows a scarce toughness, it may be necessary to crosslink together the GE macromolecules to make the network more stable.

Usually, GE is crosslinked through ionic bonds with divalent cations,[6] but in some cases, a more stable crosslinking by covalent bonds may be necessary. A covalent crosslinking may also be able to prevent the collapse of the GE structure observed in GE–hydroxyapatite composites.[4] In this article, the

Correspondence to: G. D. Guerra (giuliodante.guerra@diccism.unipi.it).

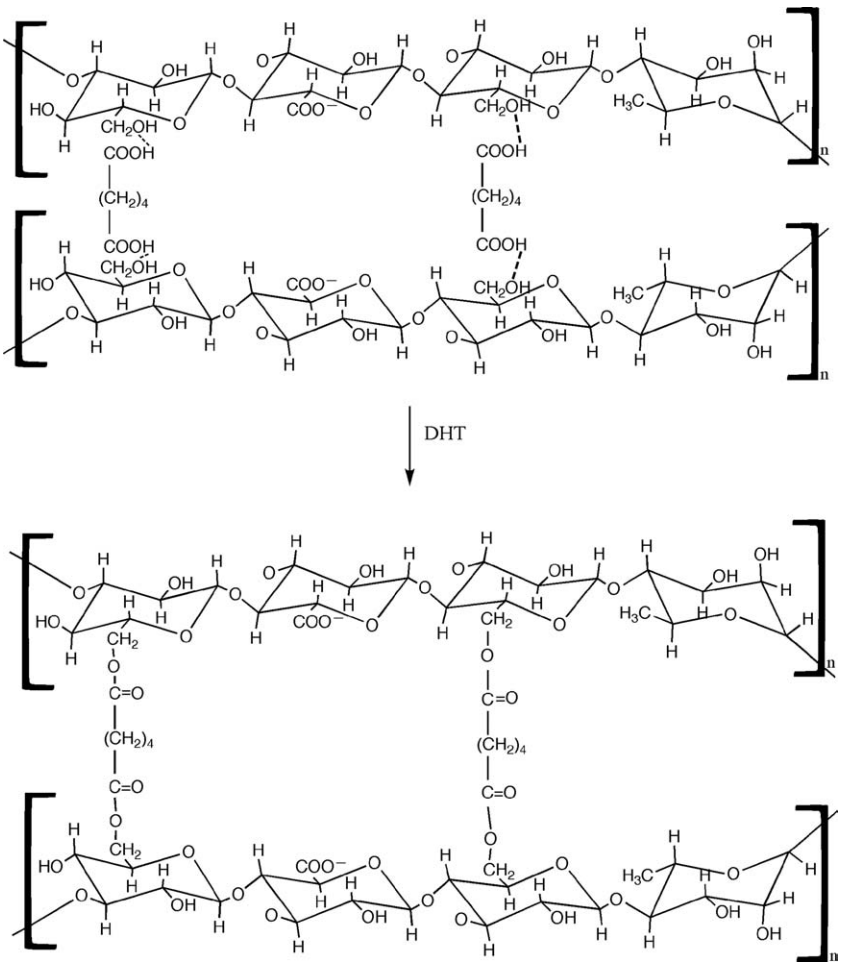

Figure 1 Very simplified scheme of the formation of adipic ester bridges between GE chains. Actually, the bridges could link together more than two macromolecules.

preparation and characterization of films of GE and 1,6-hexandioic acid [adipic acid (ADA)], both as such and as covalently crosslinked, is reported. The crosslinking of carboxyl-containing polysaccharides by ADA is generally carried out chemically through ADA dihydrazide in the presence of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride.[10] In this study, we attempted to crosslink the GE–ADA mixtures by a dehydrothermal treatment (DHT); this was a slight modification of the procedure used by Scotchford et al.[11] to crosslink collagen–poly(vinyl alcohol) blends to promote the formation of covalent ester bonds between the carboxylic groups of ADA and the hydroxyl groups of GE, according to the reaction scheme shown in Figure 1. This procedure has some advantages: it is a simpler one; it does not reduce the number of carboxyl groups in the crosslinked material, which can favor interactions with the body tissues; and, most important, it prevents the formation of byproducts such as *N*-[2-(dimethylamino)ethyl]-*N*′-ethyl-*N*′-gellanacylurea and *N*-[2-(dimethylamino)ethyl]-*N*′-ethylurea,[10] which may be harmful for human health.

The final aim was to investigate the chemical, physical, mechanical, and morphological properties of the films and their ability to favor cell adhesion and growth.

EXPERIMENTAL

Materials and methods

High-molecular-weight GE (Fluka, Buchs, SG, Switzerland) had a gel–sol transition temperature that linearly increased with the concentration in water and ranged between 30°C (0.5% w/v solution) and 45°C (2.0% w/v solution); the pH of the 1.0% solution was 7.76 at 42°C. It was examined spectrophotometrically to check for the absence of ester side groups and used without further purification. The counterions of the polyelectrolyte structure, evaluated by means of a PerkinElmer Emission Spectrometer Plasma 400 (Norwalk, CT) were present in the following amounts per gram of GE: Na^+, 15.79 mg; K^+, 10.39 mg; Ca^{2+}, 4.25 mg; and Mg^{2+}, 1.31 mg. ADA (Carlo Erba, Rodano, MI, Italy, reagent

grade (RPE) 99%) was used as such. The GE–ADA blends were prepared and thermally crosslinked as follows. A GE solution in hot water (1% w/v, about 50°C temperature) was mixed with various amounts of ADA under stirring. Films of GE–ADA with different ratios (2 : 10, 4 : 10, 6 : 10, 8 : 10, and 10 : 10) of ADA —COOH to GE —CH_2OH were prepared by a solution casting method. The DHT was carried out by a slight modification of the procedure of Scotchford et al.[11] through two dehydration steps (50°C for 3 h and 90°C for 30 min *in vacuo*) and a final crosslinking step (130°C for 18 h *in vacuo*). This three-step DHT was preferred to the single-step one used recently to crosslink collagen–chitosan blends[12] because the two steps at lower temperatures could favor the positioning of the ADA carboxyl groups close to the GE hydroxyls (see Fig. 1) without interposed water molecules. No blend containing excess ADA was prepared so that, in the ester bond formation, the less hindered primary hydroxyls of GE were preferentially involved, as shown in Figure 1.

Infrared spectroscopy

Total reflection and spotlight Fourier transform infrared (FTIR) spectra and transmission FTIR maps, obtained by the generation of thousands of spectra, were carried out by means of a PerkinElmer Spectrum One FTIR spectrometer; the apparatus was equipped with a PerkinElmer Universal ATR sampling accessory and a PerkinElmer Spectrum Spotlight 300 FTIR imaging system. The spectral resolution was 4 cm^{-1}; the spatial resolution was 6.25 nm. The spectra were the result of 16 scans. The thin samples were deposited on suitable supports, specific areas of interest were identified by means of the optical microscope, transmission spectra were collected, and IR spectral images were produced.

The Spotlight software (Perkin Elmer, Norwalk, CT) used for acquisition was also used to preprocess the spectra. The spectra were also subjected to a Savitzky Golay smoothing procedure (9 points), and the second derivative was calculated for all spectra.

For the band ratio calculation, two-dimensional images were produced from the absorption spectra with the ratio between the areas of the band at 1687 cm^{-1} and of that at 1603 cm^{-1}.

Microscopy

Scanning electron microscopy (SEM) was carried out with a JEOL T-300 instrument (Pieve Emanuele, MI, Italy). Optical microscopy images were carried out by means of the optical microscope present in the FTIR apparatus.

Thermal analysis

Differential scanning calorimetry (DSC) was carried out in triplicate with a PerkinElmer DSC7 apparatus in Al pans from 30.00 to 250.00°C at 10.00°C/min under N_2 flux. Thermogravimetric analysis (TGA) was carried out with a PerkinElmer TGA6 apparatus at a scanning rate of 10.00°C/min under N_2 flux.

Dynamic mechanical analysis (DMA)

The mechanical properties were analyzed by means of a Tritec 2000 DMA (Triton Technology, Ltd., Keyworth, Nottinghamshire, United Kingdom). The samples were put into the measurement cell under controlled humidity; then, they underwent a sinusoidal stress with a prefixed oscillation frequency and amplitude.[13] In DMA, both the storage modulus (G') and the loss tangent (tan δ) were determined. The temperature was scanned at a rate of 5°C/min.

Swelling test

The water-absorption test was carried out in triplicate by means of the following procedure. The films were dried and weighed to a constant weight; then, they were incubated for 24 h in an atmosphere saturated with water vapor at 37°C and weighed again. The percentage water uptake (Q) was calculated from the swollen weight (W_s) and dry weight (W_d) with eq. (1):

$$Q = 100(W_s - W_d)/W_d \quad (1)$$

Cell adhesion test

The cell adhesion test was performed with Murine Fibroblasts NIH-3T (Cambrex Profarmaco, Milan, Italy) with a density of 100,000 cells/mL. The cell cultures were carried out on polymer films prepared according to the following procedure. Dry samples were sterilized by washing with a 70% ethanol solution in sterile water; this was followed by UV exposure for 15 min on each sample side. We coated the films to be used as positive controls with type A porcine gelatin (Sigma, Buchs, SG, Switzerland) by leaving them in a solution (1% w/v) of the same gelatin in sterile water for 1 h at 37°C. NIH-3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (Cambrex Profarmaco, Milan, Italy) with a high glucose concentration (4.5 g/L), 10% fetal calf serum (Cambrex), 1% glutamine (Cambrex), penicillin (200 U/mL, Cambrex), and streptomycin (200 μg/mL, Cambrex). The culture was maintained in an incubator equilibrated with 5% CO_2 at 37°C. The films were seeded with a suspension of 100,000 cells/mL. After culture times of 4, 24, and 48 h, respectively, the

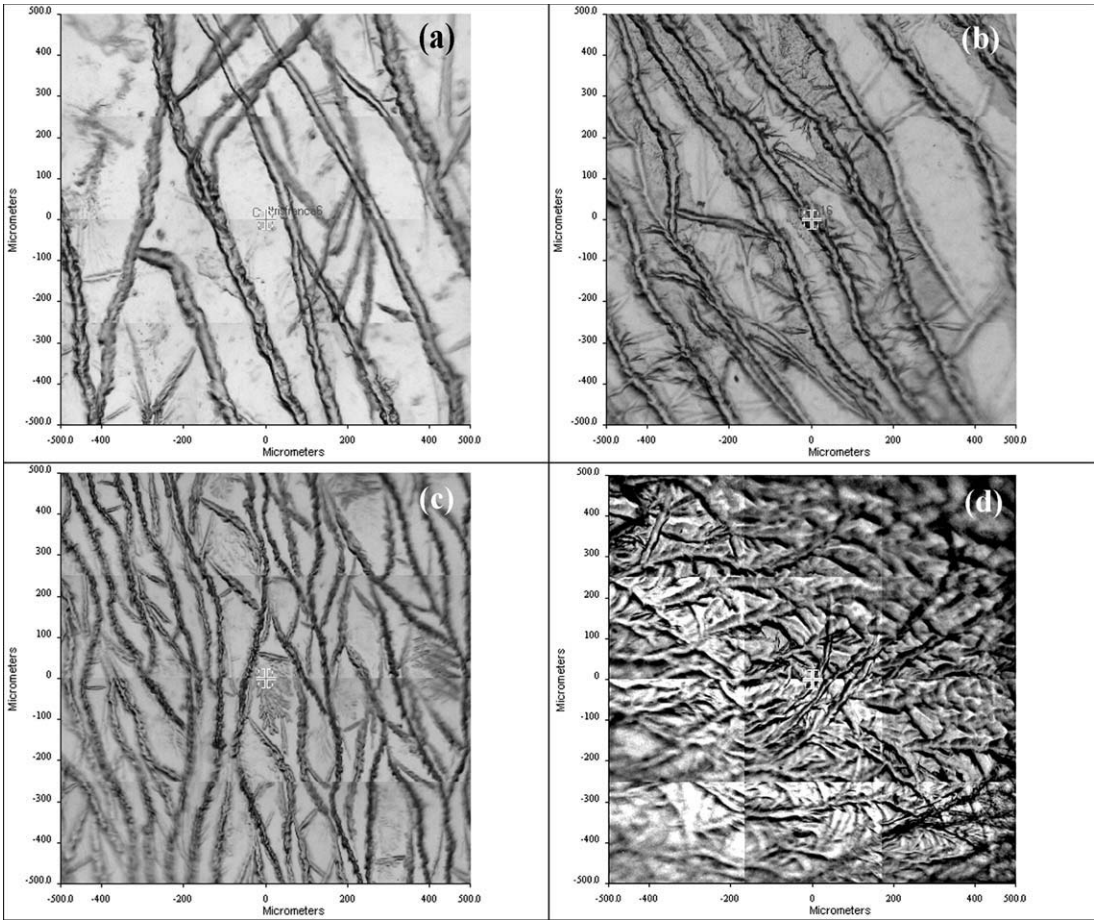

Figure 2 Optical microscopy images of the GE–ADA blends with a 6 : 10 —COOH to —CH_2OH ratio (a) before and (b) after crosslinking by DHT and a 10 : 10 —COOH to —CH_2OH ratio (c) before and (d) after crosslinking by DHT. The images were taken on the surfaces of the films, which were obtained by casting (image areas = 1 mm^2).

cells were fixed with a 4% (v/v) formaldehyde (Sigma) solution in phosphate buffered saline (Sigma) and stained a with Coomassie blue (Fluka) solution. The samples were analyzed under an optical microscope (Olympus AX 70, B&B Microscopes, Ltd., Pittsburgh, PA), and the ratio between the number of cells on the polymeric structures and the total area of the polymer substrate was calculated as an index of cell density.[14]

RESULTS AND DISCUSSION

The optical microscopy images of the sample surfaces (Fig. 2) showed the presence of a network of filamentous structures with flat regions within the meshes. The number of filaments per unit area appeared greater, as shown in Figure 2(c,d), with a higher ADA content; moreover, the DHT treatment made the network quite tighter, as shown in Figure 2(b,d). The SEM images showed that the same thick filaments were seen not only on the surfaces but also in the sections of the films, both uncrosslinked and crosslinked, as shown in Figure 3.

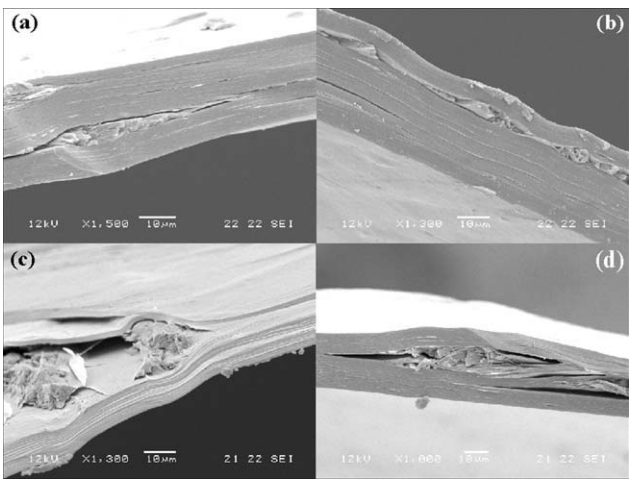

Figure 3 SEM images of the GE–ADA blends with a 4 : 10 —COOH to —CH_2OH ratio (a) before and (b) after crosslinking by DHT and a 6 : 10 —COOH to —CH_2OH ratio (c) before and (d) after crosslinking by DHT. The images were taken on the sections of the films, which were obtained by casting (dimension bars = 10 μm).

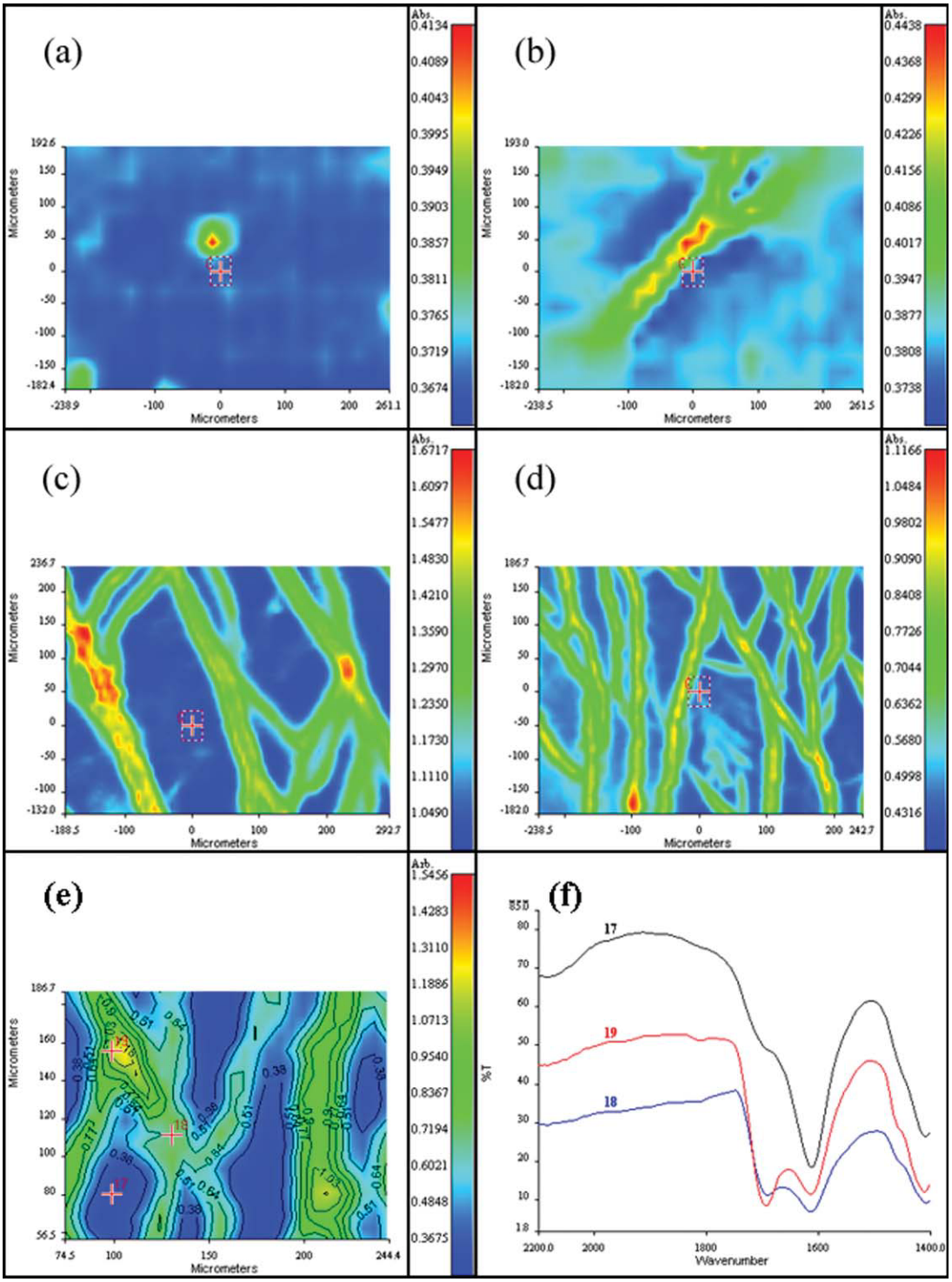

Figure 4 Spotlight FTIR maps, made in transmission mode, of the four GE–ADA blends not subjected to DHT with different —COOH to —CH_2OH ratios, namely, (a) 2 : 10, (b) 4 : 10, (c) 6 : 10, and (d) 10 : 10 and (e) 1687 to 1603-cm^{-1} band ratio map of a portion of (d), (f) spectra taken at the signed points of (e) corresponding to the band ratios of 0.38 (17), 0.54 (18), and 1.16 (19). The maps and the spectra were taken on the surfaces of the films, which were obtained by casting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The FTIR spectra of the pure components showed their characteristic absorption bands. The GE spectra showed both the O—H stretching wide band at 3308 cm^{-1} and the C—OH stretching peak at 1027 cm^{-1}, as well as the peak of the antisymmetric C—O stretching of —COO^- at 1603 cm^{-1}, whereas that of the symmetric one at 1407 cm^{-1} was visible in the spectra of both compounds. The other remarkable absorptions,

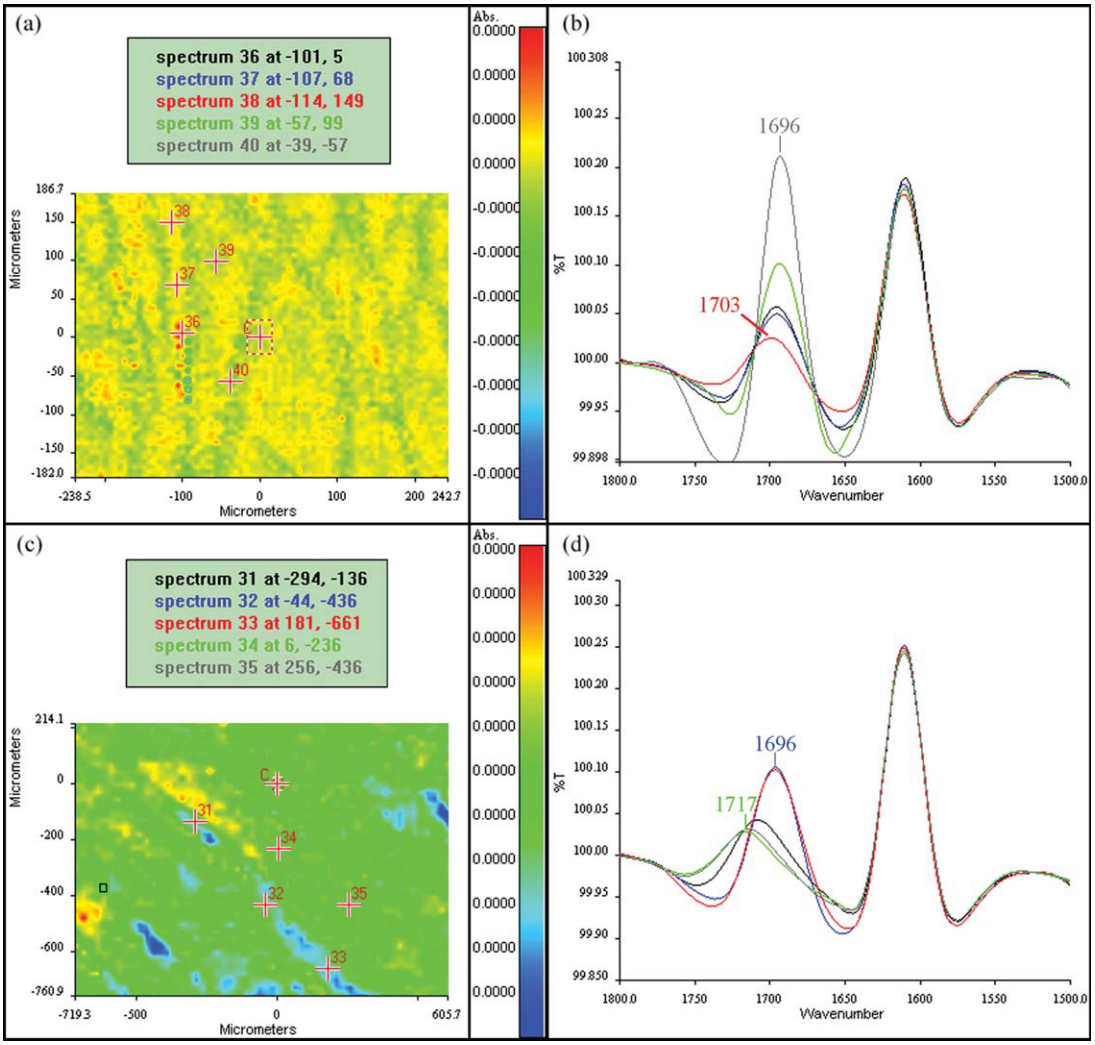

Figure 5 Spotlight FTIR second-derivative maps and spectra taken at the signed points and having the same colors as in the legends of the GE–ADA blend having a 10 : 10 —COOH to —CH_2OH ratio (a,b) not subjected to DHT and (c,d) subjected to DHT. The maps and the spectra were taken on the surfaces of the films, which were obtained by casting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

present in the spectra of ADA, were its characteristic peak at 1687 cm^{-1} and that at 919 cm^{-1}, attributable to the O—H out-of-plane bending of the dimeric carboxyl.

Imaging maps, obtained for four GE–ADA blends not subjected to DHT, with different —COOH to —CH_2OH ratios, are shown in Figure 4(a–d). As observed in the optical microscopy images, both the number and the interconnections of the filaments seemed to increase with increasing ADA content in the blends. To make the distribution of the components in the blends clearer, the maps were processed with the band ratio. The map in Figure 4(e) shows the ratios between the absorbance at 1687 cm^{-1} of the ADA carboxyl and that at 1603 cm^{-1} of the GE carboxylate anion in a portion of the map in Figure 4(d) relative to the GE–ADA blend with a 10 : 10 —COOH to —CH_2OH ratio. The spectra in Figure 4(f), taken from three points of Figure 4(e), which corresponded to band ratios of 1.16, 0.54, and 0.38, showed that the ADA peak at 1687 cm^{-1} was stronger than that at 1603 cm^{-1} on the top of a filament, whereas, at the intermediate point, their intensities were nearly inverted; finally, in the spectrum taken in a flat region, only a shoulder on the GE band at 1603 cm^{-1} was visible. In particular, the spotlight FTIR chemical imaging showed how GE and ADA were distributed in the films: on the upper of the filaments, the stronger absorption at 1696 cm^{-1} indicated a higher amount of ADA, whereas the presence of GE became more evident in the rest of the matrix, with increasing distance from the filaments (see Fig. 4). Similar results were also found for the blends with lower ADA contents.

Figure 5 shows the spotlight FTIR second-derivative maps and the spectra taken in different regions of the maps of the GE–ADA blend with a 10 : 10 —COOH to —CH_2OH ratio. Figure 5(a,b) shows the

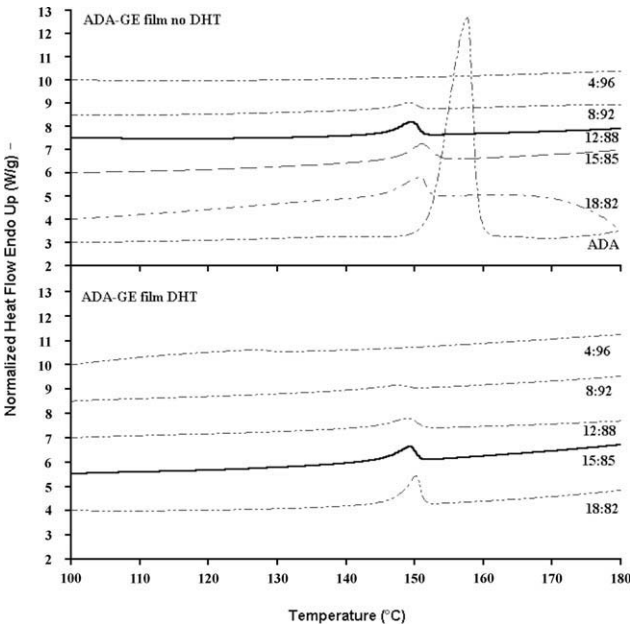

Figure 6 DSC traces for films of pure ADA and five GE–ADA blends with different ADA-to-GE weight ratios (see TABLE I) not dehydrothermally crosslinked (no DHT) and dehydrothermally crosslinked (DHT). The scanning rate was 10.00°C/min under N_2 flux.

maps and the spectra of the blends not subjected to DHT, whereas Figure 5(c,d) shows those of the subjected ones. The second-derivative spectra in Figure 5(b) point out, in the wave-number range between 1800 and 1500 cm^{-1}, the GE peaks around 1600 cm^{-1} and the ADA ones around 1680 cm^{-1}; it appeared that only slight shifts of the ADA peak toward wave numbers higher than 1687 cm^{-1} occurred, likely because of hydrogen bonds with GE. With regard to the blend subjected to DHT, the most spectra in Figure 5(d) showed great shifts of the band at 1687 cm^{-1} toward higher wave numbers and so indicated stronger interactions. Moreover, the —COO^- of GE seemed to remain unchanged for both uncrosslinked and crosslinked blends. The interactions between GE and ADA were mainly hydrogen bonds before DHT, which caused the formation of ester bonds between the ADA —COOH groups and the —CH_2OH groups of GE, as it appeared from the second-derivative spectra shown in Figure 5(b,d). Conversely, no evidence of the formation of anhydride bonds between the ADA —COOH groups and those of GE was detected from the FTIR analysis; evidently, the DHT treatment alone was not sufficient to cause such a reaction, which needs a powerful dehydrating agent to occur. The increased quantity of ADA in the blend explained the greater number of filaments in Figure 2(c) than in Figure 2(a); conversely, the higher density of the filaments in the blends subjected to DHT was attributable to the shrinkage of the network due to the formation of covalent bonds between the ADA bridges and the GE chains, which were stronger than the hydrogen bonds present in the blends without DHT.

Nearly all the DSC traces of the GE–ADA films (Fig. 6) showed in the first scan a sharp endothermic signal due to ADA melting. For the sample with a 2 : 10 ADA —COOH to GE —CH_2OH ratio (4 : 96 weight ratio), the ADA melting process was not evident. Table I reports the ADA melting temperatures; they decreased quite linearly with increasing GE content and were always lower for the blends subjected to DHT than for the those not subjected to DHT. Plotting the differences between the melting points (ΔT_m) of pure ADA and the blends not subjected to DHT versus the GE molar fraction (Fig. 7), we obtained a straight line. This linear dependence confirmed the formation of hydrogen bonds between the macromolecule and the dicarboxylic acid. The fact that the straight line intersected the ΔT_m axis at a value quite different from 0°C was due to the pure ADA melting point, which was not the thermodynamic value used by both Nishi and Wang[15] and other authors[16] to calculate the quantitative interaction parameter, but only an empirical value taken from the ADA curve in Figure 6 that was used to obtain a qualitative indication of the presence of interactions between the components of the blends.

The TGA data of the samples, evaluated from the first-derivative curves in Figure 8, are reported in Table II: only a slight decrease of the onset and maximum temperatures and no significant difference in weight loss were detected between the blends and pure GE when they were not subjected to DHT, as shown by experiments 1–6. On the contrary, experiments 7–10, carried out on DHT-subjected samples, showed slight increases in the onset and maximum temperatures with increasing ADA content, and the weight losses of the pure GE were much greater than those of the blends. A similar difference in the

TABLE I
Melting Points of ADA in the ADA–GE Films, Both not Dehydrothermally Crosslinked (No DHT) and Dehydrothermally Crosslinked (DHT), from DSC Traces

ADA : GE		Melting point (°C)	
Molar ratio	Weight ratio	No DHT	DHT
Pure ADA		158	–
10 : 10	18 : 82	151	150
8 : 10	15 : 85	151	149
6 : 10	12 : 88	150	149
4 : 10	8 : 92	149	147
2 : 10	4 : 96	nd	nd

The values plotted are the mean of three determinations; their differences from the actual data range between 1 and 3%. nd = not detectable in the DSC trace.

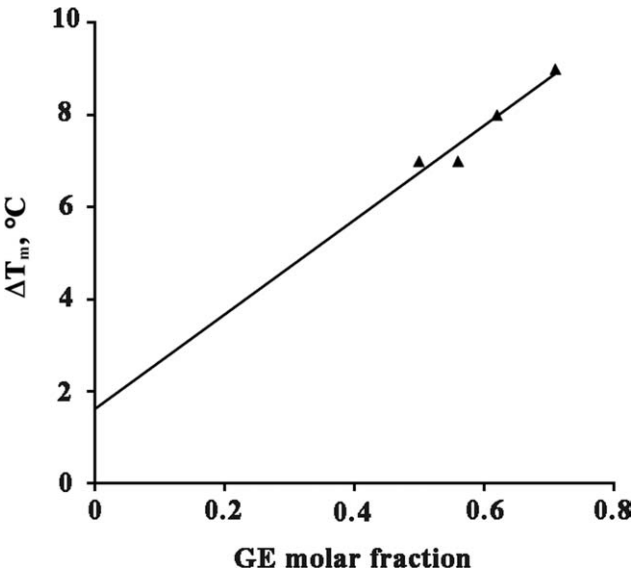

Figure 7 ΔT_m of pure ADA and the blends not subjected to DHT on the GE molar fraction in the blends. The melting points were evaluated from the DSC traces.

weight losses also appeared between the uncrosslinked blends and the corresponding crosslinked ones (see experiments 2–4 and 8–10). With regard to the TGA measurements, they showed that the thermal stability of the crosslinked materials was greater than that of the uncrosslinked ones. In particular, TGA measurements carried out on the GE–ADA blends not subjected to DHT (see Table II, experiments 1–6) showed onset and maximum degradation rate temperatures that decreased with increasing ADA content in the blends. This behavior indicated that the presence of the dicarboxylic acid made the GE macromolecules less stable toward the thermal degradation: it was likely that the interactions with ADA caused some distortions in the GE double-helix structure. No significant variation was present either in the first weight loss (Δy_1), due to the evaporation of H_2O, or in the total weight loss (Δy_{tot}), due also to the degradation of the whole structure. On the contrary, the blends crosslinked by DHT (experiments 8–10) showed degradation temperatures higher than those of the pure GE subjected to the same treatment (experiment 7). With regard to the weight losses, all of the Δy values of the GE–ADA blends subjected to DHT were lower than those of both the pure GE and the blends not subjected to DHT. All these results confirm the effectiveness of thermal crosslinking for the stabilization of the gel.

DMA showed (Fig. 9) how G' of the thermally crosslinked materials was a function of the film composition and the temperature. In the temperature range between −24 and +47°C, the higher the ADA content was, the higher G' appeared to be. At lower temperatures, the sample with a 6 : 10 ADA —COOH to GE —CH_2OH ratio had a greater G' than that with an 8 : 10 ratio; at temperatures higher than about 70°C, both the G' values of these two materials tended to the value of pure GE. On the contrary, G' of the film with a 10 : 10 ADA-to-GE ratio was much higher than those of the other ones in the whole temperature range. As a whole, the material with a 6 : 10 ADA-to-GE ratio had behavior more similar to the pure GE than to those with higher ADA contents. At temperatures higher than 30°C, the materials with ADA-to-GE ratios of both 8 : 10 and 10 : 10 had quite constant G' values; it was a signal of the thermal stabilization of these materials. The curves of the lower graph, which showed tan δ as a function of the temperature, were in substantial agreement with those of G'. In particular, the shape of the curve d confirmed that the material with a 10 : 10 ADA —COOH to GE —CH_2OH ratio was the most stable at temperatures higher than 30°C. In addition, the glass-transition temperature (T_g) values of the blends evaluated from the tan δ curves (see Fig. 9), which all were greater than 0°C, indicated behavior quite similar to that of some living tissues, such as skin. The T_g value of about 24°C, shown by sample b, with a ratio of ADA —COOH to GE —CH_2OH equal to 6 : 10, seemed not too different

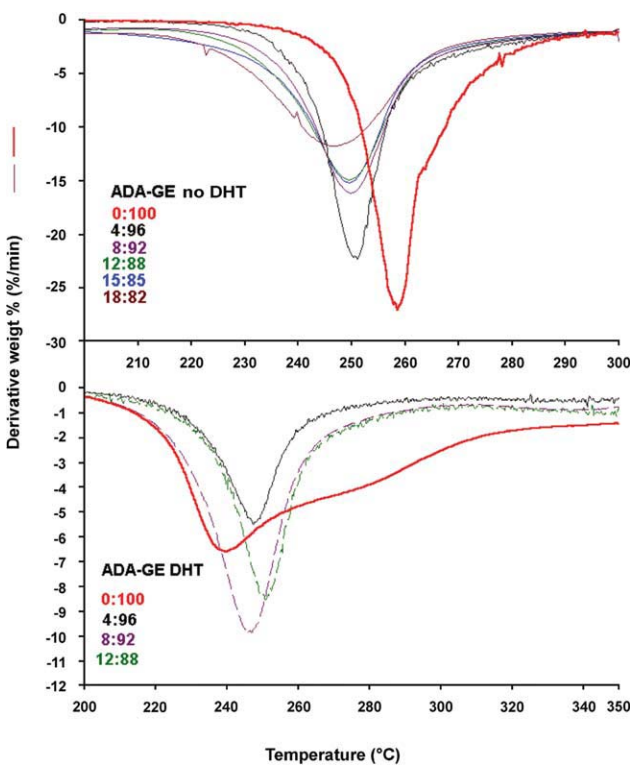

Figure 8 First-derivative TGA curves of pure GE (red curves) and the GE–ADA blends with the ADA-to-GE weight ratios listed in the legend with the same colors as the curves not dehydrothermally crosslinked (no DHT) and dehydrothermally crosslinked (DHT). The temperature scanning rate was 10°C/min. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II
Data from TGA Carried Out on the ADA–GE blends with Different ADA —COOH to GE —CH_2OH Ratios Not Subjected (Experiments 1–6) and Subjected (Experiments 7–10) to DHT

Experiment	Molar ratio	Weight ratio	Onset (°C)[a]	Maximum (°C)[b]	Δy_1 (%)	Δy_2 (%)	Δy_{tot} (%)
1	0 : 10	0 : 100	251	259	12	63	75
2	2 : 10	4 : 96	243	251	11	58	69
3	4 : 10	8 : 92	242	250	13	59	72
4	6 : 10	12 : 88	239	249	12	58	70
5	8 : 10	15 : 85	238	251	12	59	71
6	10 : 10	18 : 82	235	248	11	61	72
7	0 : 10	0 : 100	227	238	13	62	75
8	2 : 10	4 : 96	233	248	4	34	38
9	4 : 10	8 : 92	232	248	5	44	49
10	6 : 10	12 : 88	236	251	7	38	45

Δy_2 = percentage of the second weight loss. Temperature scanning rate: 10°C/min.
[a] Temperature at which the weight loss begins.
[b] Temperature of the maximum slope in the weight loss curve.

from that of about 37°C, found in the differential thermal analysis curve of the human skin stratum corneum and ascribed to a gel–liquid transition of the lipid bilayers.[17] Generally, the stability of the materials increased with ADA content; however, the high G' of that with a ratio of ADA —COOH to GE —CH_2OH equal to 10 : 10 indicated that this material may be too rigid for some medical purposes, for example, for use as a wound-healing membrane.

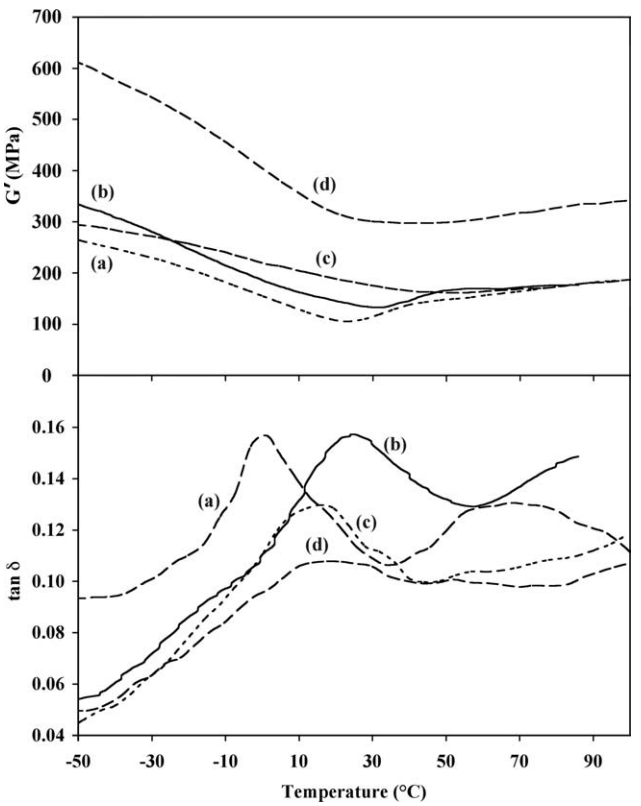

Figure 9 G' (upper graph) and tan δ (lower graph) versus temperature curves for (a) pure GE and for three GE–ADA blends crosslinked by DHT with (b) 6 : 10, (c) 8 : 10, and (d) 10 : 10 —COOH to —CH_2OH ratios. The temperature scanning rate was 5°C/min.

The swelling test, performed on DHT-crosslinked GE/ADA films, showed (Fig. 10) that the swelling degree was always lower than that of not crosslinked films; however, it showed good hydration ability. With regard to the behavior of the different samples, the best one seemed to be that of the material with a 6 : 10 ADA —COOH to GE —CH_2OH ratio. The reduced ability of the crosslinked films, with respect to those not subjected to DHT, to swell after 24 h of incubation in an atmosphere saturated with water vapor at 37°C (see Fig. 10) confirmed the increased stability of the materials. The poor decrease in the swelling with DHT observed for the pure GE was likely due to the steric hindrance for the ester bond formation between the acidic and alcoholic groups of GE. The best behavior, especially in view of the cell seeding, was shown by the samples having a ratio of ADA —COOH to GE

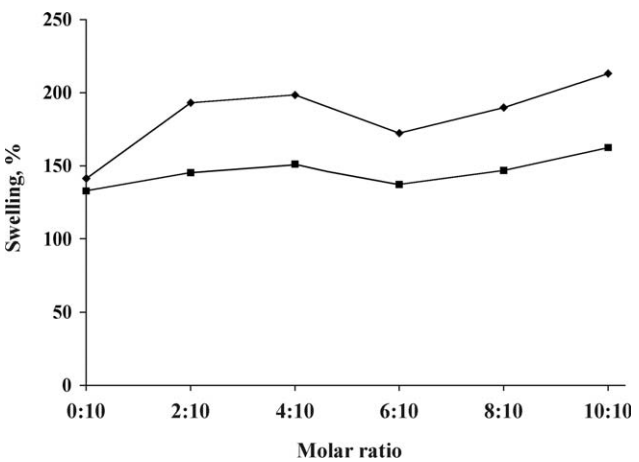

Figure 10 Swelling percentages after 24 h of incubation in an atmosphere saturated with water vapor at 37°C of pure GE and GE–ADA films with different ADA —COOH to GE —CH_2OH molar ratios: (◆) films not subjected to DHT and (■) films subjected to DHT. The values plotted are the mean of three determinations; their differences from the actual data ranged between 1 and 3%.

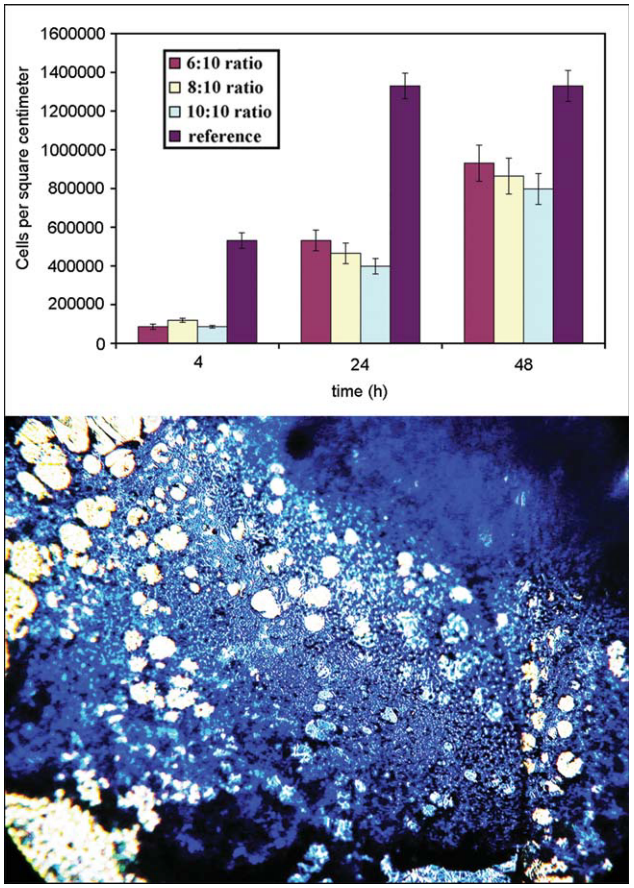

Figure 11 Upper graph: NIH-3T3 mouse fibroblasts adhesion and growth at different times after seeding for three DHT crosslinked GE–ADA films with different ADA —COOH to GE —CH_2OH molar ratios compared with a positive reference sample coated with type A porcine gelatin. Lower photo: Optical microscopy image of the film with a 6 : 10 molar ratio 48 h after seeding (actual area $\cong 2.4 \times 10^{-2}$ mm^2). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

—CH_2OH equal to 6 : 10, where the swelling of the film crosslinked by DHT was 35% lower than that of the not crosslinked one. However, the effect of DHT on the swelling was not of primary importance because the crosslinking was made mainly to prevent the collapse of the GE structure observed in GE–hydroxyapatite composites.[4]

The cell adhesion and growth on DHT-crosslinked GE–ADA films at different times is shown in Figure 11. The results indicate a good adhesion for every sample in comparison with the positive reference; a sensible increase in the cell density with time was observed, too; this indicated that the material stimulated cell proliferation. The decrease in the cell density with increasing ADA content confirmed that the best composition was the 6 : 10 ADA —COOH to GE —CH_2OH ratio. The optical image showed that the cellular adhesion visible 48 h after seeding was more evident on the matrix inside the meshes of the net present on the film surface.

CONCLUSIONS

Spectroscopic analysis helped us to ascertain the ability of DHT to covalently crosslink the GE–ADA blends and, thus, enhance the stability of the materials. The thermal analysis by both DSC and TGA also showed the greater stability of the crosslinked samples; however, DSC confirmed the presence of hydrogen bonds between ADA and GE in the uncrosslinked blends. The improved stability by DHT was also confirmed by both DMA and the swelling tests. The T_g values from the tan δ curves were not very dissimilar from that of the skin. The surface modification of the films induced by DHT seemed to direct the cellular adhesion. The fact that the crosslinked GE–ADA films were a good environment for the cellular adhesion and growth indicates that these materials might be used for the fabrication of scaffolds for tissue reconstruction.

The authors thank Giovanni Vozzi and Piero Narducci (Department of Chemical Engineering, Industrial Chemistry and Materials Science, University of Pisa) for carrying out cell cultures and obtaining SEM images, respectively, and for helpful discussions about the interpretation of the results.

References

1. Chandrasekaran, R.; Radha, A. Trends Food Sci Technol 1995, 6, 143.
2. Jagur-Grodzinski, J. React Funct Polym 1999, 39, 99.
3. Coviello, T.; Matricardi, P.; Marianecci, C.; Alhaique, F. J Controlled Release 2007, 119, 5.
4. Barbani, N.; Coluccio, M. L.; Guerra, G. D.; Krajewski, A.; Mazzocchi, M.; Ravaglioli, A. In Materials for Tissues Engineering, Chemistry and Microstructure: The Role for Ceramics, Proceedings of the 9th Periodical International Meeting and Seminar on Ceramics, Cells and Tissues, Faenza, Italy, 2004; Ravaglioli, A.; Krajewski, A., Eds.; ISTEC-CNR: Faenza, Italy, 2005; p 138.
5. Crescenzi, V.; Dentini, M.; Coviello, T.; Rizzo R. Carbohydr Res 1986, 149, 425.
6. Alhaique, F.; Santucci, E.; Carafa, M.; Coviello, T.; Murtas, E.; Riccieri, F. M. Biomaterials 1996, 17, 1981.
7. Chandrasekaran, R.; Millane, R. P.; Arnott, S.; Atkins, E. D. T. Carbohydr Res 1988, 175, 1.
8. Chandrasekaran, R.; Puigjaner, L. C.; Joyce, K. L.; Arnott, S. Carbohydr Res 1988, 181, 23.
9. Okamoto, T.; Kubota, K. Carbohydr Polym 1996, 30, 149.
10. Dulong, V.; Le Cerf, D.; Picton, L.; Muller, G. Colloids Surf A 2006, 274, 163, and references therein.
11. Scotchford, C. A.; Cascone, M. G.; Downes, S.; Giusti, P. Biomaterials 1998, 19, 1.
12. Tangsadthakun, C.; Kanokpanont, S.; Sanchavanakit, N.; Pichyangkura, R.; Banaprasert, T.; Tabata, Y.; Damrongsakkul, S. J Biomater Sci Polym Ed 2007, 18, 147.
13. Ferry, J. D.Viscoelastic Properties of Polymers; Wiley: New York, 1980.
14. Vozzi, G.; Flaim, C. J.; Bianchi, F.; Ahluwalia, A.; Bhatia, S. Mater Sci Eng C 2002, 20, 43.
15. Nishi, T.; Wang, T. T. Macromolecules 1975, 8, 909.
16. Seggiani, M.; Lazzeri, L.; Giusti P. J Mater Sci Mater Med 1994, 5, 877.
17. Bouwstra, J. A.; De Vries, M. A.; Gooris, G. S.; Bras, W.; Brussee, J.; Ponec, M. J Controlled Release 1991, 15, 209.