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Preparation and characterization of chitosan/poly(vinyl alcohol) chemically crosslinked blends for biomedical applications

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ABSTRACT

In this study the development and characterization of novel polymer blends based on chitosan and poly (vinyl alcohol) and chemically crosslinked by glutaraldehyde for possible use in a variety of biomedical applications is reported. Mechanical properties of hybrids were evaluated by stress–strain tensile tests. Also, the microstructure, morphology and crystallinity of the blended hydrogels were characterized through X-ray diffraction, Fourier transform infrared spectroscopy and scanning electron microscopy analysis. Moreover, biocompatibility, cytotoxicity and cell viability were also performed by swelling test in simulated body fluid, MTT assay with cell culture. The relative crystallinity of pure chitosan film was reduced from approximately 23% to 18% when the polymeric network was reticulated by glutaraldehyde. A similar trend was also observed for PVA films. It was found that by increasing the chitosan content relative to PVA the swelling index of the blend has decreased from about 200% (pure PVA) to approximately 80% (50/50 chitosan/PVA), reflecting the reduction in the mobility of the polymer network and the hydrophilic behavior of the blend. The mechanical properties of the polymers were also significantly altered by changing blend composition and chemical crosslinking. Chitosan films have shown values of 50 MPa and 45% for maximum tensile strength and tensile elongation, respectively. It was verified that the average blend toughness has decreased about 40% by increasing the chitosan concentration from 25% (14.8 MJ/m^3) to 50% (8.9 MJ/m^3) related to a brittle characteristic of chitosan. In addition, the obtained results of elastic moduli were 0.1 GPa for PVA and 0.8 GPa for chitosan. The tested hydrogels clearly show adequate cell viability, non-toxicity and suitable mechanical properties which can be tailored to for potential use in skin engineering applications.

1. Introduction

Biomaterials for tissue engineering or drug delivery have seen numerous advances in recent years as researchers continue to develop and modify polymeric materials to meet the demanding needs of these biomedical applications. In this sense, many types of synthetic and natural polymers have been synthesized and employed as drug delivery vehicles or tissue scaffolding, roles that typically require precise and controllable polymer properties for successful function (Davis & Anseth, 2002; Hollister, 2005; Lavik, Klassen, Warfvinge, Langer, & Young, 2005; Wang, Williams, Yang, & Elisseeff, 2004a; Wang, Turhan, & Gunasekaran, 2004b). Degradable analogs of these biopolymers have received particular attention by reducing the need for device removal surgery. Recent results have demonstrated that an appropriate breakdown of the synthetic biomaterial can improve healing and increase tissue function, by controlled release of large molecular weight biomolecules when compared to non-degradable hydrogel constructs (Davis & Anseth, 2002; Drury & Mooney, 2003; Kim & Mooney, 1998). In addition to degradation, the ability to tailor the physical properties of degradable hydrogels polymers is an integral part of biopolymer design as properties such as fluid content, permeability, and mechanical strength have been shown to influence drug release, as well as cellular growth and function, critical parameters for successful tissue engineering or delivery applications (Burdick, Chung, Jia, Randolph, & Langer, 2005; Butler, Goldstein, & Guilak, 2000; Cowin, 2000; Lavik et al., 2005). In the continually expanding library of natural and synthetic polymers available for biomedical applications, block copolymers of chitosan (CHI) and poly(vinyl alcohol) (PVA) have emerged as one of the more promising biodegradable materials due to their highly controllable chemical and

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physical properties (Costa & Mansur, 2007; Mansur & Costa, 2008; Mansur, Oréfice, & Mansur, 2004; Muzzarelli & Muzzarelli, 2002; Peppas & Mongia, 1997).

Chitosan a [(1 → 4) 2-amino-2-deoxy-β-D-glucan] is obtained by alkaline deacetylation of chitin. Chitosan is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. The sugar backbone consists of β-1,4-linked D-glucosamine with a high degree of *N*-acetylation, a structure very similar to cellulose, except that the acetylamino group replaces the hydroxyl group on the C-2 position. Thus, chitosan is poly(*N*-acetyl-2-amino-2-dexoxy-D-glucopyranose), where the *N*-acetyl-2-amino-2-deoxy-D-glucopyranose (or Glu-NH_2) units are linked by (1 → 4)-β-glycosidic bonds (Hejazi & Amiji, 2003). Chitosan is degraded by enzymatic hydrolysis (Suh & Matthew, 2000), however its tensile strength and elasticity is not suitable for some biomedical applications such as wound dressing and skin tissue replacement. Chitosan joined to other polymers opened a window of research for altering or tailoring the property of interest. Blend systems with PVA hydrogel has been explored for medical and pharmaceutical application due to the advantage of non-toxic, non-carcinogenic and bioadhesive properties (Mansur et al., 2004). Chemical crosslinking is a highly versatile method to create and modify polymer nanostructure, where properties can be improved, such as mechanical, thermal, and chemical stabilities. In addition degradation is regulated (Mansur & Mansur, 2007).

The chitosan-based hydrogels present degradation kinetics apparently related to the degree of crystallinity which is controlled mainly by the degree of deacetylation (DD) (Suh & Matthew, 2000). PVA with different degrees of hydrolysis when blended with chitosan may produce hydrogels and hybrids with more flexible and less brittle aspect besides the biodegradability. Hence, by combining hydrophilic polymers of different origins, one synthetic (PVA) and the other natural (CHI) a class of hybrid organic–organic (O–O) network can be produced with properties not present in either one separately (Mansur & Costa, 2008). Arvanitoyannis and collaborators (Arvanitoyannis, 1999; Arvanitoyannis, Kolokuris, Nakayama, Yamamoto, & Aiba, 1997) have investigated chitosan-based blends with PVA using plasticizers (sorbitol and sucrose) to modify mechanical properties, for instance tensile strength and elongation. Furthermore, the effect of altering the ratio from chitosan to PVA has been extensively characterized with regard to the crystallinity and gas permeability of the blends produced.

This paper aims to prepare and comprehensively investigate the physical, chemical and mechanical properties of polymer blends based on chitosan/PVA and chemically crosslinked with glutaraldehyde. To our knowledge, this is the first report where such a system (PVA with very low degree of hydrolysis) was synthesized and extensively characterized by morphological, spectroscopic and mechanical aspects with different polymer contents, regarding to the hybrid network formation (polymer–polymer), swelling performance and also biocompatibility behavior. Moreover, cell viability was also investigated using an MTT reducing assay in order to evaluate the ability of these hybrids to support cell growth and proliferation.

2. Materials and methods

2.1. Materials

All salts and reagents used were of analytical degree and Milli-Q water was used in all solutions (18.0 MΩ). Poly (vinyl alcohol-co-vinyl acetate) (PVA) supplied from Sigma–Aldrich Chemical (Milwaukee, Wisconsin, USA) (Cat.#360627) with 80% degree of hydrolysis and molar weight MW = 9000–10,000 g/mol (Fig. 1A). Chitosan (Aldrich Chemical) powder, medium molecular weight, M_W = 161,000 g/mol, degree of deacetylation, DD = 75.6%, and viscosity 1406 m.Pas (1% in 1% acetic) were used without further purification (Fig. 1A). Glutaraldehyde (GA) or 1,5-pentane-dial (Aldrich Chemical) used as chemical crosslinking reagent was purchased as a 25% (wt%) aqueous solution.

2.2. Methods

2.2.1. Chitosan and PVA solution preparation

Briefly, PVA hydrogels were prepared by fully dissolving 5.0 and 10.0 g of polymer powder without further purification in 100 mL of Milli-Q water, under magnetic stirring, at temperature of 75 ± 2 °C, as previously reported by our group (Costa, Costa, Oréfice, & Mansur, 2007; Costa & Mansur, 2007; Mansur & Costa, 2008; Mansur et al., 2004). PVA 5% and 10% (wt%) solutions were let to cool down to room temperature and the pH was corrected to (2.00 ± 0.05) with 1.0 M HCl (Sigma).

Chitosan hydrogels (CHI) were produced in a similar procedure by fully dissolving 2.5 g in 250.0 mL of Milli-Q water with 2% of CH_3COOH (Sigma), under magnetic stirring for 48 h.

2.2.2. Chitosan, PVA and blends films preparation

Different quantities of PVA were added into the 1.0% chitosan solution to obtain chitosan/PVA molar ratios of (0:1), (1:3), (1:1), (3:1) and (1:0) and pH was corrected to (4.00 ± 0.05) with 1.0 M NaOH solution. The mixture was kept under stirring for 5 min until the PVA and chitosan completely formed a clear solution. Then, the crosslinker reagent (glutaraldehyde) was slowly added under constant stirring. The final concentration of glutaraldehyde in the gel solution precursors was 1% and 5% (wt%). Further in the sequence, the solution was poured into plastic moulds (polyethylene, round-plate shape, diameter = 85 mm, height = 10 mm) and allowed to dry for 72–120 h at room temperature (~25 °C) in the desiccator (silica gel replaced every 24 h) followed by additional 24 h in an oven at 40 °C. Chitosan/PVA samples chemically crosslinked were identified by (X:Y:Z) that is, X as chitosan content, Y as PVA content and Z as glutaraldehyde (wt%). For instance, sample identified as (1:3:1) represents the following proportion of reagents: 25% chitosan, 75% PVA and crosslinked with 1.0% GA (wt%). The hydrogel samples were cut into 5 mm × 5 mm pieces and soaked in simulated body fluid (SBF) solution at pH 7.4 for swelling and degradation assays. Samples were evaluated at 30 min, 2, 4, 24, 96 and 192 h. At the end of each soaking period, the remaining solution excess on the gels was wiped with a lint-free tissue paper, and dried at 40 °C in an oven for 24 h. The dried gel was stored in a desiccator before all following characterization procedures, such as Fourier transform infrared spectroscopy (FTIR), SEM and XRD.

2.3. Characterization

2.3.1. Qualitative assessment

Qualitative visual observations were made taking into account the solubility, miscibility and phase segregation of the blends. The average film thickness produced was assessed with a Mitotoyo (±10 μm) micrometer.

2.3.2. Scanning electron microscopy (SEM)

The morphology of the films obtained was assessed by scanning electron microscopy (SEM), (JSM 6360LV,JEOL/Noran), the microscope was attached to a dispersive energy spectrometer (EDS). The images were obtained using an accelerating voltage of 10–15 kV. Before examination the samples were sprayed with a fine layer of gold using a low deposition rate, refrigerated and placed at the maximum distance from the target to prevent damage to them.

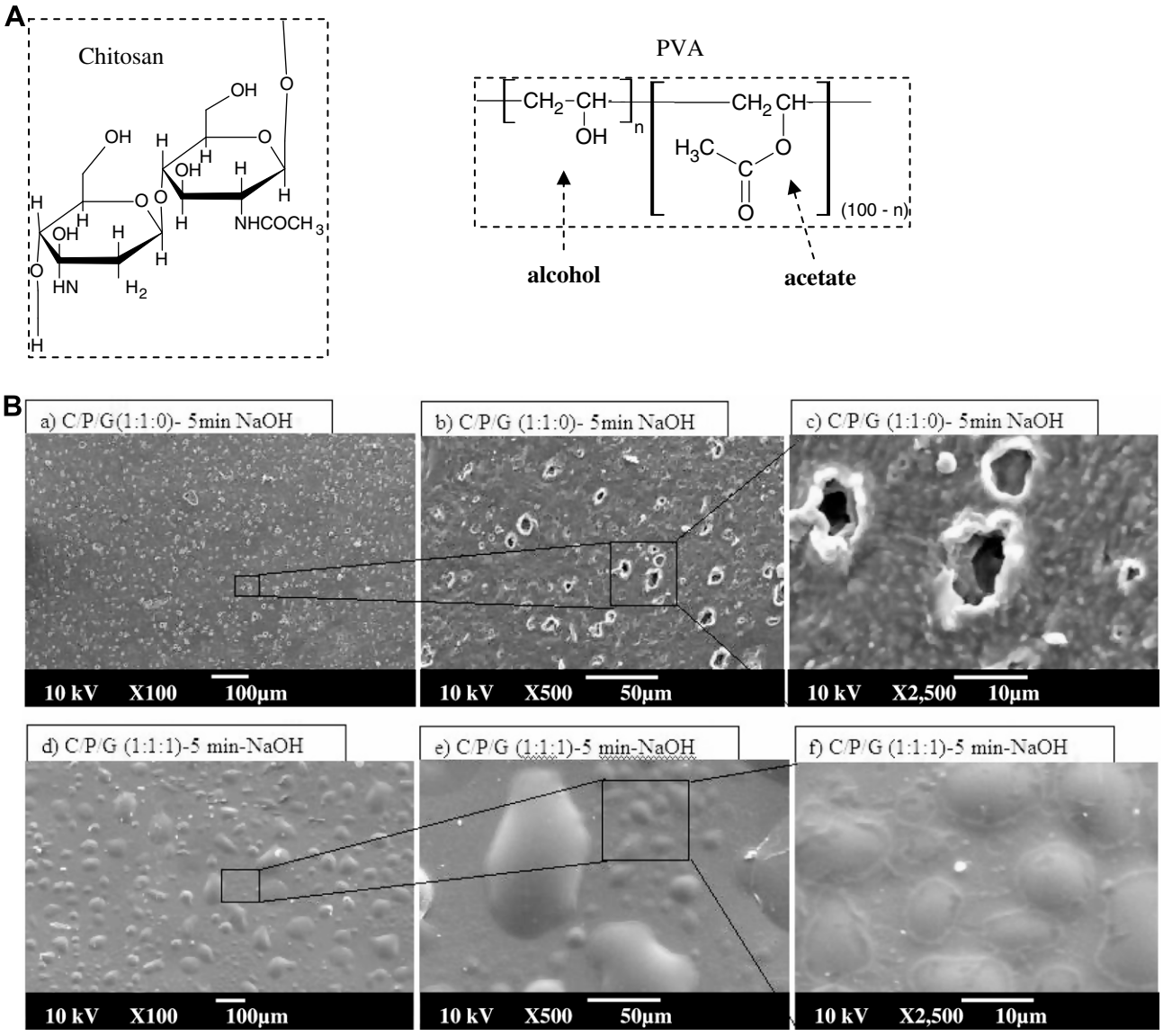

Fig. 1. (A) Chemical representation of structures of (a) chitosan and (b) PVA. (B) SEM images presenting PVA segregation in blends after 0.5 M NaOH immersion.

2.3.3. Fourier transform infrared spectroscopy (FTIR)

FTIR was used to characterize the presence of specific chemical groups in the hydrogels. CHI, PVA films and hydrogels blends crosslinked with GA (CHI/PVA/GA) were obtained as thick films (75 ± 25 µm) and analyzed by FTIR using ATR (attenuated total reflection) modes. FTIR spectra were obtained in the wavenumber range from 4000 to 650 cm^{-1} during 64 scans, with 2 cm^{-1} resolution (Paragon 1000, Perkin-Elmer, USA). The FTIR spectra were normalized and major vibration bands were identified associated with the main chemical groups.

2.3.4. Crystallinity by X-ray diffraction (XRD)

X-ray diffraction characterization (XRD) patterns were obtained from hydrogel CHI/PVA and PVA/Chi/GA samples prepared as crushed powders and films produced from pure polymer precursors of PVA and chitosan (PHILIPS, PW1710) using CuKα radiation with $\lambda = 1.54056$ Å (2θ range from 3.03° to 89.91°). PVA diffraction patterns were analyzed based on the monoclinic unit cell (Bunn, 1948) and chitosan patterns on the orthorhombic and monoclinic unit cell (Clark & Smith, 1936; Mazeau, Winter, & Chanzy, 1994; Ogawa, Yui, & Okuyama, 2004). Narrow peaks identified within the scan range were confirmed using previously published literature (Andrade, Barbosa-Stancioli, Mansur, Vasconcelos, & Mansur, 2006; Cho, Lyoo, Chvalum, & Blackwell, 1999; Costa & Mansur, 2008; Costa et al., 2008c; Mansur et al., 2004).

2.3.5. Mechanical properties of chitosan–PVA hydrogel characterization

The tensile strength of the hydrogel samples was determined using a uniaxial tensile testing device (EMIC DL 3000, Brazil) equipped with a 50 N load cell and the crosshead speed was 5 mm s^{-1}. The average value of the stress at break and elongation at break was set as the representative value. Each result was taken from 4 (replicates, $n = 4$) rectangular specimens with dimensions of 60 mm × 4 mm (length × width), according to ASTM D882-02 (2002) standard, cut from the films obtained by molding. The thickness of films produced were (75 ± 25 µm) depending on the blend composition, resulting on sample strips with average cross-section area of 0.3–0.5 mm^2. Samples used were previously inspected and any defect such as air bubbles, holes or tears and having average thickness variation superior than 5% were rejected.

2.3.6. Acellular biocompatible test in vitro

2.3.6.1. Swelling test. Fluid absorption studies are of great importance for a biodegradable material. For fluid-uptake measurements, all the specimens of the chitosan/PVA hydrogels with molar ratios of 0:1, 1:3, 1:1, 3:1 and 1:0 were prepared as described in the previous section, were weighed (W_0) before being immersed in SBF at 37 °C. After immersion for different time periods, the samples were carefully removed from the medium and, after wiping off water excess on the surface with filter paper, they were weighed for the determination of the wet weight (W_f) as a function of the immersion time (Mansur, Sadahira, Souza, & Mansur, 2008; Oréfice, Hench, & Brennan, 2000). SBF absorption (S) is given by using the Eq. (1):

$$S = \frac{W_f - W_0}{W_0} \times 100 \quad (1)$$

Each SBF absorption experiment was repeated three times and the average value was taken to validate the results.

2.3.6.2. Neutralization procedures. Phosphate buffered saline (PBS) was used in the procedure to neutralize any remaining cytotoxic groups of non-reacted glutaraldehyde crosslinker. The Chitosan/PVA films were immersed in polyethylene flasks with 75 mL solution without cells and with an area/volume ratio ranging from 0.5 to 1.0 cm^{-1}. The flasks were placed in an incubator with controlled temperature of 37 °C for 2.5 h. Later the samples were washed in de-ionized water, and dried at 40 °C for 48 h. All the samples submitted to the cytotoxicity experiment have been previously sterilized by exposure to saturated ethylene oxide vapour.

2.3.6.3. Viability of VERO cells. As previously reported by our group (Costa, Mansur, Barbosa-Stancioli, Pereira, & Mansur, 2008a; Costa, Oliveira, Oréfice, Mansur, & Pereira, 2008b; Costa et al., 2008c; Costa & Mansur, 2008), the cell viability was analyzed using 3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyl tretrazolium bromide (MTT) as a substrate. Briefly, 5×10^4 cells were seeded on matrices samples within a 96-well plate. The cells were incubated at 37 °C in humidified atmosphere containing 5% CO_2. After 24 h incubation, supernatant of each well was replaced with MTT diluted in serum free medium and the plates incubated at 37 °C for 4 h. After that, SDS 10%/HCl 0.04 N solution was added to the supernatant and plates were re-incubated for more 24 h and after extensive pipetation, 200 μL was transferred to a clean 96-well plate, where absorbance was measured at 595 nm using ASYS EXPERT PLUS spectrometric microplate reader. For analysis, all data were expressed as average ± standard deviation for number of 4 replicates ($n = 4$). One way ANOVA was used to access statistical significance of results. Post tests Dunnett multiple comparison test and Neuman–Keuls were carried out at a level of 95% significance.

3. Results and discussion

3.1. Qualitative assessment

The films were (75 ± 25 μm) thick and no heterogeneities were observed regarding to solubility, miscibility and phase segregation when the Chi/PVA blends with different proportions were visually inspected. Highly uniform yellowish optically transparent films were obtained.

3.2. Scanning electron microscopy (SEM)

The scanning electron microscopy images presented very similar morphological aspects for the Chi/PVA samples at different polymer ratios, showing the formation of uniform and continuous films. At higher magnification some scattered voids were verified (not shown), which are likely to be caused by a few phase separation that may have occurred due to different crosslinking kinetics of chitosan compared to PVA (Costa & Mansur, 2008; Don, King, Chiu, & Peng, 2006). In order to have a more in-depth understanding on that, Chi/PVA (1:1) samples were etched in alkaline medium (NaOH, 0.5 M) for 5 min, before and after chemical crosslinking aiming to preferentially remove the PVA not crosslinked from blend. In fact, that actually has occurred as can be observed from SEM results shown in Fig. 1. Blends not crosslinked (Fig. 1B-a–c) clearly indicated material removal by etching solution, leaving voids and holes on the structure. Even though, at high pH (~13.5) and short immersion time (~5 min) some negligible alkaline etching of crosslinked PVA may occur due to acetal covalent bonds cleavage. On the other hand, the Chi/PVA (1:1) samples crosslinked by glutaraldehyde (Fig. 1B-d–f) did not show any evidence of surface holes formation or material leaching, nevertheless some effect of phase segregation was detected with "droplet-like" forms onto these Chi/PVA blends after etching. In summary, it is suggested that polymers, PVA and chitosan, prior to chemical crosslinking have their chains mostly physically entangled in the hydrogel network, but formed a chemically bonded hydrogel after glutaraldehyde crosslinking has taken place (Wang et al., 2004a, 2004b).

3.3. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used to assess the polymer chemical groups (chitosan and PVA) and investigating the formation of crosslinked networks from the blends with glutaraldehyde. Fig. 2A shows the FTIR spectra relative to the chitosan, PVA and [chi/PVA] blends. Fig. 2A–a spectrum of pure chitosan shows peaks around 893 and 1156 cm^{-1} corresponding to saccharide structure (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996; Wang et al., 2004a, 2004b). In spite of several peaks clustering in the amide II peak range from 1510 to 1570 cm^{-1}, there were still strong absorption peaks at 1658 and 1322 cm^{-1}, which are characteristic of chitosan and have been reported as amide I and III peaks, respectively. The sharp peaks at 1383 and 1424 cm^{-1} were assigned to the CH_3 symmetrical deformation mode. The broad peak at 1030 and 1080 cm^{-1} indicates the C—O stretching vibration in chitosan. Another broad peak at 3447 cm^{-1} is caused by amine N—H symmetrical vibration, which is used with 1650 cm^{-1} for quantitative analysis of deacetylation of chitosan. Peaks at 2800 and 2900 cm^{-1} are the typical C—H stretch vibrations (Brugnerotto et al., 2001; Costa & Mansur, 2007, 2008; Rao, Naidu, Subha, Sairam, & Aminabhavi, 2006; Shigemasa et al., 1996; Wang et al., 2004a, 2004b). The IR spectra of the CHI/PVA blended films presented in Fig. 2A–b, Fig. 2A–c and A–d are different from that of the chitosan because of the ionization of the primary amino groups. There are two distinct peaks at 1408 and 1548–1560 cm^{-1}. Formation of the 1548–1560 cm^{-1} peak is the symmetric deformation of $-NH_3^+$ resulting from ionization of primary amino groups in the acidic medium whereas the peak at 1408 cm^{-1} indicates the presence of carboxylic acid in the polymers. The peaks at 1700–1725 cm^{-1} are characteristic of the carboxylic acid. In the present study, the presence of carboxylic dimmer was due to the acetic acid used for dissolving the chitosan (Wang et al., 2004a, 2004b). The peak at 1210–1300 cm^{-1} is due to the C—H vibration. Hence, there is a significant reduction of intensities from the main absorption bands related to chitosan, for instance amine region (1500–1650 cm^{-1}), as its content was decreased from 100% (pure chitosan, Fig. 2A–a), 75% (Fig. 2A–b), 50% (Fig. 2A–c), 25% (Fig. 2A–d) and 0% (pure PVA, Fig. 2A–e). The FTIR spectrum of pure PVA is shown in Fig. 2A–e, where all major peaks related to hydroxyl and acetate groups were observed. More specifically, the broad

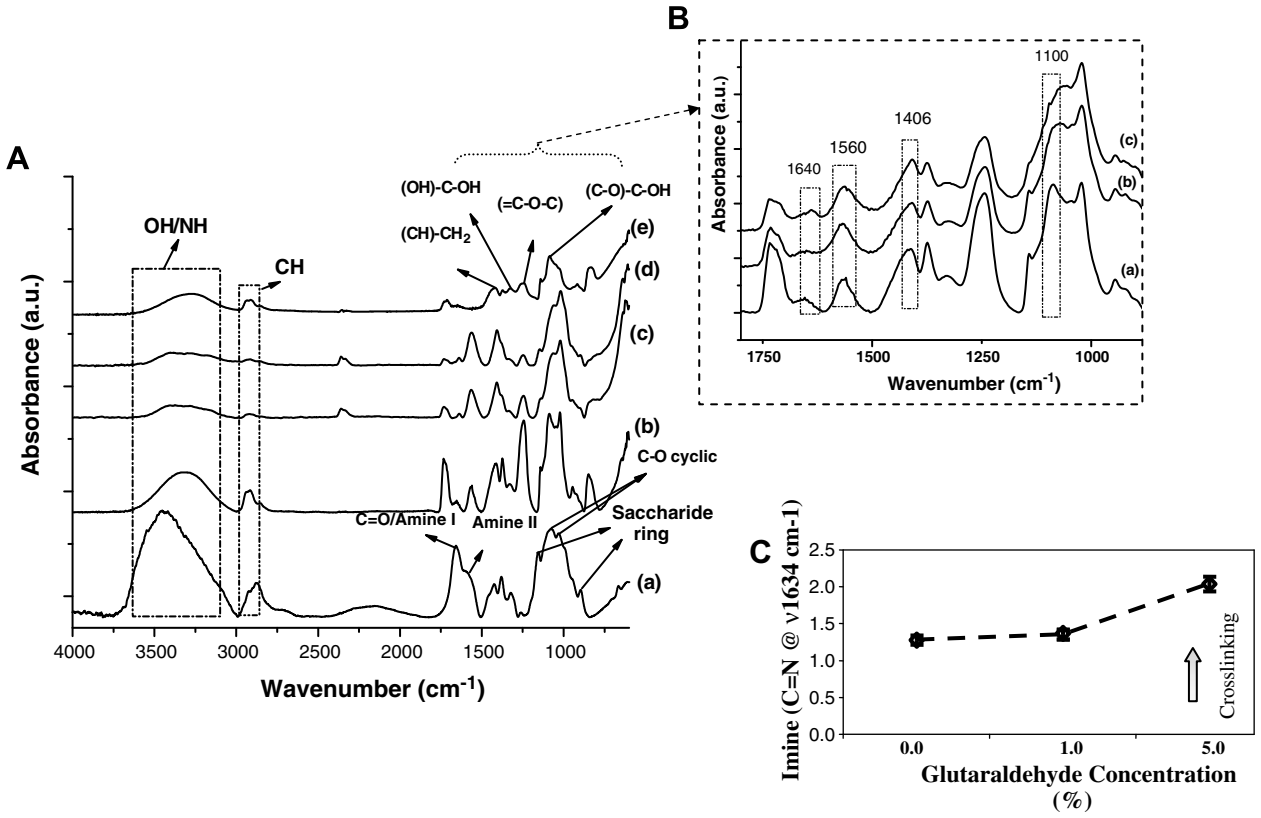

Fig. 2. (A) FTIR spectra of the chitosan (a), C/P/G(1:3:0) (b), C/P/G(1:1:0) (c), C/P/G(3:1:0) (d) and PVA (e) bands without chemical crosslinking. (B) FTIR spectra of the C/P(1:3) bands without (a) and chemical crosslinking with 1% (b) and 5% (c) GA. (C) Evolution of vibration band from imine group (C=N) with the concentration of glutaraldehyde.

band observed between 3550 and 3200 cm^{-1} is associated with the stretching O—H from the intermolecular and intramolecular hydrogen bonds. The vibrational band observed between 2840 and 3000 cm^{-1} refers to the stretching C—H from alkyl groups and the peaks between 1750 and 1735 cm^{-1} are due to the stretching C=O and C—O from acetate group remaining from PVA (saponification reaction of polyvinyl acetate) (Mansur & Costa, 2008; Mansur et al., 2008; Suh & Matthew, 2000).

Fig. 2B shows the FTIR spectra of CHI/PVA blend with a proportion of 25% Chitosan and 75% PVA (curve-a), at two concentrations of GA chemical crosslinker, 1% (curve-b) and 5% (curve-c). It can be noted the bands at 1110, 1406, 1638 and 1650 cm^{-1} mainly associated with PVA, and also the presence of peaks related to carboxylic acid and the imines formed by the crosslinking reaction by glutaraldehyde of amine groups from chitosan. Moreover, an increase in the intensity and a shift in the band associated with the bend vibration of the CH_2 (1406 cm^{-1}) group was observed. As expected, because the blend crosslinking reaction was conducted at pH (4.00 ± 0.05), covalent chemical bonds have preferentially occurred in the chitosan amine groups and less in the PVA hydroxyl groups (Brugnerotto et al., 2001; Costa & Mansur, 2007; Mansur & Costa, 2008; Rao et al., 2006; Shigemasa et al., 1996; Wang et al., 2004a, 2004b). Chemical crosslinking of the chitosan/PVA blends can be explained by the Schiff base formation as verified by the 1634 and 1550 cm^{-1} bands associated with the C=N and NH_2 groups, respectively (Costa & Mansur, 2008; Rokhade, Patil, & Aminabhavi, 2007; Wang et al., 2004a, 2004b). All chitosan-derived blends have shown a relative increase on their imine (—C=N—) band (ν = 1634 cm^{-1}) and simultaneous drop on the amine (—NH_2) band after chemical crosslinking with glutaraldehyde. The imine group was formed by the nucleophilic reaction of the amine from chitosan with the aldehyde. Fig. 1C shows the evolution of imine groups as the glutaraldehyde concentration is increased.

The PVA reaction with GA resulted in significant alterations in the bands regarding to hydroxyls (O—H), normally associated with the acetal bridge formation (Mansur et al., 2008).

3.4. Crystallinity by X-ray diffraction

The chitosan diffractogram (Fig. 3A–a) shows two peaks, one of high intensity at 19.8° and one of low intensity at 37.7° resulting from the crystalline phase, which were associated with the reflections (200) and (314), respectively. Also, a broad region under these peaks ranging from approximately 8° to 80° is related to the predominant amorphous phase. Hence, the crystallinity was estimated by calculating the ratio between these two areas, that is approximately 17%. These finding are endorsed by the literature (Arvanitoyannis, 1999; Ogawa, Yui, & Miya, 1992; Okuyama, Noguchi, Hanafusa, Osawa, & Ogawa, 1999), chitosan presents an orthorhombic unit cell of dimensions a = 0.826 nm, b = 0.95 nm and c (fiber axis) = 1.043 nm with 2-fold helical chains stabilized by hydrogen bond with the *gauche-trans* orientation. There are direct hydrogen bonds between adjacent chains along the a-axis, which makes a sheet structure parallel to the ac-plane and no hydrogen bond found between the sheets. There were two antiparallel chains per unit cell and no water of crystallization (Mazeau et al., 1994). In summary, chitosan and chitosan-derived networks usually exhibit a semi-crystalline structure due to free-energy balance caused by hydrogen bonding formation. The PVA GH = 80% diffraction pattern (Fig. 3A–b) presented three major peaks characteristic of polymer crystallinity at 19.35° (strong), 22.47° (medium) and 40.28° (weak). On the contrary, under these peaks, ranging from approximately 10° to 75°, the broad amorphous region was evidenced.

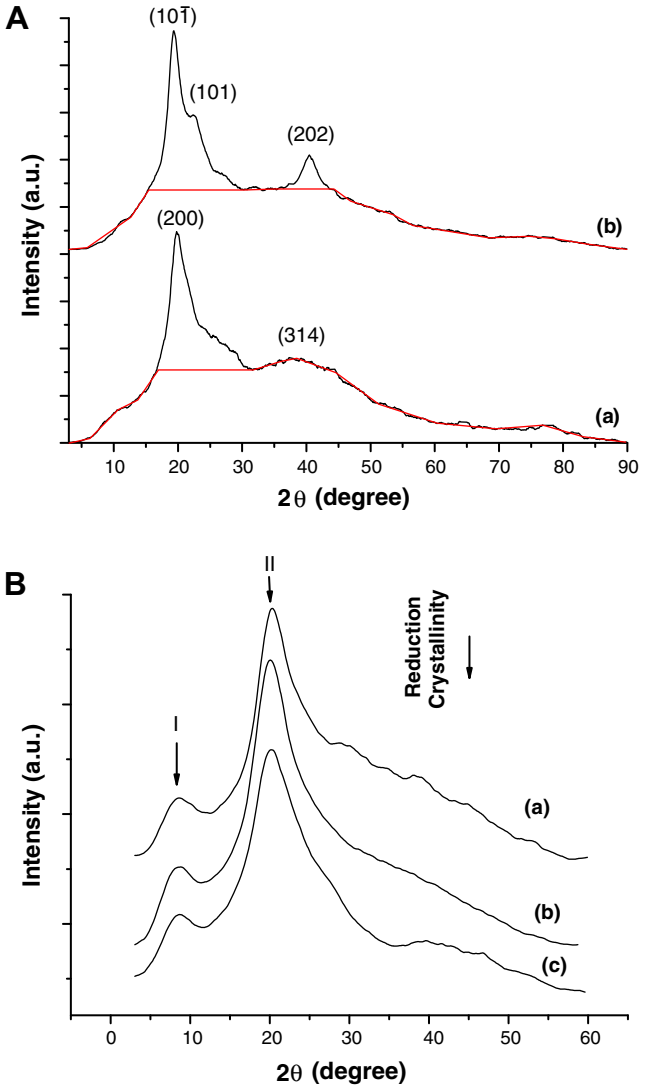

Fig. 3. (A) XRD patterns of (a) chitosan and (b) PVA films. (B) Diffractogram of pure chitosan (a), and crosslinked with GA (1% and 5%) (b) (c) respectively.

The average crystallinity of pure PVA film was estimated at 23%. Again, these results are in agreement with previous investigation of similar polymeric system, where sharp crystalline reflections, with a strong maximum reflections at ($2\theta = 19.4°$) and a shoulder at ($2\theta = 20°$), typical of crystalline atactic PVA, were reported (Ricciardi, Auriemma, Rosa, & Lauprétre, 2004). The diffraction pattern indicates that the sample was organized in the PVA semi-crystalline structure. That means, crystalline domains characterized by chains in a trans-planar conformation, packed in a monoclinic unit cell with $a = 0.781$ nm, $b = 0.252$ nm (chain axis), $c = 0.551$ nm and $\beta = 91.42°$ (Bunn, 1948).

Fig. 3B shows the diffractogram of pure chitosan and chitosan films reticulated with GA. The areas under peaks I and II decreased slightly by raising the GA chemical crosslinking concentration. The average crystallinity index (ratio of the areas) of pure chitosan films of approximately 23% (Fig. 3B–a) was reduced to about 18% when the polymer blends were reticulated by 1.0% and 5.0%, (Fig. 3B–b) and (Fig. 3B–c). So, the crosslinking inhibits close packing of the polymer chains by reducing the degree of freedom in the 3-D conformation, limiting or even preventing the formation of crystalline regions (Beppu, Vieira, Aimoli, & Santana, 2007). This reduction of crystallinity would play a crucial role on influencing the blend degradability, water absorption and swelling, as it is endorsed by results in following sections.

3.5. Mechanical properties of chitosan–PVA hydrogel

Fig. 4 shows the tensile strength response recorded for samples at different [chi/PVA] ratios, pure PVA (Fig. 4a), C/P/G(1:3:0) (Fig. 4b), C/P/G(1:1:0) (Fig. 4c), C/P/G(3:1:0) (Fig. 4d) and pure chitosan (Fig. 4e). As expected, it was observed that the blends presented intermediate mechanical properties between the pure components. Table 1 summarizes the overall experimental results for mechanical properties evaluated for chitosan/PVA blends. In addition, the obtained results of elastic moduli were 0.1 GPa for PVA and 0.8 GPa for chitosan. Except for the C/P/G(1:1:0), made of equal molar fraction from chitosan and PVA (50:50), that has performed unexpectedly different (Fig. 4c), which is most probably related to a phase segregation that has occurred during the drying process, as reported by other researchers (Don et al., 2006). Chitosan films have indicated values of 49.6 MPa and 44.9% for maximum tensile strength and tensile elongation, respectively. The result of tension is higher than that published by Chen and Hwa (1996), which have found tensile strength value of 23 MPa. The difference is attributed to the processing during hydrogel preparation such as pH and drying temperature, in the present work pH 4.0 and dried at room temperature compared to Chen's pH 4.5 and dried at 50 °C. In addition the membrane formation time used in this work was shorter than the process used in the present work. Longer film formation time, such as carried in our study, tend to favor molecule re-arrangements as the drying process occurs, resulting on the formation of a more ordered structure, increasing the crystallinity, thus raising the maximum tension. Thus, it is important to point out that some processing parameters, for instance the degree of deacetylation of chitosan, solution pH, solvent drying profile, use of plasticizer and blending procedure, will interact to influence the overall mechanical properties. This will explain the wide variations found in tensile strength and tensile elongation ranging from 1.4 to 57.2 MPa and from 3.5% to 115%, respectively (Arvanitoyannis et al., 1997; Bahrami, Kordstani, Mirzadeh, & Mansoori, 2003; Chen & Hwa, 1996; Liu et al., 2005; Rao, Sridhar, Wey, & Krishnaiah, 2007). In summary, as far as mechanical property is concerned, the results obtained for the Chi/PVA developed in this research is suitable for potential application as skin replacement,

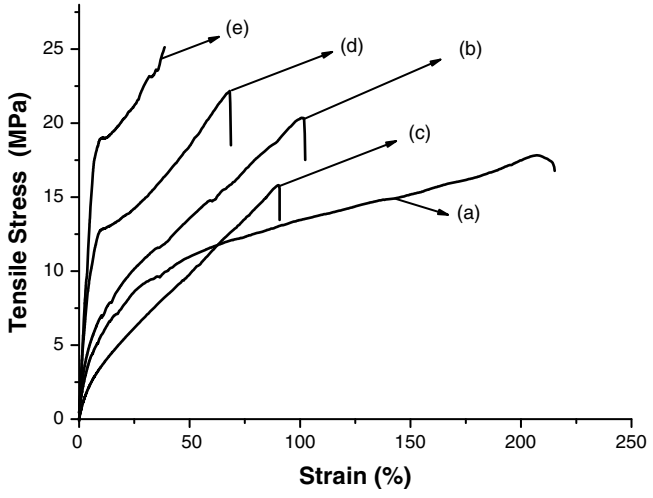

Fig. 4. Mechanical properties (Tensile strength × Strain) of Chitosan/PVA blends without chemical crosslinking; (a) pure PVA, (b) Chi/PVA (1:3), (c) Chi/PVA (1:1), (d) Chi/PVA (3:1) and (e) pure Chitosan films.

Table 1
Mechanical properties of chitosan (C)/PVA (P) blends.

[GA]	C/P(0:1)		C/P(1:3)		C/P(1:1)		C/P(3:1)		C/P(1:0)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Tensile strength (MPa)										
0	20.0	2.9	18.0	1.6	17.3	3.7	25.3	5.1	49.6	9.5
1	17.1	2.2	13.3	2.6	12.2	3.0	21.8	4.4	33.9	2.2
5	18.4	1.9	10.1	1.5	12.7	2.4	17.0	4.7	28.0	2.4
Strain (%)										
0	248	29	107	7	100	8	83	9	45	5
1	269	28	30	7	48	12	17	3	34	8
5	254	19	30	9	36	9	6	3	17	7
Toughness (MJ/m^3)										
0	34.2	7.2	14.8	6.9	8.9	3.1	14.0	4.1	16.8	3.0
1	30.5	5.4	2.9	1.2	2.4	1.7	1.6	1.3	8.4	3.8
5	27.4	4.3	1.9	0.7	3.4	1.3	1.4	1.3	3.8	1.7

which has typical values in the range of 2.5–16 MPa (Bahrami et al., 2003; Cervera et al., 2004; Xu, Wen, Lu, & Seffen, 2008).

Despite attracting the attention of several research groups around the world, chitosan application in some biomedical fields has being limited due to concerns of its brittle behavior. Therefore, toughness is a key property in assessing the potential performance of chitosan-based blends. Fig. 5 shows the toughness values estimated from the area under the stress–strain curves until rupture obtained for all samples, pure chitosan, pure PVA and their blends. In addition, these results are compared to their respective crosslinked system at two GA concentration, 1% and 5%. It was verified that sample toughness decreased about 40% by increasing the chitosan content, i.e. 14.8 MJ/m^3 of C/P/G(1:3:0) to 8.9 MJ/m^3 for the C/P/G(1:1:0) and subsequently raising to 16.8 MJ/m^3 for sample C/P/G(1:0:0; pure chitosan). Hence, there was a recovery when the chitosan content was increased from 50% to 100%, most likely associated with a less disordered network without PVA. Also, chemical crosslinking with GA also sharply reduced toughness values from samples, mainly for those with higher chitosan content. Such a trend can be explained that by increasing the network crosslinking, in other words the glutaraldehyde concentration. The polymer chains are covalently linked, consequently becoming more rigid and brittle and showing less flexibility (Bahrami et al., 2003; Wang et al., 2004a, 2004b). These findings are endorsed by a similar behavior that has been reported by Wang et al. (2004a, 2004b), where the fracture compressive strength and toughness of hydrogels tend to increase as the PVA content is raised in the investigated blend.

3.6. Swelling test

Swelling experiments were conducted with chi/PVA blends, with different polymer proportions and crosslinked by GA. A typical swelling behavior is shown in Fig. 6 performed for Chi/PVA blend [25:75] before and after chemical crosslinking with 1% and 5% of glutaraldehyde content. Briefly, the observed pattern indicated an initial rapid mass uptake, usually in approximately 30 min, followed by mass stabilization over a 192 h period. Visual inspection of the samples also shows appreciable volume increase. The results have revealed a strong influence of e-crosslinking on the swelling volume, from about 700% in chi/PVA sample before crosslinking, it dropped to 400% and 200%, with 1.0% and 5% glutaraldehyde, respectively. That fact is attributed to a more rigid network formed by the inter–intra polymer chain reactions that have occurred, reducing the flexibility and number of hydrophilic groups of hydrogel which is unfavorable to the swelling rate. So, these results are corresponding to the hydrogel mechanism. Before GA reaction, the PVA chains are physically entangled with the chitosan chains, forming a hydrogel network. In the sequence, when the GA content was increased the chemical crosslinking has occurred, forming covalent bonds among chains, fixing and reducing polymer mobility, which resulted in a lower swelling rate, which in this case was less than half of the blend without chemical crosslinking.

The effect of chitosan to PVA ratio was also analyzed and the results are presented in Fig. 7. It was verified that the swelling behavior is notably influenced by the chitosan content in the blend, crosslinked at 5.0% GA, where the swollen mass reduced by

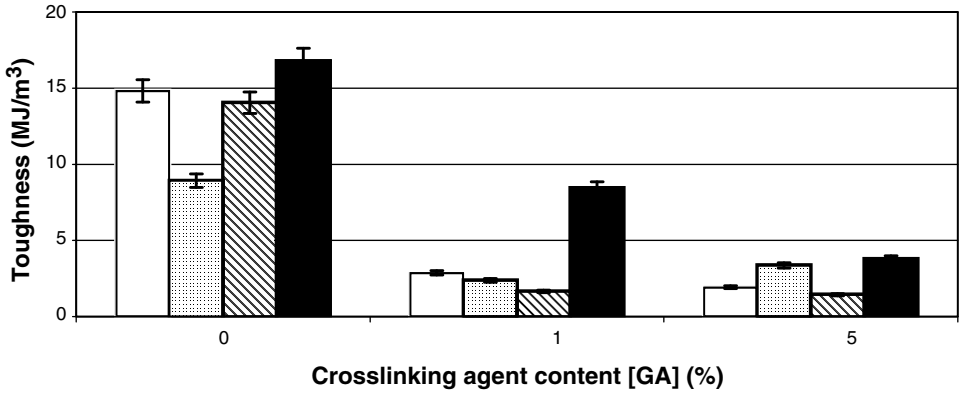

Fig. 5. Evaluation of toughness from Chitosan/PVA blends at different polymer ratios and varying the GA crosslinking concentration, 0.0, 1.0 and 5.0 wt%; legend: blank, C/P(1:3); dotted, C/P(1:1); dashed, C/P(3:1); solid, C/P(1:0).

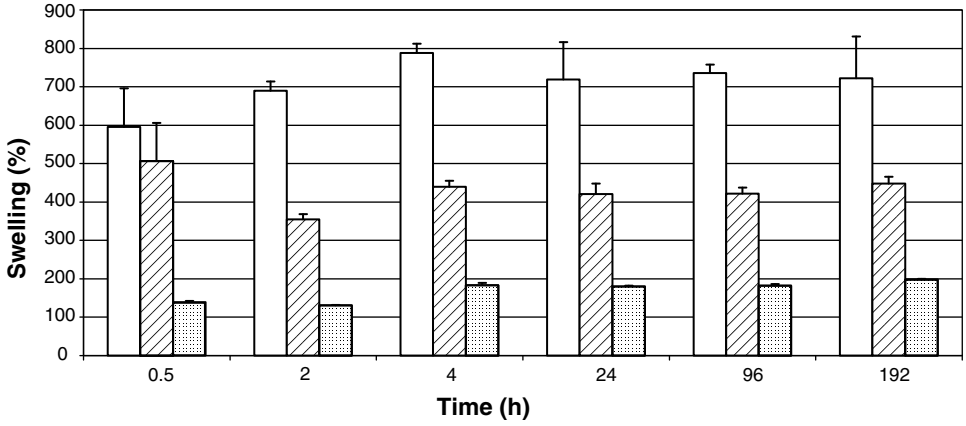

Fig. 6. Evaluation of swelling degree of the C/P(1:3) blends with GA crosslinking content 0.0, 1.0 and 5.0 wt%. Legend: blank, C/P/G(1:3:0); dashed, C/P/G(1:3:1); dotted, C/P/G(3:1:5).

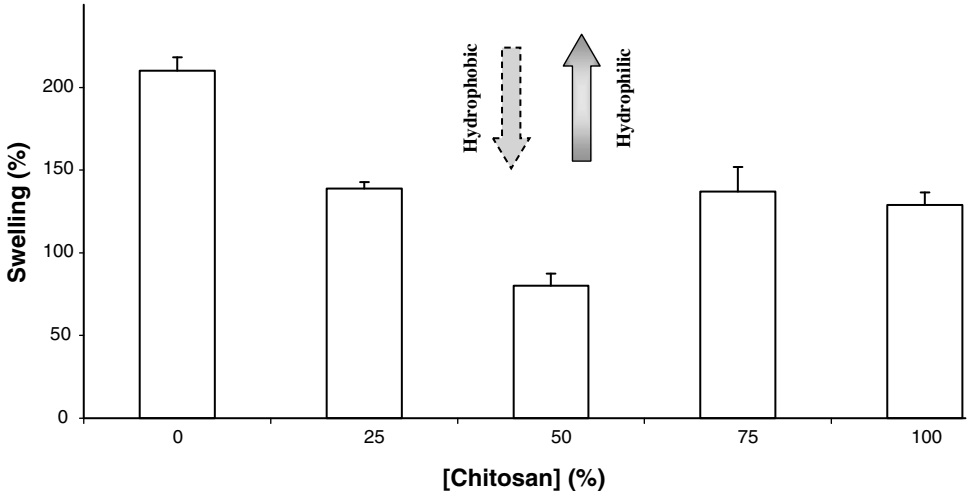

Fig. 7. Swelling degree of chitosan, PVA and CHI/PVA blends with GA crosslinking content 5.0 wt% after swelling for 192.0 h.

increasing the chitosan concentration and reaching a minimum value at [chi/PVA] = 50:50. The swelling degree reduced from 200% (pure PVA) to 100% (50:50 chi/PVA), then raised to about 140% at chi/PVA ratios of 75:25 and 100% chitosan. These results are supported by understanding the crosslinking reaction which has occurred in the blended hydrogels, where the amine groups of chitosan are more reactive to glutaraldehyde than hydroxyls of PVA. The minimum value observed at 50:50 (Fig. 7) is probably related to the overall balance between amine and hydroxyl crosslinking which is caused by the formation of a rigid structure amongst

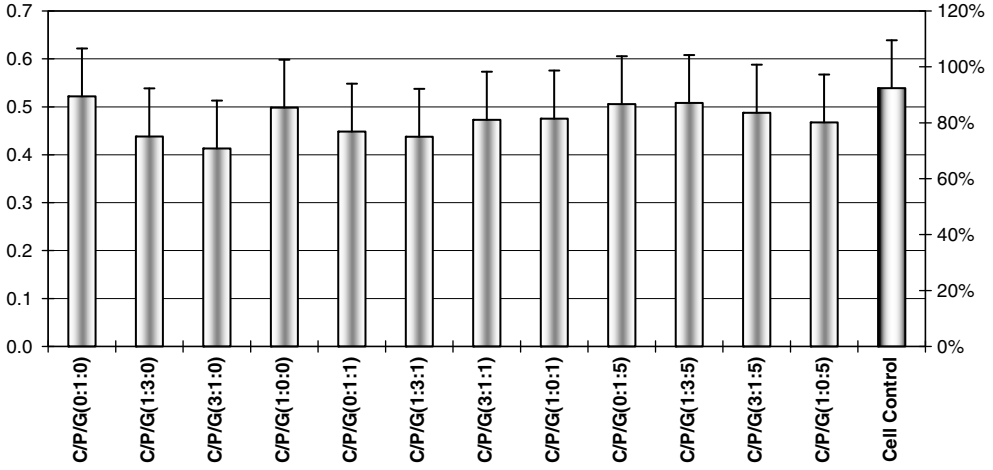

Fig. 8. Viability and spreading of VERO cell on different matrices. Biocompatibility assay for cell viability was carried out for VERO cells seeded on CHI/PVA/GA matrices (MTT test).

the PVA/chi chains, reducing drastically their possibility of solution uptake. Despite the present research being different from other reported chitosan studies, similar trends regarding to the swelling behavior of PVA and chitosan supported these findings, where PVA has a swelling degree above 500% and chitosan of about 200%, depending of course of the solution medium, pH, temperature and so forth (Berger et al., 2004; Claper, Skeie, Mullins, & Guymon, 2007; Grupta & Jabrail, 2007; Wang et al., 2004a, 2004b; Zhang et al., 2007).

3.7. Chitosan/PVA biocompatibility

Cell viability was measured using MTT assay and represents the active mitochondrial enzymes present in a cell capable of reducing MTT. In this study the viability assay was measured at 24 h interval after cell seeding. The ability of the CHI/PVA matrices (crosslinked or not) to support viability and proliferation shows that these samples evaluated exhibited comparable biocompatibility (Fig. 8). Although it is possible to observe in Fig. 8 that the cell viability numbers varying from approximately 78% to 97% (comparing to VERO cell control as 100%), this difference was not statistically significant ($p > 0.05$), and can be inferred that all the matrices produced merit *in vivo* testing. Cytotoxicity tests using cell cultures have been accepted as the first step in identifying active compounds and for biosafety testing. Cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, metastasis and tissue integration of biomaterials (Costa et al., 2008a, 2008c; Dunn & Zicha, 1995; Mansur & Costa, 2008).

4. Conclusion

Novel chitosan/PVA blends were synthesized and chemically crosslinked in this work. The results have shown that by altering the proportion of chitosan to PVA, associated with different concentration of crosslinker, the overall properties from hydrogels can be tailored. The systems investigated have indicated a significant reduction on the swelling behavior as the chitosan content was increased and also as the amount of crosslinking reagent was raised. As a consequence, from the mechanical point of view, the tensile strength was increased by the formation of a more rigid network. Moreover, all systems evaluated have proven to be non-toxic and biocompatible. In summary, these blends based on chitosan/PVA offer a broad range of choices to be potentially used in biomedical applications such as biomaterial, drug delivery vehicles and skin tissue engineering.

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