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Properties and biocompatibility of chitosan films modified by blending with PVA and chemically crosslinked

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Abstract In the present work we report the synthesis, characterization, and preliminary biocompatibility of polymer blends based on Chitosan and poly(vinyl alcohol) (PVA) with low degree of hydrolysis and chemically crosslinked by glutaraldehyde for potential application on skin tissue repairing. The microstructure and morphology of the blended hydrogels were characterized through Fourier Transform Infrared spectroscopy (FTIR) and Scanning electron microscopy (SEM/EDX) analysis. Hydrogels were investigated by swelling as preliminary in vitro test using simulated body fluid. In addition, biocompatibility, cytotoxicity, and cell viability were assessed via MTT assay with VERO cell culture and cell spreading-adhesion analysis. It was found that by increasing the chitosan to PVA ratio, simulated body fluid uptake of the material was significantly altered. All the tested hydrogels have clearly presented adequate cell viability, non-toxicity, and suitable properties which can be tailored for prospective use in skin tissue engineering.

1 Introduction

Chitosan can be considered as one of the most investigated materials in recent years. Chitosan [(1 → 4) 2-amino-2-deoxy-β-D-glucan] is obtained by the alkaline deacetylation of chitin, that is a naturally abundant mucopolysaccharide, and the supporting material of crustaceans and insects. So, chitosan molecule is also a polysaccharide, specifically a copolymer of *N*-acetyl-*D*-glucosamine, and *D*-glucosamine, which is a material resembling cellulose in its solubility and low chemical reactivity. The non-toxicity, high biocompatibility, and antigenicity of chitosan have driven its potential applications in biomedical field. The biodegradability of chitosan brings its utility as biomaterial, in tissue engineering, membranes and drug delivery systems. However, it has been of limited use as skin repairing biomaterial mainly due to the brittle behavior [1–4]. In addition, for specific medical applications such as in skin tissue replacement on wounds and burns, chitosan requires initial relatively low solubility accompanied by a predictable kinetics of biodegradability of the polymer. These biomaterials should ideally resemble the lost tissue where the outer surface is almost insoluble and the inner surface in contact with the supporting tissue undergoes dynamically and progressively biodegradation leading to complete resorption, as soon as they are no longer needed. However such complex behavior has never been produced and many challenges are yet to be overcome. One path to be pursuit is the use of natural or synthetic polymers, separately or blended, with grafted or crosslinked networks, in order to match the required properties. In other words, the polymer blended crosslinked system may present differential degradation behavior under physiological fluid conditions, where part of polymeric network may undergo to fast solvation and another portion may experience slow degradation by de-polymerization. Hence, chitosan joined to other polymers opened a window of research for altering or tailoring the property of interest [4–7].

In the present research we have developed a novel system by blending chitosan with poly(vinyl alcohol-co-vinyl acetate) (PVA-80%) at different ratios followed by chemical crosslinking with glutaraldehyde (GA), aiming

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for modulating degradability to be potentially used as wound dressing as skin tissue substitute. To our knowledge, this is the first report where hybrid blends (biopolymer/synthetic polymer) based on chitosan and copolymer (PVA-80%) were synthesized at different proportions and lately chemically crosslinked followed by an in-depth characterization.

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 = 9,000–10,000 g/mol. Chitosan powder (Cat.#419419, Sigma-Aldrich Chemical, Milwaukee, Wisconsin, USA), high molecular weight, MW = 161,000 g/mol, degree of deacetylation, DD = 75.6%, was used without further purification. GA or 1,5-pentane-dial (Cat.#49630, Sigma-Aldrich Chemical, Milwaukee, Wisconsin, USA) 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 g of polymer powder without further purification in 100 ml of Milli-Q water, under magnetic stirring, at temperature of 75 ± 2°C (solution A), as previously reported by our group [7–9]. PVA 5% solution was 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 (solution B).

2.2.2 Chitosan, PVA and blends films preparation

Different quantities of PVA (solution A) were added into the 1.0% chitosan solution (solution B) to obtain chitosan/PVA mass 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 (GA) was slowly added under constant stirring. The final concentration of GA in the gel solution precursors was 1% and 5% (wt%). Further in the sequence, the solution was poured into plastic moulds and let drying for 72–120 h at room temperature, and finally dried at 40°C for 24 h (constant weight). Chitosan/PVA samples chemically crosslinked were identified by (X:Y:Z) that is, X as chitosan content, Y as PVA content, and Z as GA (wt%). For instance, sample identified as Chi/PVA/GA (1:3:1) represents the following proportion of reagents: 25% chitosan, 75% PVA and crosslinked with 1.0% GA (wt%). The dried gel was stored in a desiccator before all subsequent characterization procedures.

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 was measured 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 coupled to a dispersive energy spectrometer (EDS). The images were obtained using an accelerating voltage of 10–15 kV. Before examination, the samples were coated with Au to make them conductive. As the solubility of chitosan is much lower than PVA in alkaline medium, some blends were etched by alkaline solution (NaOH, 0.5 M) aiming to have PVA domains removed from the sample and chitosan-rich regions revealed for SEM evaluation. A similar approach was used with chemically crosslinked samples.

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 100–200 μm thick films and analyzed by FTIR using ATR (attenuated total reflection) Modes. FTIR spectra were collected with wavenumber ranging from 4,000 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 and associated with the main chemical groups.

2.3.4 Swelling and degradation tests in physiologically-mimicking conditions

In vitro studies on the mechanism of biomineralization have been documented to extensively confirm preliminary

biocompatibility behavior of materials. In other words, it would reliably reproduce the expected performance of in vivo experiments. This study generally uses a protein-free acellular simulated body fluid medium (SBF or Kokubo solution) with pH (7.40) and ionic composition (Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mM) equal to those in blood plasma, indicating the compositional and structural dependences of material bioactivity [9]. Therefore, the chitosan/PVA hydrogel samples were cut into 5×5 mm^2 pieces and soaked in simulated body fluid 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. Before the swelling and degradation assays, samples were immersed in PBS to neutralize any remaining non-reacted groups from GA crosslinker.

2.3.4.1 Swelling test Fluid absorption studies are of paramount importance for preliminary analysis of biodegradable materials. 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 [9]. SBF absorption (S) is given by 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.

2.3.4.2 Degradation test The degradation study of the hydrogel was carried out in vitro by incubating the samples in SBF at pH: 7.4 and 37°C for different periods. In order to find out the degradation index (D_i), triplicate samples were dried in a chamber at 40°C and weighed after their weight stabilized. After drying, the samples were placed in recipients with SBF solution using the area/volume ratio = 0.1 cm^{-1}. The samples were kept at (37 ± 1°C) in a water bath. At each measurement, the samples were then removed from the recipient and weighed after drying at 40°C and constant weight being verified. So, the degradation index was calculated based on the mass loss using the eq. 2:

$$D_i = \frac{W_0 - W_t}{W_0} \times 100 \quad (2)$$

where: W_0 denotes the original weight, while W_t is the weight after the immersion time (t). Each degradation experiment was repeated three times and the average value was considered.

2.3.5 Cytocompatibility, cell viability and bioactivity assays on blends

2.3.5.1 Neutralization procedures Phosphate Buffered Saline (PBS) was used in the procedure to neutralize any remaining cytotoxic groups of non-reacted GA crosslinker. The Chitosan/PVA films were immersed in polyethylene flasks with 75 ml PBS 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 steam of ethylene oxide.

2.3.5.2 Chitosan/PVA biocompatibility and spreading assay As previously reported by our group [9, 10], African green monkey kidney VERO cells, a fibroblastic cell line, were used for the experiments of cell biocompatibility MTT (3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyl tretrazolium) and adhesion assays. It is worth to point out that a fully detailed description of the MTT is beyond the major goal of this research as it has been extensively reported in the literature. 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 supernatant and plates were re-incubated for more 24 h and after exhaustive pipetation, 200 µl was transferred to a clean 96-well plate, where absorbance was measured at 595 nm using ASYS EXPERT PLUS (ASYS Hitech, Austria) spectrometric microplate reader. For analysis, all data were expressed as average ± standard deviation for number of four replicates ($n = 4$). One way ANOVA was used to access statistical significance of results. Post tests Dunnett Multiple Comparison Test and Neman-Keuls were carried out at a level of 95% significance.

2.3.5.3 Cell adhesion-spreading assay Cell viability was evaluated by spreading and attachment assays in order to examine their morphology, adhesion, and spreading

behavior. VERO cells were plated at 6×10^4 density on the hosting scaffold; Cell spreading was evaluated by scanning electron microscopy of the specimens after culturing for 2 h. Before microscopy analysis, specimens were fixed with 2% GA for 16 h and dehydrated by passing through a series of alcohol solutions (ethanol-water). Then, they were dried in nitrogen flowing reactor for 4 h and outgassed in vacuum desiccator for 12 h. Finally, prior to SEM assessment samples were coated with a thin layer of sputtered Au to make them conductive.

3 Results and discussion

3.1 Qualitative assessment

The films were (75 ± 25 μm) thick and no heterogeneity was 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 with average thickness in the 50–100 μm range.

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 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 (data not shown) [9, 11]. 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 not crosslinked phase. In fact, that actually has occurred as it can be observed from SEM results shown in Fig. 1. Blends not crosslinked (Fig. 1a, b, and c) clearly indicated material removal by etching solution, leaving voids and holes on the structure. On the other hand, the Chi/PVA (1:1) samples crosslinked by GA (Fig. 1d, e, and 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 GA crosslinking has taken place as it can seen by FTIR in Sect. 3.3 [11, 12].

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 GA. Figure 2 shows the FTIR spectra relative to the chitosan, PVA and [Chi/PVA] blends. Figure 2a spectrum of pure chitosan shows peaks around 893 and 1,156 cm^{-1} corresponding to saccharide structure, as it is the repeating unit from chitosan, similar to chitin and cellulose [7, 9, 11–14]. In spite of several peaks clustering in the amide II peak range from 1,510 to 1,570 cm^{-1}, there still were strong absorption peaks at 1,658 and 1,322 cm^{-1}, which are characteristic of chitosan and have been reported as amide I and III peaks, respectively. The sharp peaks at 1,383 and 1,424 cm^{-1} were assigned to the CH_3 symmetrical deformation mode. The broad peak at 1,030 and 1,080 cm^{-1}

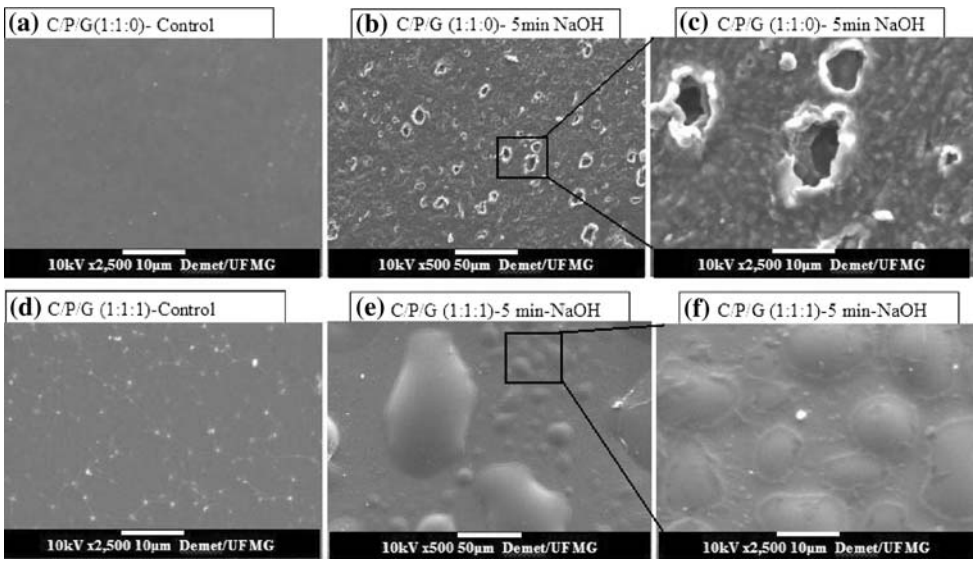

Fig. 1 SEM images with PVA segregation in blends after 0.5 M NaOH immersion

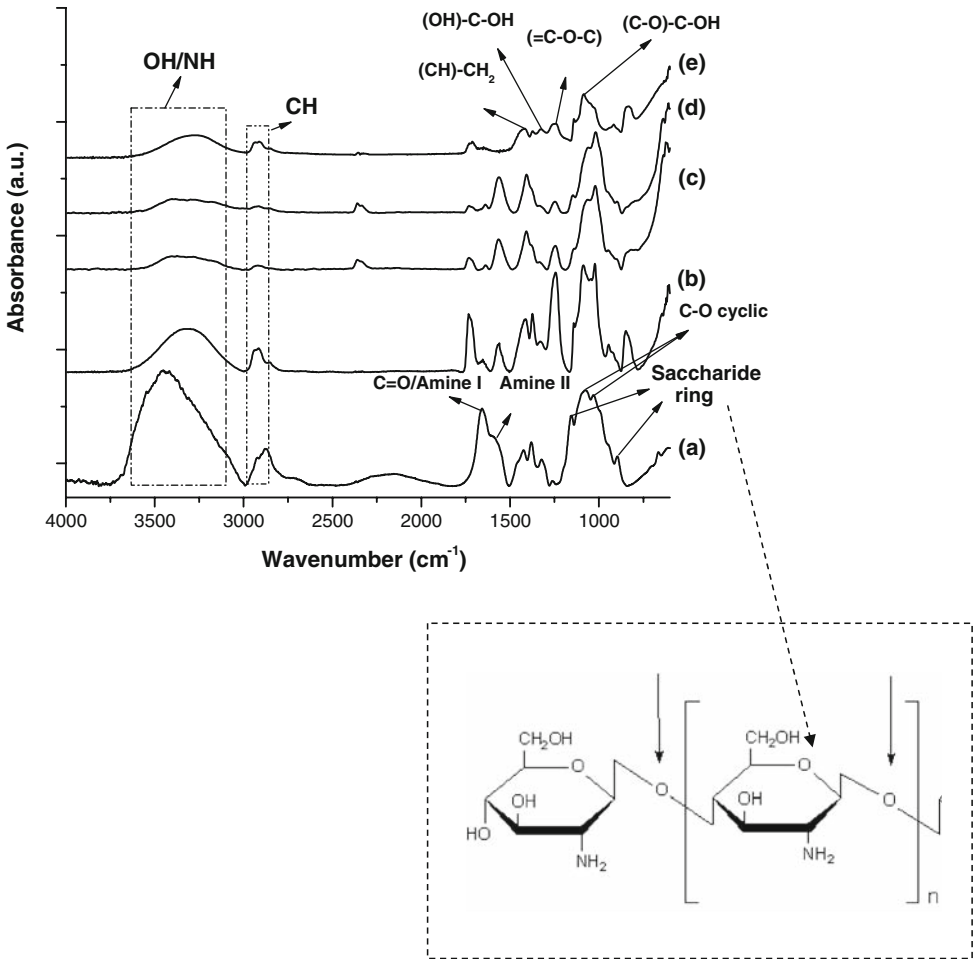

Fig. 2 FTIR spectra of (a) chitosan, (b) Chi/PVA/GA (1:3:0), (c) Chi/PVA/GA (1:1:0), (d) Chi/PVA/GA (3:1:0), and (e) PVA vibrational bands without chemical crosslinking

indicates the C–O stretching vibration in chitosan. Another broad peak at 3,447 cm^{-1} is caused by amine N–H symmetrical vibration, which is used with 1,650 cm^{-1} for quantitative analysis of deacetylation of chitosan. Peaks at 2,800 and 2,900 cm^{-1} are the typical C–H stretch vibrations [7, 9, 11–14]. The IR spectra of the Chi/PVA blended films (Fig. 2b, c, and d) are different from that of the chitosan because of the ionization of the primary amino groups. There are two distinct peaks at 1,408 and 1,548–1,560 cm^{-1}. Formation of the 1,548–1,560 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 1,408 cm^{-1} indicates the presence of carboxylic acid in the polymers. The peaks at 1,700–1,725 cm^{-1} are characteristic of the carboxylic acid dimmer. In the present study, the presence of carboxylic dimmer was due to the acetic acid used for dissolving the chitosan [7, 9, 11–14]. Also, in the blends, some contribution is given by the presence of acetates (carbonyls, C=O, at 1,700–1,720 cm^{-1}) from the chitosan and PVA structures, i.e., deacetylation degree and hydrolysis degree, respectively. The peak at 1,210–1,300 cm^{-1} is due to the C–H vibration. Hence, it can be undoubtly observed a significant relative reduction of intensities from main absorption bands related to chitosan (spectra normalized, background subtracted), for instance amine region (1,500–1,650 cm^{-1}), as its content was decreased from 100% (pure chitosan, Fig. 2a), 75% (Fig. 2b), 50% (Fig. 2c), 25% (Fig. 2d) and 0% (pure PVA, Fig. 2e). In chi/PVA blends, the spectra region from 3,200 cm^{-1} up to 3,500 cm^{-1} was wider than pure polymers mostly attributed to the overlapping signals from two groups, hydroxyls (–OH, chitosan, and PVA) and amino (–NH, chitosan). Figure 4e shows the FTIR spectrum of PVA. All major peaks related to hydroxyl and acetate groups were observed. More specifically, the broad band observed between 3,550 and 3,200 cm^{-1} is associated with the stretching O–H from the intermolecular and intramolecular hydrogen bonds. The vibrational band observed between 2,840 and 3,000 cm^{-1} refers to the stretching C–H from alkyl groups and the peaks between 1,750–1,735 cm^{-1} are due to the stretching C=O and C–O from acetate group remaining from PVA (saponification reaction of polyvinyl acetate) [12–14].

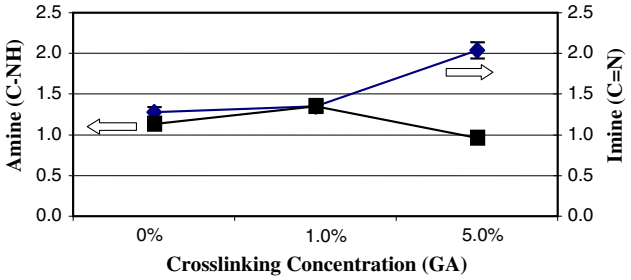

Fig. 3 Evaluation of chemical crosslinking of chitosan/PVA blend via FTIR spectroscopy with different concentration of GA. Left *Y*-axis amine band decrease (squares); Right *Y*-axis imine band increase (circle)

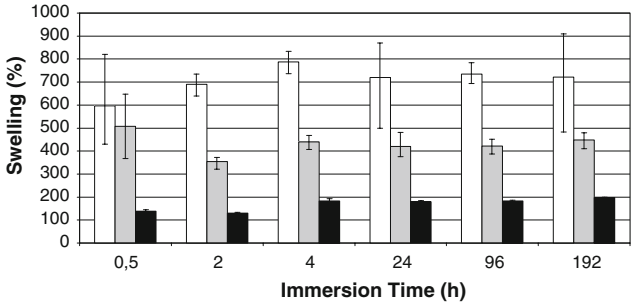

Fig. 4 Evaluation of swelling degree of the Chi/PVA (1:3) blends with GA crosslinking content 0.0, 1.0, and 5.0 wt%. Legend: blank = Chi/PVA/GA (1:3:0); dashed = Chi/PVA/GA (1:3:1); solid = Chi/PVA/GA (3:1:5)

Furthermore, all chitosan-derived blends have shown a relative increase on their imine (–C=N–) bands and simultaneous drop on the amine ($–NH_2$) vibrational band after chemical crosslinking with GA. The imine group was formed by the nucleophilic addition reaction of the amine from chitosan with the aldehyde giving a hemiaminal (–C(OH)(NHR)–) followed by an elimination of water (Schiff base). Schiff base (or azomethine), named after Hugo Schiff, is a functional group that contains a carbon-nitrogen double bond with the nitrogen atom connected to an aryl or alkyl group, but not hydrogen. Schiff bases are of the general formula R1R2-C=N-R3 [9, 11–14]. As presented in Fig. 3, the major IR vibrational bands associated with imine, amine and reference were plotted which have given evidence of an increase on the network crosslinking as the GA concentration was increased. Hence, it reasonable to assume that in fact, the crosslinking mechanism occurred via covalent bond formation, preferably with chitosan amine groups reacting with bi-functional aldehyde (GA).

3.4 Swelling and degradation tests in physiologically-mimicking conditions

3.4.1 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. 4 performed for Chi/PVA blend [25:75] before and after chemical crosslinking with 1% and 5% of 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 the 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% GA, 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 the lower swelling rate, in case, less than half of that blend without chemical crosslinking.

The effect of chitosan to PVA ratio was also analyzed and the results are presented in Fig. 5. It was verified that the swelling behavior is especially influenced by the chitosan content in the blend, crosslinked at 5.0% GA, where the swollen mass reduced by 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 GA than hydroxyls of PVA. The minimum value observed at 50:50 (Fig. 5) is probably related to the overall balance between amine and hydroxyl crosslinking which is caused by the formation of a rigid structure amongst the Chi/PVA chains, reducing drastically their

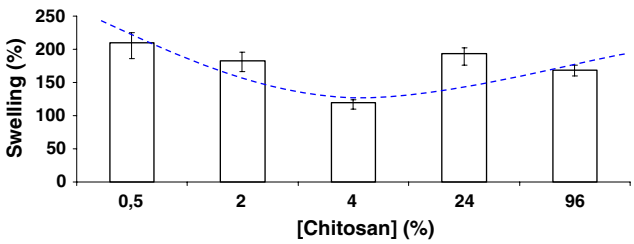

Fig. 5 Swelling degree of chitosan, PVA, and CHI/PVA blends with GA crosslinking content 5.0 wt% after swelling for 192.0 h

possibility of solution uptake. Despite of 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 [7, 9–14].

3.4.2 Degradation test

Figure 6 presents the typical result for degradation behavior of the Chi/PVA (1:3) blends using the SBF immersion method, without and with the addition of 1% and 5% GA as chemical crosslinking. It was clearly observed that when the GA content increased, degradation in SBF decreased, suggesting higher density of chemical crosslinking among polymer chains [12, 15, 16]. These findings were endorsed by the SEM images (Fig. 1) which have shown that the degradation was evident in the blend without GA and where covalent bonds are much more difficult to be broken compared to physical crosslinked network. Moreover, it was inferred from the statistical analysis at significance level of 1% that in the Chi/PVA(1:3) blend the GA contents altered the degradation property in SBF at 37 ± 1°C. The widely used term "degradation" is a process where the deterioration in the properties of the polymer takes place due to different factors like, radiation, chemical, thermal, mechanical, among others. Therefore degradation may be a result of chemical activities such as solvation, desorption, dissociation, hydrolysis, dissolution, oxidation, reduction, and photolysis, as well as physical activities that may erode a polymeric blend matrix such as diffusion, abrasion, cracking, peeling, mechanical breakage, or any combination of these chemical and physical activities. For that reason, in the present study, the polymer mass loss is thought to occur primarily through two methods, solvation and de-polymerization of chains. This assumption is supported by FTIR results which have indicated the preference of polymer crosslinking via NH_2 (chitosan) over hydroxyls (PVA). Therefore, mixed degradation kinetics is expected for blended network, with PVA physically crosslinked undergoing to fast solvation and chitosan-PVA covalently crosslinked would have a slow degradation via de-polymerization.

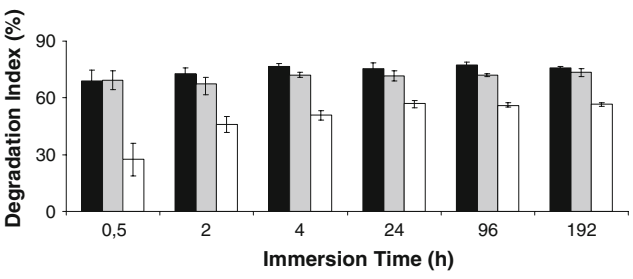

Fig. 6 Degree of degradation of the Chi/PVA(1:3) blends with GA crosslinking content 0.0, 1.0, and 5.0 wt% ($n = 3$). Legend: solid = Chi/PVA/GA (1:3:0); dashed = Chi/PVA/GA (1:3:1); blank = Chi/PVA/GA (1:3:5)

3.5 Chitosan/PVA biocompatibility test in vitro with cells

3.5.1 VERO cell culture

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 all viability and proliferation shown that these samples evaluated exhibited comparable biocompatibility (Fig. 7). It is rather important to emphasize that despite of being reported cytotoxic, GA reacts under acid condition with both amine and hydroxyls groups from chitosan and PVA. Yet, any remaining aldehyde group is almost immediately blocked by aminoacids and proteins present in living organisms sera. Although it is possible to observe in Fig. 7 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 shows to be promissory to be tested in vivo assays. Cytotoxicity tests using cell cultures have been accepted as the first step in identifying active compounds and for biosafety testing [7, 9, 12, 17].

3.5.2 Chitosan/PVA biocompatibility and spreading assay

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

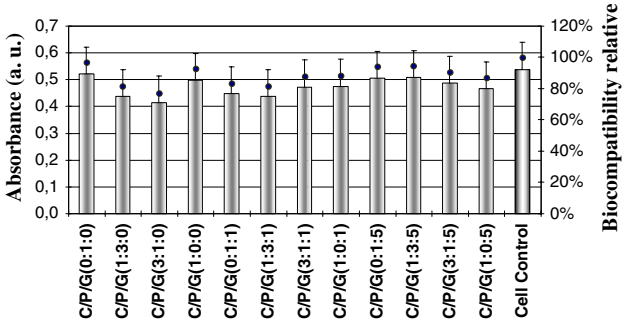

Fig. 7 Viability of VERO cell on different matrices. Biocompatibility assay for cell viability was carried out for VERO cells seeded on CHI/PVA/GA matrices (MTT test)

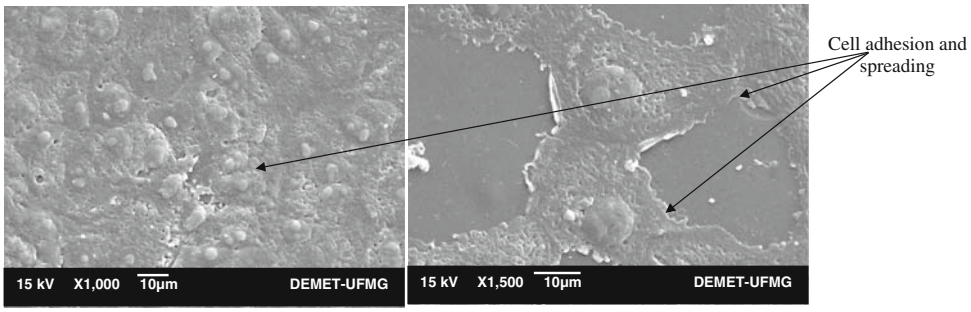

Fig. 8 Adhesion and spreading of VERO cells for biocompatibility assay. VERO cells seeded on CHI/PVA/GA matrices. SEM image illustrating typical morphology of cells seeded on Chi/PVA/GA (1:3:1) blend

ability of the Chi/PVA matrices (crosslinked or not) to support all viability and proliferation shown that these samples evaluated exhibited comparable biocompatibility, Fig. 8 Although we can see on figure viability numbers reaching from 77% to 97% (comparing to VERO cell control as 100%), this difference was not significant ($P > 0.05$), and can be inferred that all the matrices produced shows to be promissory to be tested in vivo assays. These assumptions are supported by the SEM results (Fig. 8) showing the VERO cell seeded on matrix with good adhesion and spreading morphology regular for this fibroblastic lineage. Since cellular attachment, adhesion, and spreading belong to the first phase of cell/material interactions, the quality of this phase will influence proliferation and differentiation of cells on biomaterials surfaces. Based on the SEM results obtained in our work, one may attribute the VERO cell spreading and adhesion verified on the chitosan/PVA blends to be a reliable proof of biocompatibility and non-cytotoxicity of samples. According to the literature [15, 17–21], cell spreading are usually divided into three main interaction levels: (a) not spread: cells were still spherical in appearance, protrusions or lamellipodia were not yet produced; (b) partially spread: at this stage, cells began to spread laterally at one or more sides, but the extensions of plasma membrane were not completely confluent; and (c) fully spread. The last model (c) would represent the best result for material cell hosting. Furthermore, the cell line used as a model in this research plays a role in producing many of the components essential to connective tissue, for example extracellular components such as glycosaminoglycans and, in fibrous tissue, collagen. Promoting the attachment of fibroblast cells would aid in integrating soft connective tissue to the implant, improving vascularity at the implant surface and decreasing the chance of fibrous encapsulation. In summary, cell adhesion and spreading are of paramount importance in living biology processes and are involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, metastasis, and tissue integration of biomaterials [7, 10, 17–21]. In fact, further investigation should be carried out before a clinical use of the proposed system as skin tissue repairing. Some very recent researches have been published where PVA-chitosan membranes were studied aiming to fulfill the requirements of biomaterials for wound dressing, skin and vascular tissue engineering [22–24]. Nevertheless, many challenges need to be overcome to meet the necessities for replacing such complex tissues, mainly associated with their functions and properties under physiological conditions in living organisms.

4 Conclusion

In the present research, chitosan/PVA blends were synthesized and chemically crosslinked with bi-functional aldehyde. The results have shown that by altering the proportion of chitosan to PVA, associated with different crosslinker concentration, the overall properties from hydrogels can be modified. 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. This fact was attributed to the formation of a more rigid network. Moreover, cell biocompatibility assays have proven that all systems evaluated are non-toxic, biotolerant, and biocompatible. In summary, these developed blends based on chitosan/PVA have broadened the number of choices of biomaterials to be potentially used in biomedical applications such as biomaterial, drug delivery vehicles and skin tissue engineering.

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