J Mater Sci: Mater Med (2008) 19:889–898
DOI 10.1007/s10856-007-3212-5

Genipin-crosslinked chitosan/gelatin blends for biomedical applications

Valeria Chiono · Ettore Pulieri · Giovanni Vozzi · Gianluca Ciardelli · Arti Ahluwalia · Paolo Giusti

Received: 25 October 2006 / Accepted: 6 June 2007 / Published online: 1 August 2007

Abstract Blends between chitosan (CS) and gelatin (G) with various compositions (CS/G 0/100 20/80, 40/60, 60/40 100/0 w/w) were produced, as candidate materials for biomedical applications. Different amounts of genipin (0.5 wt.% and 2.5 wt.%) were used to crosslink CS/G blends, promoting the formation of amide and tertiary amine bonds between the macromolecules and the crosslinker. The effects of composition and crosslinking on the physico-chemical properties of samples were evaluated by infrared analysis, thermogravimetry, contact angle measurements, dissolution and swelling tests. Mechanical properties of crosslinked samples were also determined through stress–strain and creep tests: samples stiffness increased with increasing the crosslinker amount and the CS content. Blend composition affected mouse fibroblasts adhesion and proliferation on substrates, depending on the crosslinker amount. Finally, crosslinked CS/G blends containing 80 wt.% G were found to support neuroblastoma cells adhesion and proliferation which made them promising candidates for uses in the field of nerve regeneration.

V. Chiono (✉) · G. Vozzi · G. Ciardelli · P. Giusti
Department of Chemical Engineering, Industrial Chemistry and Materials Science, University of Pisa, Via Diotisalvi 2, Pisa 56126, Italy
e-mail: valeria.chiono@ing.unipi.it

E. Pulieri · G. Vozzi · A. Ahluwalia
Centro "E. Piaggio", University of Pisa, Via Diotisalvi 2, Pisa 56126, Italy

G. Ciardelli
Department of Mechanics, Politecnico in Turin, Corso Duca degli Abruzzi 24, Turin 10129, Italy

P. Giusti
C.N.R. Institute for Composite and Biomedical Materials, Via Diotisalvi 2, Pisa 56126, Italy

Introduction

Tissue engineering generally makes use of biocompatible and biodegradable scaffolds with mechanical properties closely matched to those of the target tissue. A control of biological activity at a molecular level, which is a highly desired feature for a scaffold, can be obtained using natural polymers. Polysaccharides have recently received a great interest as materials for scaffold fabrication, since their carbohydrate moieties interact with or are integral component of many cell adhesion molecules and matrix glycoproteins [1]. Chitosan (CS) is a polysaccharide obtained from *N*-deacetylation of chitin and it is a copolymer of D-glucosamine and *N*-acetyl-D-glucosamine. CS was found to suppress the growth of fibrous cells and the formation of scabs, and to facilitate the growth of endothelium cells, capillary vessels and cardiac muscle cells [2]. Yuan et al. [3] showed that CS membranes and fibers supported the adhesion, migration and proliferation of Schwann cells (SCs) [4], whereas Itoh et al. [5] used hydroxyapatite-coated CS tubes adsorbed with laminin-1 or laminin peptides as scaffolds for peripheral nerve reconstruction. Blends between CS and a protein have been previously used for nerve repair, having similar mechanical properties to those of nerve tissues [6]. The in vitro evaluation of interactions between nerve cells and CS/protein (albumin, collagen) or CS/peptide (poly-L-lysine) substrates was performed using foetal mouse cerebral cortex cells and PC12 cells [7]: cell adhesion was higher for CS/poly-L-lysine membranes due to their positive surface charge, whereas cell differentiation levels were higher for

CS/collagen blend. In this work we produced blends between CS and gelatin (G), a protein derived from collagen, with the aim to obtain a material with suitable mechanical properties for the production of scaffolds, in particular for peripheral nerve regeneration. Gelatin is obtained from collagen by thermal denaturation or physical and chemical degradation. Gelatin has been widely used for tissue engineering applications due to its antigenicity, biodegradability and biocompatibility and has found uses in the fields of artificial skin, neuronal regeneration and bone grafts [8–10]. The main limitation of G for the production of tissue substitutes arises from its rapid dissolution in aqueous environments. Blending G with a synthetic or a natural polymer can provide a simple method for increasing the stability of G-based scaffolds in aqueous media and improving their mechanical properties [11, 12]. Chemical and physical crosslinking methods have also been used to increase G stability in aqueous media. Physical crosslinking methods for G include microwave energy [13], dehydrothermal treatment (DHT) [14, 15] and UV-irradiation [16, 17].

The main advantage of physical methods is that they do not cause potential harm, whereas their main drawback arises from the difficulty to obtain the desired crosslinking degree. Commonly used chemical crosslinkers include aldehydes (formaldehyde, glutaraldehyde, glyceraldehyde) [18], polyepoxy compounds [19] and carbodiimides [20].

The main limitation in the use of chemical crosslinkers arises from the presence of some unreacted crosslinker inside the scaffolds and from the risk of formation of toxic products by reaction between the substrate and the crosslinking agent during in vivo biodegradation. For this reason, much interest has been recently addressed toward naturally derived crosslinking agents, with a low toxicity. Genipin is a natural crosslinking reagent which has recently been used for its ability to crosslink CS and proteins containing residues with primary amine groups, particularly G and soy protein isolates [21]. Genipin is obtained from geniposide, an iridoid glucoside isolated from the fruits of *Genipa Americana* and *Gardenia jasminoides Ellis*. Genipin have found applications in herbal medicine [22], whereas the dark blue pigments obtained by the reaction of genipin with primary amines have been used in the fabrication of heat, pH and light resistant food dyes [23].

Genipin has been recently used as a crosslinking agent of CS particles for drug delivery [24–26] and of G conduits for peripheral nerve regeneration [27].

Studies have been carried out to compare genipin with other widely used crosslinking reagents such as glutaraldehyde, formaldehyde and epoxy compounds, which assessed genipin lower cytotoxicity and higher biocompatibility [28–31]. It has also been reported that genipin-fixed tissues display a comparable mechanical strength and resistance against enzymatic degradation as the glutaraldehyde-fixed tissues [32].

Crosslinking mechanism consists of two reactions, involving different sites on genipin molecule [33]. The first step is the nucleophilic attack of the genipin C3 carbon atom from a primary amine group to form an intermediate aldehyde group. The just formed secondary amine reacts with the aldehyde group to form a heterocyclic compound. The following step is a nucleophilic substitution reaction that involves the replacement of the ester group on the G molecule by a secondary amide linkage. The reaction is complicated by the oxygen radical-induced polymerization of genipin that occurrs once the heterocyclic compound has formed, giving the gel a blue colour.

In this work, we produced genipin-crosslinked CS/G blends with various weight ratios between blend components, with the aim to find out the optimal composition supporting nerve cells' adhesion and proliferation. We investigated the blends physico-chemical properties of the blends (using thermogravimetric analysis, TGA; Fourier transform infrared-attenuated total reflectance spectroscopy, FTIR-ATR; scanning electron microscopy, SEM) and their functional characteristics with reference to their potential application in tissue engineering (studied using wettability and cell culture tests). Mechanical properties (stress–strain and creep tests) of crosslinked samples were also measured in a dry state by means of a home-made apparatus based on an isotonic force transducer. Efficacy of genipin-crosslinking was evaluated by means of swelling and dissolution measurements carried out in media simulating physiological conditions. Fibroblasts adhesion tests performed on crosslinked cast films showed the biocompatibility of genipin-crosslinked samples.

Materials and methods

Materials

Chitosan derived from crab shell with 85% deacetylation degree was purchased from Aldrich. Type B gelatin from bovine skin (G; 75 Bloom gel strength; M_n = 62,260 g/mol) [34] was supplied from Aldrich. Genipin (GP) was purchased from Wako Chemicals. All commercially available solvents and reagents were of analytical grade and used without further purification.

Crosslinking of gelatin samples

Gelatin was dissolved in demineralised water at 50 °C, obtaining 10% (w/v) solutions. GP was added to G solutions at different weight percentages: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5% (w/w). Each mixture was kept at 50 °C under

moderate stirring until a gel started to form as suggested by visual inspection and by the viscosity increase of the mixture which caused a decrease of the effective rotation rate of the magnetic stirrer. Then, it was cast on a Petri dish and left drying at room temperature for 48 h. Dried samples were washed several times in demineralised water to remove GP residues. Crosslinked G samples with 0.5% (w/w) and 2.5% (w/w) GP contents were coded as: G-0.5 GP and G-2.5 GP, respectively.

Crosslinking of CS/G blends

One gram of CS was dissolved in a 1% (v/v) acetic acid (Aldrich) solution in water and its acid-insoluble fraction was removed by filtration. Gelatin was dissolved in demineralised water at 50 °C, obtaining a 10% (w/v) solution. Known amounts of the two solutions were poured together and stirred for 2 h at 50 °C. Blends containing 20 wt.%, 40 wt.%, 60 wt.% and 100 wt.% CS were produced and here referred to as CS/G 20/80, CS/G 40/60, CS/G 60/40 and CS, respectively.

GP was added to each solution at 0.5% (w/w) and 2.5 % (w/w) concentrations. Each mixture was kept under agitation at 50 °C till a gel started to form. Then, it was cast on a Petri dish and left drying at room temperature for 48 h. Dried cast films were neutralised with a 0.1 M NaOH (Merck) solution in demineralised water, rinsed several times in demineralized water until pH was neutral and then dried in a vented oven for 48 h at 37 °C. Crosslinked samples using 0.5% (w/w) and 2.5% (w/w) were respectively coded as CS-0.5 GP, CS/G 20/80-0.5 GP, CS/G 40/60-0.5 GP and CS/G 60/40-0.5 GP, and CS-2.5 GP, CS/G 20/80-2.5 GP, CS/G 40/60-2.5 GP and CS/G 60/40-2.5 GP.

Methods

Thermogravimetric analysis (TGA)

Thermal degradation was measured using a Perkin–Elmer TGA 6 Thermogravimetric Analyzer equipment under a nitrogen atmosphere. The experiments were performed at a 10 °C/min heating rate in the 30–800 °C temperature range.

Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)

The Fourier transform infrared attenuated total reflectance spectroscopy (FTIR-ATR) spectra were recorded at room temperature in a Perkin–Elmer System 1600 FTIR Spectrometer in the range 4000–600 cm^{-1} at a resolution of 4 cm^{-1}.

Contact angle measurements

Static contact angles of both crosslinked and uncrosslinked films were measured at room temperature in a KSV instrument equipped with a CAM 200 software for data acquisition. Sessile drop method was applied, using a 5 μL double distilled water droplet. For each angle reported, at least five measurements on different surface locations were averaged.

Swelling and dissolution tests

Film samples (1×1 cm^2) were weighed and then put in phosphate buffered saline (PBS, Sigma) at pH 7.4 at 37 °C. Swollen films were drawn at various times (1, 3, 6, 24, 48 h), dried superficially by gentle contact with a filter paper and weighed again. The swelling percentage was calculated as:

$$\%S_w = [(W_s - W_i)/W_i] \times 100 \quad (2)$$

where W_i and W_s are the sample weights before and after swelling, respectively. After drying at 37 °C for 48 h in a vented oven, samples were weighed again and the solubility percentage was calculated as:

$$\%\,S = [(W_i - W_d)/W_i] \times 100 \quad (3)$$

where W_d is the dried sample weights after the dissolution test. Each test consisted of three replicate measurements and result was expressed as an average value.

Mechanical properties

The tensile properties of GP-crosslinked CS/G films were measured using an isotonic transducer (model 7006, UGO Basile Biological Research Apparatus, Italy), where the applied force has a resolution of 1 mN. Test specimens were dry cast films with 2 cm length, 0.5 cm width and a height of around 100 μm. Images of each scaffold were recorded through an optical microscope (Olympus AX 70) equipped with a digital camera (ccD Camera HIRES, DTA; Italy), to obtain data on initial dimensions and geometry. The traction force was applied along the length of the samples. Three specimens for each composition were tested. The Young's modulus of each sample was calculated from the slope of the initial linear portion of the stress-strain curve.

Creep measurements were performed on samples of the same kind, using the same instrument as for tensile tests. In creep experiments, the load corresponding to 10% of the maximum elastic deformation of the specimens was 'instantaneously' applied to each sample and then kept constant for a period of 1 h, during which the developing strain $\gamma(t)$ was measured. After load removal, the recoverable strain was monitored for further 60 min (creep-recovery measurement).

The creep compliance $J(t)$, which is the normalised strain with respect to the applied stress, was plotted against time to study the creep and creep-recovery behaviour of samples.

Fibroblasts and neuroblastoma cells cultures

Cells adhesion and proliferation tests were performed on cast films, using NIH-3T3 mouse fibroblasts and S5Y5 neuroblastoma cells. Both types of cells were separately cultured in Dulbecco's modified Eagle's medium (DMEM; Cambrex, Italy) with high glucose, 10% fetal calf serum (Cambrex, Italy), 1% glutamine (Cambrex, Italy), penicillin (200 U/mL) (Cambrex, Italy) and streptomycin (200 μg/mL) (Cambrex, Italy). Typical seeding densities were about 100,000 cells/mL. After an appropriate washing and sterilisation cycle [35], samples were placed singly in a 24 well plate (Nunc, USA). Samples were precoated either with type A G (Sigma, Italy; 1 wt.% in PBS) in the case of fibroblasts, or with poly-L-lysine (Sigma; 0.1 wt.% in PBS) in the case of neuroblastoma cells. 1 mL of adhesion factor solution was added to each well, left in incubation for 1 h at 37 °C and then aspired. The measurement of cell adhesion was performed on three samples for each time (4, 24, 48 h for fibroblasts and 1, 2, 3 days for neuroblastoma cells). After each culture time, culture medium was removed and substrates with attached cells were rinsed with PBS. Attached cells were fixed by addition of 4% (v/v) formaldehyde (Sigma, Italy) solution in PBS for 10 min and then stained with Coomassie blue (Fluka, Italy) solution for 10 min. Samples were analysed under an optical microscopy (Olympus AX 70) 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 [36]. Both cells of a reference sample and cells seeded on scaffolds were examined for comparison to evaluate the effect of scaffold topography on cell growth. Poly-L-lysine and type A G were selected as a control, respectively for tests using neuroblastoma cells and mouse fibroblasts.

Statistical analysis

Student's t-test was performed for statistical evaluation of data, with $p < 0.05$ considered significant.

Results

Choice of GP optimum concentration

In a previous work [37], the water stability of CS/G blends was found to decrease with increasing the G content, which suggested that crosslinking was necessary for CS/G samples for long-term biomedical applications. The optimal GP content for CS/G blends was selected on the basis of crosslinking data for pure G, which is the blend component with higher water solubility. Figure 1 shows gelling time for G-genipin solutions as a function of the crosslinker amount. The gelling time decreased with increasing the crosslinker content from 0.5% (w/w) to 2.5% (w/w), then reaching a plateau. This finding suggested that crosslinking of G with higher GP amounts than 2.5% (w/w) did not affect significantly G gelling time and, therefore, its crosslinking degree. A GP amount equals to 2.5% (w/w) was identified as the optimal content for the crosslinking of CS/G blends. For comparison, CS/G samples were also crosslinked using 0.5% (w/w) GP.

Physico-chemical properties

Thermogravimetric analysis (TGA)

Decomposition traces of crosslinked and uncrosslinked CS/G samples displayed two weight loss phenomena: water evaporation at 50–150 °C and polymer pyrolysis in the 200–500 °C temperature range. Table 1 collects the maximum degradation rate temperatures (T_ds) of all samples, derived from the corresponding derivative termogravimetric (DTG) traces. Figure 2 shows exemplary DTG curves for crosslinked and uncrosslinked CS/G 60/40 samples. Gelatin samples were more stable than CS samples, both in a crosslinked and uncrosslinked form. DTG curves of uncrosslinked and crosslinked CS/G blends showed that pyrolysis of each component occurred separately: pyrolisis peaks of blend components were partly overlapped with values of T_ds similar to those of pure components. Values of T_d did not change significantly upon crosslinking. As a

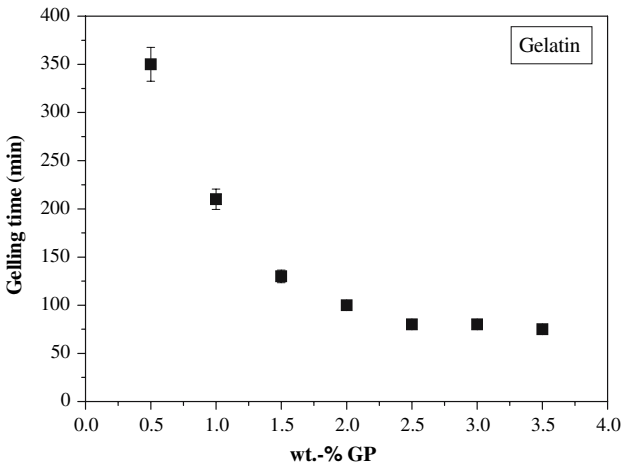

Fig. 1 Gelatin gelling time as a function of genipin content. Reported data are the averaged values on three measurements. Bars indicate standard deviations

Table 1 Maximum degradation rate temperature (T_ds) for crosslinked and uncrosslinked samples

Samples	T_d (°C)		
	Uncrosslinked	Crosslinked	
		0.5% (w/w)	2.5% (w/w)
CS	277–295	297.5	292
CS/G 60/40	281; (343)[a]	286.5; (345)[a]	291; 337
CS/G 40/60	(284)[a]; 336.6	290; 338	290; 336
CS/G 20/80	(282)[a]; 327	(280)[a]; 328	(280)[a]; 326
G	331	338	

[a] Values in brackets indicate the temperature of the "shoulder" of the recorded degradation peaks

conclusion, the use of 0.5% and 2.5% (w/w) genipin did not affect significantly the heat resistance of crosslinked samples.

Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)

Figure 3a, b show FTIR-ATR spectra of CS/G and CS/G-2.5 GP, respectivey. FTIR-ATR spectrum of CS showed typical adsorption bands at 3400–3500 cm^{-1} due to the partially overlapped characteristic bands of amine and alcohol stretching vibrations, at 1587 cm^{-1} and at 1414 cm^{-1} respectively due to the amide and amine bending vibrations and to O–H bending vibrations, at 1647 cm^{-1} due to amide C=O stretching vibrations. Bands at 1151 cm^{-1} (C–O–C asymmetric stretching) and at 1027 cm^{-1} (C–O stretching vibrations) are typical of CS saccharide structure [38].

The FTIR-ATR spectrum of G showed the characteristic bands of amide I and amide II, respectively at 1629 cm^{-1} and 1538 cm^{-1}, and that of amide III at 1238 cm^{-1}, caused by C–N stretching vibrations, amide N–H in-plane bending vibrations and CH_2 wagging vibrations [39]. Bands due to N–H and O–H stretching vibrations overlapped in the adsorption peak at 3282 cm^{-1}. FTIR-ATR spectra of CS/G blends showed the typical adsorption bands of each component.

FTIR-ATR spectra of crosslinked CS/G samples only showed slight variations with respect to those of uncrosslinked samples. More precisely, FTIR-ATR spectra of crosslinked samples displayed a slight increase of the ratio between the intensities of the adsorption bands at 1630–1640 cm^{-1} (amide C=O stretching vibrations) and at 1540–1550 cm^{-1} (amine and amide N–H bending vibrations). These findings, which were detected for samples crosslinked with a relatively high GP amount (2.5 wt.%), were attributed to the formation of amide and tertiary amine linkages between CS and G macromolecules, through the reaction of primary amine moieties with the crosslinker. FTIR-ATR spectra of CS/G-0.5 GP were substantially unchanged with respect to their uncrosslinked counterparts, probably due to their low crosslinking degree.

FTIR-ATR spectra of crosslinked and uncrosslinked CS samples were similar: this behaviour was attributed to the formation of a reduced number of crosslinks between CS macromolecules in the form of GP oligomers. CS crosslinking took place in a more acidic environment which slowed down the reaction times [26], favouring GP oligomerisation.

Contact angle analysis

Cell adhesion, which depends on various parameters, is known to be maximised on surfaces with an intermediate wettability (with 50–70° contact angle) [40]. The static

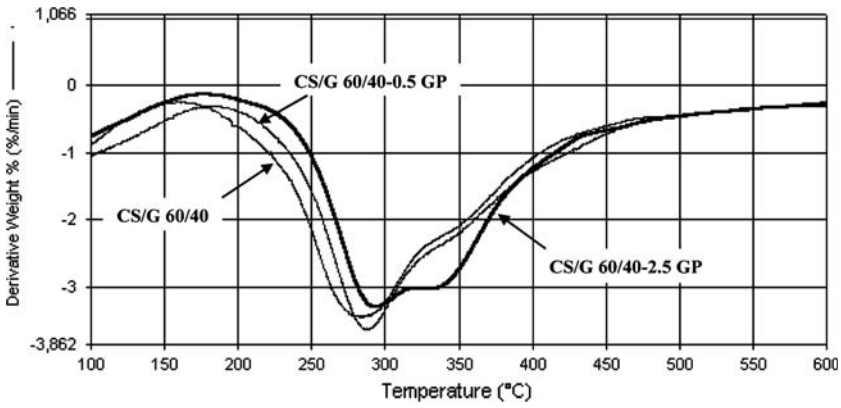

Fig. 2 Derivative thermogravimetric (DTG) curves for uncrosslinked and crosslinked CS/G 60/40 blends

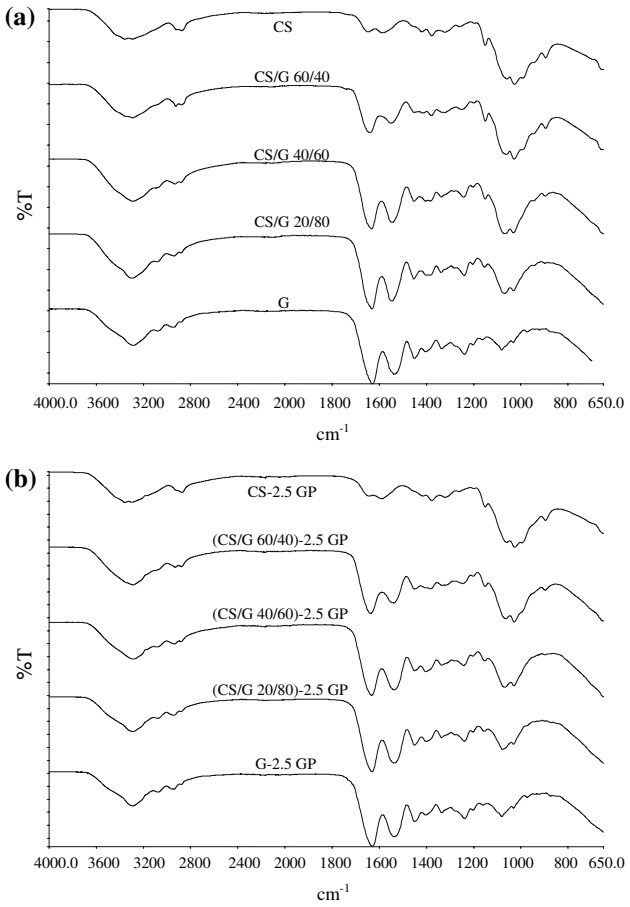

Fig. 3 FTIR-ATR spectra of CS/G (**a**) and CS/G-2.5 GP (**b**) blends

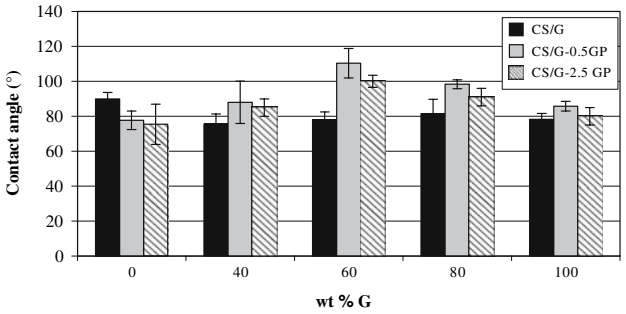

Fig. 4 Water contact angles for uncrosslinked and GP-crosslinked CS/G film samples. Column heights correspond to the mean values, whereas bars indicate standard deviations (n = 5).

water contact angles of both crosslinked and uncrosslinked CS, G and blend films are reported in Fig. 4. Uncrosslinked samples displayed a slightly hydrophilic behaviour with contact angles in the 78–90° range. Crosslinked CS samples showed a slightly higher hydrophilicity when compared to uncrosslinked CS samples ($p < 0.05$). This finding was in agreement with what reported by Jin et al. [24] and was attributed to the formation of a porous network: the more acidic conditions for CS crosslinking were supposed to slow down the kinetic of the second step (a nucleophilic substitution reaction) involved in the crosslinking mechanism, favouring the formation of GP oligomers [26]. Surface wettability of CS-0.5 GP and that of CS-2.5 GP samples were not significantly different ($p > 0.05$).

On the other hand, contact angles of crosslinked G and CS/G samples slightly increased with respect to their uncrosslinked counterparts, due to the decreased concentration of hydrophilic moieties, as a consequence of the crosslinking reactions. However, differences between the water contact angle of samples crosslinked either with 0.5 (w/w) or 2.5 (w/w) GP were not statistically significant ($p > 0.05$). Some GP-crosslinked blends were hydrophobic (CS/G 40/60-0.5 GP; CS/G 40/60-2.5 GP; CS/G 20/80-2.5 GP; CS/G 20/80-0.5 GP) or poorly hydrophilic (CS/G 60/40-2.5 GP; CS/G 60/40-0.5 GP) with contact angles close to 90°. Hydrophilicity of crosslinked blend samples slightly decreased with increasing the G amount: this behaviour could be attributed to the chemical properties of the formed GP oligomer crosslinks, which molecular weight presumably increased with increasing the CS content as a consequence of the decreasing pH of the reaction medium [26]. Samples with high CS content (40 wt.% G) were crosslinked in a medium with a lower pH, allowing the formation of a crossliked network with a more "open" structure, which was supposed to slightly reduce the hydrophobic behaviour of crosslinked blends.

Hydrophobic behaviour, generally, is not optimal for cell adhesion. Anyway cell attachment is affected also by a number of substrate surface characteristics, such as surface chemistry, charge density, roughness and morphology, which can counterbalance the effects of a not-optimal surface wettability.

Swelling and dissolution tests

Figure 5a, b reports the swelling and dissolution degrees as a function of time for uncrosslinked CS/G samples, respectively. Swelling and dissolution behaviour of uncrosslinked samples increased with increasing the G content: G and CS/G 20/80 samples dissolved within 1 h. The swelling degree of CS/G 60/40 and 40/60 blends displayed a maximum at 3 h (about 225 wt.%) and at 1 h (260 wt.%), respectively. The abscissa of the maximum of swelling data shifted toward lower times with increasing the G amount, due to the lower stability of G-rich samples. Dissolution degree of CS/G 60/40 and 40/60 increased with increasing time, reaching a value of 54 wt.% and 82 wt.%, respectively after 24 h ($p < 0.05$ CS/G 60/40 vs. CS/G 40/60). The stability of CS/G samples in aqueous media increased

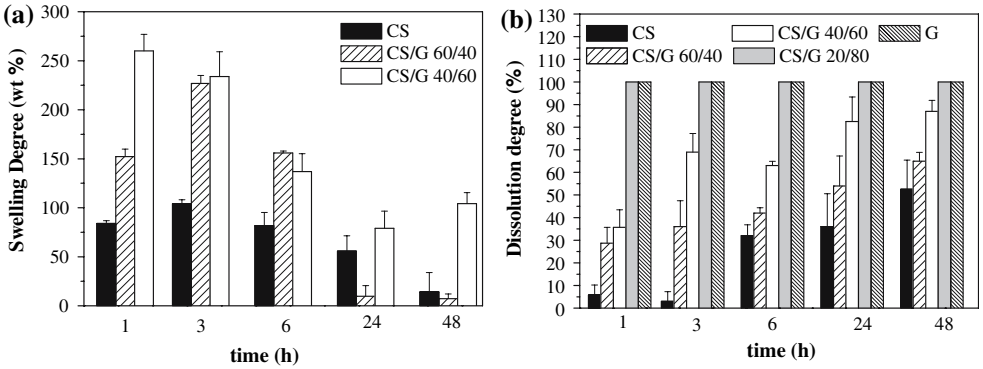

Fig. 5 Swelling (**a**) and dissolution (**b**) behaviour of CS/G samples as a function of time. Measurements were carried out in PBS at pH 7.4 at 37 °C. Data are averaged on three measurements. Bars indicate standard deviation ($n = 3$)

with increasing the CS amount, due to the insolubility of CS in media having a higher pH than 6.5. Differences between dissolution degrees of CS and CS/G 60/40 were not statistically significant after 24–48 h ($p > 0.05$). Figure 6a, b respectively shows the swelling and the dissolution degrees of crosslinked G film samples as function of the GP amount after 24 h. For lower GP contents than 2% (w/w), samples completely dissolved at a shorter time than 24 h. Swelling and dissolution degrees of G decreased with increasing the GP amount from 2.0% (w/w) to 2.5% (w/w) and kept substantially unchanged for higher GP contents than 2.5% (w/w). This result confirms that water stability of G (and therefore its crosslinking degree) did not vary appreciably using higher GP contents than 2.5% (w/w). Figure 7a, b reports swelling and dissolution degrees for crosslinked CS/G film samples after 24 h, respectively. The swelling degree of blends decreased with increasing the GP content from 0.5% (w/w) to 2.5% (w/w) ($p < 0.05$), whereas that of CS increased with increasing the GP amount ($p < 0.05$). These findings could be attributed to the prevalence of GP oligomerisation reactions for pure CS, due to the more acidic characteristics of the reaction environment [26]. A higher genipin oligomerisation degree led to a porous network with higher swelling properties. The dissolution degree of crosslinked CS samples was close to 0 wt.% after 24 h, independently from the GP amount. For CS/G-0.5 GP, dissolution degrees increased with increasing G amount, and were found to be much higher than those of the corresponding compositions crosslinked with 2.5% (w/w) GP ($p < 0.05$). The dissolution degree of CS/G-2.5 GP samples had a lower dependence on the G amount with respect to samples crosslinked with 0.5 (w/w) GP. Differences between dissolution degrees of CS/G 60/40-2.5 GP, CS/G 20/80-2.5 GP and G-2.5 GP were not statistically significant ($p > 0.05$). G-2.5 GP only lost 14.8 wt.% after 24 h immersion in PBS. In conclusion, crosslinking treatment using 2.5 wt.% GP allowed the obtainment of samples with higher water stability.

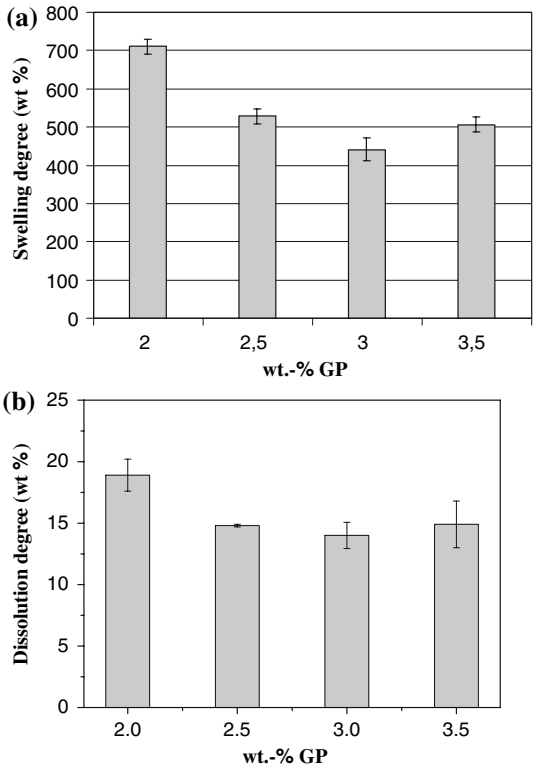

Fig. 6 Swelling (**a**) and dissolution (**b**) degrees for gelatin cast film samples as a function of the genipin amount. Measurements were carried out in PBS at pH 7.4, at 37 °C after 24 h. Column heights correspond to the mean values. Bars indicate standard deviations ($n = 3$)

Mechanical properties

Figure 8 shows the elastic moduli of GP-crosslinked samples in a dry state, as a function of G content and GP amount. At each composition, elastic moduli of samples increased with increasing the crosslinker amount, due to a higher crosslinking density which made samples stiffer. However, differences between the elastic modulus of samples crosslinked with 0.5 wt.% and 2.5 wt.% GP amount were statistically significant only for samples

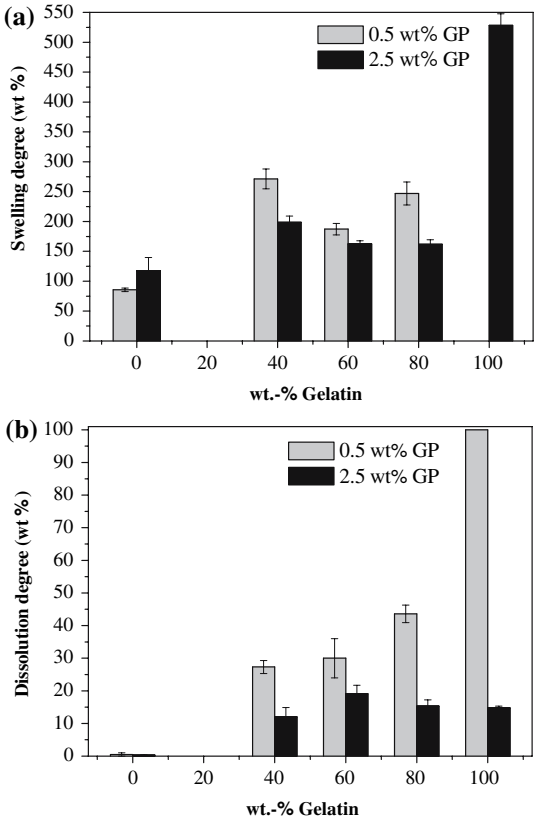

Fig. 7 Swelling (**a**) and dissolution (**b**) degrees of CS/G samples crosslinked with 0.5 and 2.5 wt.% GP as a function of composition. Measurements were carried out in PBS at pH 7.4, at 37 °C after 24 h. Column heights correspond to the mean values. Bars indicate standard deviations (n = 3)

containing 80 wt.% G. On the other hand, for blends crosslinked with the same GP amount, elastic modulus decreased with increasing the G content.

Figure 9 reports creep and creep-recovery curves for CS/G-0.5 GP samples normalised with respect to the applied stress (called compliance curves), as exemplary of the creep and creep-recovery behaviour of GP-crosslinked samples. Creep and creep-recovery curves for CS/G-2.5 GP blends were close to those of CS/G-0.5 GP blends and therefore they were not reported. Instantaneous compliance of the samples increased with increasing the G amount: this behaviour was in agreement with the Young modulus measurements which showed an increasing stiffness of the samples with increasing the CS amount.

Compliance slightly increased with time, especially for G rich samples. In particular, after a very short transient response (due to the viscoelastic properties of materials), the compliance settled to an approximately constant value, which was only slightly higher than the instantaneous compliance.

The load removal caused a quick decrease of the strain, which could be related to the elastic response of samples.

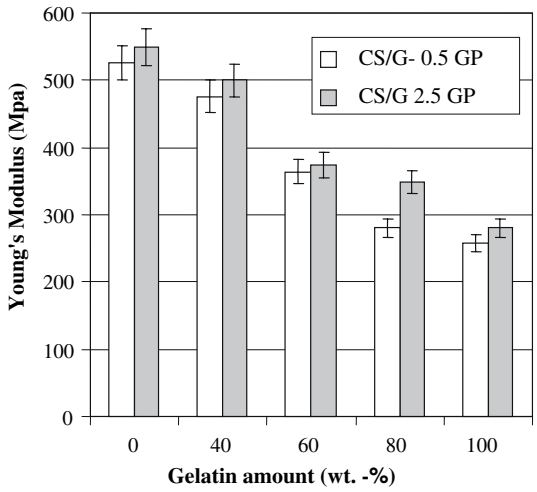

Fig. 8 Elastic moduli for GP-crosslinked CS/G samples in a dry state, as a function of composition and GP amount. Columns correspond to the average values, whereas bars indicate standard deviations (n = 3). For each composition, differences between the elastic moduli of samples crosslinked with 0.5 wt.% and 2.5 wt.% GP were not statistically significant, with the exception of blends containing 80 wt.% G ($p < 0.05$)

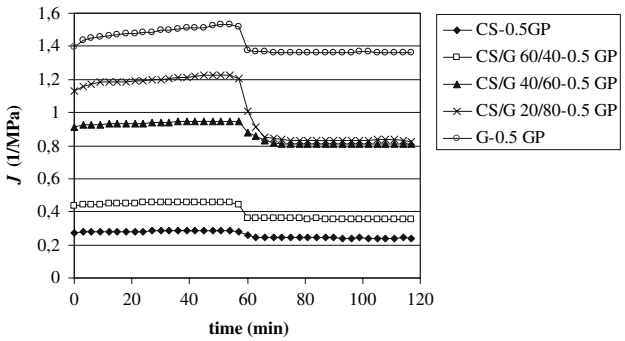

Fig. 9 Creep and creep-recovery behaviour of dry CS/G samples, crosslinked with 0.5 wt.% GP. Reported data are averaged on three measurements

The strain decreased with time and recovery was not complete after 1 h.

Cell culture tests

Figure 10a, b respectively shows cell density data for cast film samples crosslinked either with 0.5% (w/w) or 2.5% (w/w) GP at 4, 24 and 48 h, as compared to a G control layer. According to the results from dissolution tests, it is reasonable to assume that samples swelled, partially dissolved and mainly released G, during cell adhesion and proliferation tests. Swelling and dissolution behaviour of samples affected cells adhesion and proliferation.

For all samples, cell attachment increased with increasing cell culture time and was lower than for G control layer.

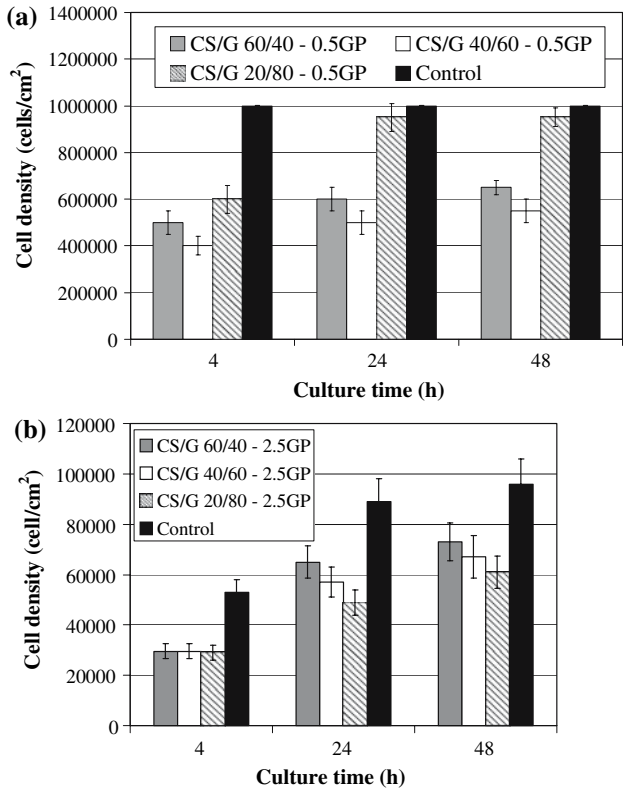

Fig. 10 Fibroblast density data for CS/G cast film samples crosslinked with 0.5 w/w (**a**) and 2.5 w/w GP (**b**) as a function of culture time. Column heights correspond to the mean values. Bars indicate standard deviation ($n = 9$). For CS/G-0.5 GP samples, cell density data were significantly different at each culture time ($p < 0.05$). For CS/G-2.5 GP samples, differences between cell density were significant only for CS/G 60/40-2.5 GP vs. CS/G 20/80-2.5 GP at 24–48 h ($p < 0.05$)

More precisely, cell density data for the different CS/G-2.5 GP blends were similar after 4 h, whereas they showed a slight decrease with decreasing CS content after 24–48 h. After 24–48 h, cell density on CS/G 60/40-2.5 GP and CS/G 40/60-2.5 GP samples, as well as that on CS/G 40/60-2.5 GP and CS/G 80/20-2.5 GP samples were not significantly different ($p > 0.05$). On the other hand, cell density of CS/G 60/40-2.5 GP blend significantly differed from that of CS/G 20/80-2.5 GP after 24–48 h.

For CS/G-0.5 GP blend substrates, cell density decreased with increasing G content from 40 wt.% to 60 wt.%, whereas its maximum value was found for CS/G 20/80-0.5 GP blend. Fibroblasts adhesion and proliferation was affected by surface chemistry of films (which depends on composition and processing) as well as by partial dissolution of poorly crosslinked film samples, which influenced surface composition, and interfered with cell attachment through G releases.

Cells tests using neuroblastoma cell line were only performed on CS/G blends crosslinked with a high GP amount (2.5 wt.%) as these experiments were preliminary to future trials in the field of the peripheral nerve regeneration, where scaffolds with a low degradation rate are usually required.

Neuroblastoma cells adhesion and proliferation was poor for CS/G 60/40-2.5 GP and CS/G 40/60-2.5 GP (data not shown), whereas good results were obtained for CS/G 20/80-2.5 GP samples (Fig. 11). The behaviour of a certain substrate towards cell adhesion and proliferation is affected by the selected cell line, as the mechanisms of adhesion and proliferation are cell-specific [41–43]. Once the biocompatibility of substrates has been assessed by fibroblasts attachment tests, experiments using specific cell lines may supply information about suitability of materials for the regeneration of a particular tissue. Neuroblastoma cells adhesion and proliferation tests showed that CS/G 20/80-2.5 GP scaffolds had the highest affinity towards nerve cells and could be suitable in the field of nerve repair. In the future, in vivo tests will be carried out concerning the use of CS/G 20/80-2.5 GP blend as an inner coating of guides for the repair of the peripheral nerve system.

Conclusions

In this work, genipin-crosslinked CS/G blends were produced with various compositions. Gelatin content had a strong influence on the physico-chemical properties of blends, mainly due to the decreased water stability of samples with increasing the G amount. Genipin crosslinking was expected to cause the formation of tertiary amide and ester bonds between macromolecules, which had an influence on many properties such as swelling and dissolution degrees, wettability and mechanical resistance. Crosslinking degree increased with increasing the GP amount, as suggested by the higher water stability and mechanical stiffness of the highly crosslinked samples.

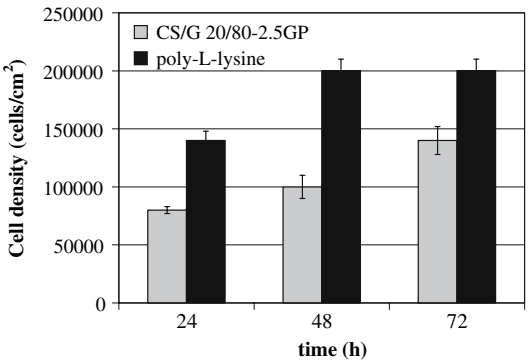

Fig. 11 Neuroblastoma cells density as a function of time for CS/G 20/80-2.5 GP as compared to poly-L-lysine. Column heights correspond to the mean values. Bars indicate standard deviations ($n = 9$)

GP crosslinking reduced wettability, dissolution and swelling degree of G and blend samples, whereas, for CS, the lower pH during crosslinking probably led to the formation of a porous crosslinked network with increased wettability and swelling behaviour. The formation of chemical bonds between macromolecules through crosslinking was assessed by FTIR-ATR analysis, only for the highest crosslinked blend samples (2.5 wt.%). Crosslinked samples were found to be biocompatible: in particular, blends containing 80 wt.% G well-supported neuroblastoma cells adhesion and proliferation, resulting optimal candidates for future trials in the field of peripheral nerve regeneration.

References

1. W. W. MINUTH, M. SITTINGER and S. KLOTH, *Cell. Tissue Res.* **291** (1998) 1
2. D. M. BARNES, *Science* **230** (1985) 1024
3. Y. YUAN, P. ZHANG, Y. YANG, X. WANG and X. GU, *Biomaterials* **25** (2004) 4273
4. R. P. BUNGE, *J. Neural.* **241** (1994) S 19
5. S. ITOH, I. YAMAGUCHI, M. SUZUKI, S. ICHINOSE, K. TAKAKUDA, H. KOBAYASHI, K. SHINOMIYA and J. TANAKA, *Brain Res.* **993** (2003) 111
6. M. CHENG, J. DENG, F. YANG, Y. GONG, N. ZHAO and X. ZHANG, *Biomaterials* **24** (2003) 2871
7. M. CHENG, W. CAO, Y. GAO, Y. GONG, N. ZHAO and X. ZHANG, *J. Biomater. Sci. Polym. Ed.* **14** (2003) 1155
8. F. -H. LIN, C. -H. YAO, J. -S. SUN, H. -C. LIU and C. -W. HUANG, *Biomaterials* **19** (1998) 905
9. P. -R. CHEN, M. -H. CHEN, J. -S. SUN, M. -H. CHEN, C. -C. TSAI and F. -H. -F. -H. LIN, *Biomaterials* **25** (2004) 5667
10. J. MAO, L. ZHAO, K. D. YAO, Q. SHANG, G. YANG and Y. CAO, *J. Biomed. Mater. Res.* **64A** (2003) 301
11. Y. HANG, S. ONYERI, M. SIEWE, A. MOSHFEGHIAN and S. V. MADIHALLY, *Biomaterials* **26** (2005) 7616
12. E. CHIELLINI, P. CINELLI, E. GRILLO FERNANDES, el-R. -S. KENAWAY and A. LAZZERI, *Biomacromolecules* **3** (2001) 806
13. M. A. VANDELLI, M. ROMAGNOLI, A. MONTI, M. GOZZI, P. GUERRA, F. RIVASI and F. FORNI, *J. Control. Release* **96** (2004) 67
14. V. YANNAS and A. V. TOBOLSKY, *Nature* **215** (1967) 509
15. H. UEDA, T. NAKAMURA, M. YAMAMOTO, N. NAGATA, S. FUKUDA, Y. TABATA and Y. SHIMIZU, *J. Control. Release.* **88** (2000) 55
16. M. M. WELZ and C. M. OFNER III, *J. Pharm. Sci.* **81** (1992) 85
17. L. MA, C. GAO, Z. MAO, J. SHEN, X. HU and C. HAN, *J. Biomater. Sci. Polym. Ed.* **14** (2003) 861
18. G. A. DIOGENIS, T. B. GOLD and V. P. SHAH, *J. Pharm. Sci.* **83** (1994) 915
19. T. MIYADA, T. TARA and Y. NOISHIKI, *Clin. Mater.* **9** (1992) 139
20. A. J. KUIJPERS, G. H. M. ENGBERS, J. KRIJGSVELD, S. A. J. ZAAT, J. DANKERT and J. FEIJEN, *J. Biomater. Sci. Polym. Ed.* **11** (2000) 225
21. A. BIGI, G. COJAZZI, S. PANZAVOLTA, N. ROVERI and K. RUBINI, *Biomaterials* **23** (2002) 4827
22. T. AKAO, K. KOBASHI and M. ABURABA, *Biol. Pharm. Bull.* **17** (1994) 1573
23. Y. S. PAIK, C. M. LEE, M. H. CHO and T. R. HAHN, *J. Agric. Food Chem.* **49** (2001) 430
24. J. JIN, M. SONG and D. J. HOURSTON, *Biomacromolecules* **5** (2004) 162
25. F. -L. MI, Y. -C. TAN, H. -F. LIANG and H. -W. SUNG, *Biomaterials* **23** (2002) 181
26. F. -L. MI, H. -W. SUNG, S. -S. SHYU, C. -C. SU and C. -K. PENG, *Polymer* **44** (2003) 6521
27. Y. -S. CHEN, J. -Y. CHANG, C. -Y. CHENG, F. -J. TSAI, C. -H. YAO and B. -S. LIU, *Biomaterials* **26** (2005) 3065
28. H. W. SUNG, R. N. HUANG, L. L. H. HUANG and C. C. TSAI, *J. Biomater. Sci. Polym. Ed.* **10** (1999) 63
29. C. C. TSAI, R. N. HUANG, H. W. SUNG and H. C. LIANG, *J. Biomed. Mater. Res.* **52** (2000) 58
30. H. W. SUNG, D. M. HUANG, W. H. CHANG, L. L. HUANG, C. C. TSAI and I. L. LIANG, *J. Biomater. Sci. Polym. Ed.* **10** (1999) 751
31. H. W. SUNG, D. M. HUANG, W. H. CHANG, R. N. HUANG and J. C. HSU, *J. Biomed. Mater. Res.* **46** (1999) 520
32. H. W. SUNG, R. N. HUANG, L. L. H. HUANG, C. C. TSAI and C. T. CHIU, *J. Biomed. Mater. Res.* **42** (1998) 560
33. M. F. BUTLER, Y. -F. NG and P. D. A. PUDNEY, *J. Polym. Sci. Part A: Polym. Chem.* **41** (2003) 3941
34. C. ABRUSCI, A. MARTIN-GONZALEZ, A. DEL AMO, T. CORRALES and F. CATALINA, *Polym. Degrad. Stability.* **86** (2004) 283
35. G. CIARDELLI, V. CHIONO, G. VOZZI, M. PRACELLA, A. AHLUWALIA, N. BARBANI, C. CRISTALLINI and P. GIUSTI, *Biomacromolecules* **6** (2005) 1961
36. G. VOZZI, C. J. FLAIM, F. BIANCHI, A. AHLUWALIA and S. N. BHATIA, *Mater Sci Eng.* **C20** (2002) 43
37. E. PULIERI, V. CHIONO, G. CIARDELLI, G. VOZZI, A. AHLUWALIA, C. DOMENICI, F. VOZZI and P. GIUSTI, *J. Biomed. Mater. Res. Part A*. (in press)
38. C. PENICHE, C. ELVIRA and J. S. ROMAN, *Polymer* **39** (1998) 6549
39. A. SIONKOWSKA, M. WISNIEWSKI, J. SKOPINSKA, C. J. KENNEDY and T. J. WESS, *Biomaterials* **25** (2004) 795
40. P. B. van WACHEM, T. BEUGELING, J. FEJIEN, A. BANTJES, J. P. DETMERS and W. G. van AKEN, *Biomaterials* **6** (1985) 403
41. J. M. BELLON, L. A. CONTRERAS, C. SABATER and J. BUJAN, *World J. Surg.* **21** (1997) 402
42. R. HAIGH, N. FULLWOOD and S. RIMMER, *Biomaterials* **23** (2002) 3509
43. G. CIARDELLI and V. CHIONO, *Macromol. Biosci.* **6** (2006) 13