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Preparation and characterization of genipin cross-linked porous chitosan–collagen–gelatin scaffolds using chitosan–CO_2 solution

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ABSTRACT

Novel porous scaffolds composed of chitosan, collagen and gelatin were prepared by the multistep procedure involving final freeze-drying and characterized. To eliminate the need for residual acid removal from the material after drying, carbon dioxide saturation process was used for chitosan blend formulation. The use of CO_2 for chitosan dissolution made the scaffold preparation process more reproducible and economically sustainable. Genipin was applied to stabilize the structure of the scaffolds and those crosslinked at a level of 7.3% exhibited a homogenous porous structure (33.1%), high swelling capacity (27.6 g/g for wound exudate like medium; 62.5 g/g for water), and were stable under cyclic compression. The values of other investigated parameters: dissolution degree (30%), lysozyme-induced degradation (5% after 168 h), good antioxidant properties (DPPH, ABTS, Fe^{2+} assays) and especially very low in vitro cytotoxicity against fibroblasts (103%, MTT assay), were highly advantageous for possible biomedical applications of the novel materials.

1. Introduction

In recent years, dynamic development of the medical wound dressing market has been observed. Many polymers have been extensively investigated as potential materials for wound covering, realizing that an ideal wound dressing should present several specific properties for the intended application, including capacity to absorb wound exudates, maintenance of the moisture, protection from secondary infection, provision of adequate gaseous exchange, provision of thermal insulation free from particulate or toxic contaminants, mechanical durability, flexibility, and non-toxicity (Purna & Babu, 2000; Stashak, Farstvedt, & Othic, 2004). The use of natural polymers such as chitosan, collagen and gelatin in the construction of wound healing devices is quite attractive, mostly because of their biocompatibility, possibility of various chemical modifications, and relatively low cost of production (in many cases directly from renewable sources) (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Hoyer et al., 2012; Jayakumar, Prabaharan, Sudheesh, Nair, & Tamura, 2011).

Although chitosan processing technology is well known and often used by the manufactures, there are still some inconveniences, that could be avoided in order to facilitate the production of chitosan based materials. All known methods of scaffold fabrication from chemically unmodified chitosan require prior dissolution of chitosan in an acidic medium such as acetic, citric, formic, hydrochloric, lactic, and tartaric acid, among which acetic acid is most commonly used (Leffler & Müller, 2000). Standard scaffold fabrication procedures demand a step for residual acid removal from the final product, which is necessary because of the potentially harmful effects of organic acids on various cell populations as well as the impediment of the epithelialization process that is important in wound healing (Miller, Creazzo, & Witt, 1992). In most cases this is done by washing with water or alcohol solutions (Lin, Tan, Marra, Jan, & Liu, 2009; Nandagiri et al., 2011; Yan et al., 2010). This step is followed by additional drying of the scaffolds in order to obtain a dry product. Such procedures often lead to the loss of the original structure of the formulated materials as well as the partial dissolution of the scaffold, rendering the procedure less reproducible.

To avoid these inconveniences we developed an alternative method of fabrication of chitosan based scaffolds, where chitosan is dissolved upon saturation of an aqueous chitosan suspension with gaseous CO_2 under mild conditions: atmospheric pressure and room temperature. As CO_2 dissolves in water, the pH decreases

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below 5 due to the formation of bicarbonate and carbonic acid, which is sufficient to dissolve a properly pretreated chitosan. The use of CO_2 in chitosan processing has been already shown by the Japanese research group in 2001 (Sakai, Hayano, Yoshioka, & Yoshioka, 2001) but to date it was only applied for coating of cellulose-derived materials (Sakai et al., 2002).

Herein, we describe the use of gaseous CO_2, to prepare a chitosan blend for the fabrication of chitosan–collagen–gelatin scaffolds with new properties. Although such composites do not exist in Nature, it has been shown that they together and individually provide favourable physicochemical properties for the purpose of wound healing (Muzzarelli, 2009a). A natural non-cytotoxic compound, genipin, was chosen as a cross-linking agent to stabilize the structure of the scaffold. The functional properties of the novel materials indicate a potential for biomedical applications, in particular fabrication of wound dressings.

2. Experimental

2.1. Materials

Chitosan (low molecular weight) was purchased from "Sigma–Aldrich (St. Louis, MO, USA)". Genipin was purchased from Challenge Bioproducts (Taiwan). Lysozyme (70,000 U/mg), ninhydrin solution (2, w/v), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt [ABTS $(NH_4)_2$], and ferrozine were obtained from "Sigma–Aldrich (St. Louis, MO, USA)". MilliQ water was used for the preparation of all aqueous solutions. All other reagents were of analytical grade or higher.

Fish proteins used in this work were extracted from salmon skin (*Salmo salar*). The extraction was performed according to the procedures described earlier in our research group (Kołodziejska, Skierka, Sadowska, Kołodziejski, & Niecikowska, 2008; Sadowska, Kołodziejska, & Niecikowska, 2003). Freeze-dried protein samples (CHRIST Alpha 1-4 LD Plus) stored at 4 °C were used in the formulation process.

2.2. Methods

2.2.1. Scaffold development

Each of the polymeric blends was formulated separately (chitosan and protein) (Fig. 1). The gelatin–collagen blend was prepared as follows: 1.2 g of gelatin was suspended in 20 ml of distilled water, left until swollen (2 h, room temperature) (2.A) and heated at 50 °C until dissolved (3.A). Collagen (0.6 g) was added to a gelatin solution cooled to <12 °C, and the mixture was homogenized (4.A) just before adding to the chitosan blend. The chitosan blend was prepared by first dissolving 1.5 g of chitosan in 100 ml of 0.5 M acetic acid (2.B) and then dropwise adding 0.5 M sodium hydroxide under continuous mechanical stirring, until a pH of 7.5 was reached (3.B). The chitosan precipitate was separated by centrifugation (10,000 rpm, 30 min), washed twice with distilled water, and centrifuged again (4.C). The resulting precipitate was suspended in distilled water in an amount necessary to obtain a total weight of 80 g, and the mixture was then homogenized to obtain a homogeneous colloidal solution (5.C). Next, the solution was saturated with CO_2 for 3 h at room temperature with the use of a hollow shaft stirrer (6.C). To intensify the saturation process, small pellets of dry ice were added to the solution during the saturation process. The genipin ethanolic solution was added to a final genipin concentration of 0.5% (w/w) to the total weight of polymer (7.C). The mixture was stirred for 10 min and then cooled to <12 °C (8.C). Subsequently, the 20 ml collagen and gelatin solution, prepared as a mixture as described above, was added to the chitosan–genipin blend, and intensive stirring was continued for 10 min. (9). The resulting blend was optionally foamed (11) and poured into moulds (12 cm × 12 cm × 0.4 cm) (11), incubated for 24 h at 12 °C (12), frozen at −20 °C (13), and freeze-dried (14). Sponges with 1%, 2% (w/w) of genipin, and without genipin were prepared in the same manner. Sponges were conditioned at room temperature and <90% humidity for 3 h (15) and stored in closed plastic bags awaiting characterization.

2.2.2. Morphological examination

The cross-sectional morphology of the scaffolds was studied by scanning electron microscopy (SEM, Philips-FEI XL 30 ESEM). A 1-mm piece of the sponge was fixed on the SEM sample holder, and images were collected in water vapour atmosphere (133.3 Pa, 12–15 kV). The average cross-sectional area of the pores was calculated using image-processing tools of the Photoshop CS5 software. The porosity of the chitosan–protein scaffold was evaluated using trimmed samples of 5 cm^2 × 0.5 cm in ethanol (Yang et al., 2010), according to the following formula:

$$P(\%) = \frac{V_c}{V_m} \times 100 = \frac{(W_{24} - W_0) \times \rho}{V_m} \times 100$$

where V_m is the total volume of the chitosan scaffold (cm^3), V_c is the pore volume of the chitosan scaffold (cm^3), W_{24} is the weight (g) of the chitosan scaffold after incubation with ethanol for 24 h, W_0 is the original weight (g) of the chitosan scaffold, and ρ is the density of ethanol (0.808 g/cm^3).

2.2.3. Cross-linking degree

The cross-linking degree of each scaffold was determined using the ninhydrin assay (Yuan et al., 2007). The 2.5 mg test sample was heated with 1 ml of 2% (w/v) ninhydrin for 20 min at 100 °C. The solution was cooled to room temperature, diluted with 50% isopropanol, and the absorbance of the solution at 570 nm was then read with a spectrophotometer (UV-VIS Lambda Bio 20). Glycine solutions of various known concentrations were used as standards, and sponges that had been prepared without genipin were used as control materials. Each determination was performed in triplicate.

2.2.4. Swelling/dissolution tests

The swelling capacity of the scaffolds was evaluated by weighing the scaffolds before (w) and after (w_1) placing the scaffolds in an aqueous solution containing a salt composition similar to that of wound exudate according to EN 13726-1, prepared by adding 8.298 g of NaCl and 0.368 g of $CaCl_2 \cdot 2H_2O$ to 1l of distilled water. The samples were incubated at 37 °C for 24 h, withdrawn from the medium, and weighed after removal of the surface fluid using a filter paper. The samples were then freeze-dried and weighed again (w_2). The percentage of fluid absorption was defined as the ratio of the weight increase ($w_1 - w_2$) relative to the weight remaining after freeze-drying (w_2). Each value was calculated as the mean of 3 independent measurements. To show the influence of the ionic strength of the solution, a similar test was performed using distilled water as the reference medium. The dissolution of the scaffold (in percent) is defined as the ratio of the weight decrease of the scaffold ($w - w_2$) relative to the initial weight (w). Each sample was measured in triplicate.

2.3. Water vapour transmission rate (WVTR)

The WVTR was determined according to ASTM method E96-90 by monitoring the mass of evaporated water in the test sample and by measuring the weight loss from a water-filled homemade permeability cup. The permeability cups were filled with 20 g of deionised distilled water, and the test samples were fixed onto the opening of the cup. Next, the permeability cups were weighed and

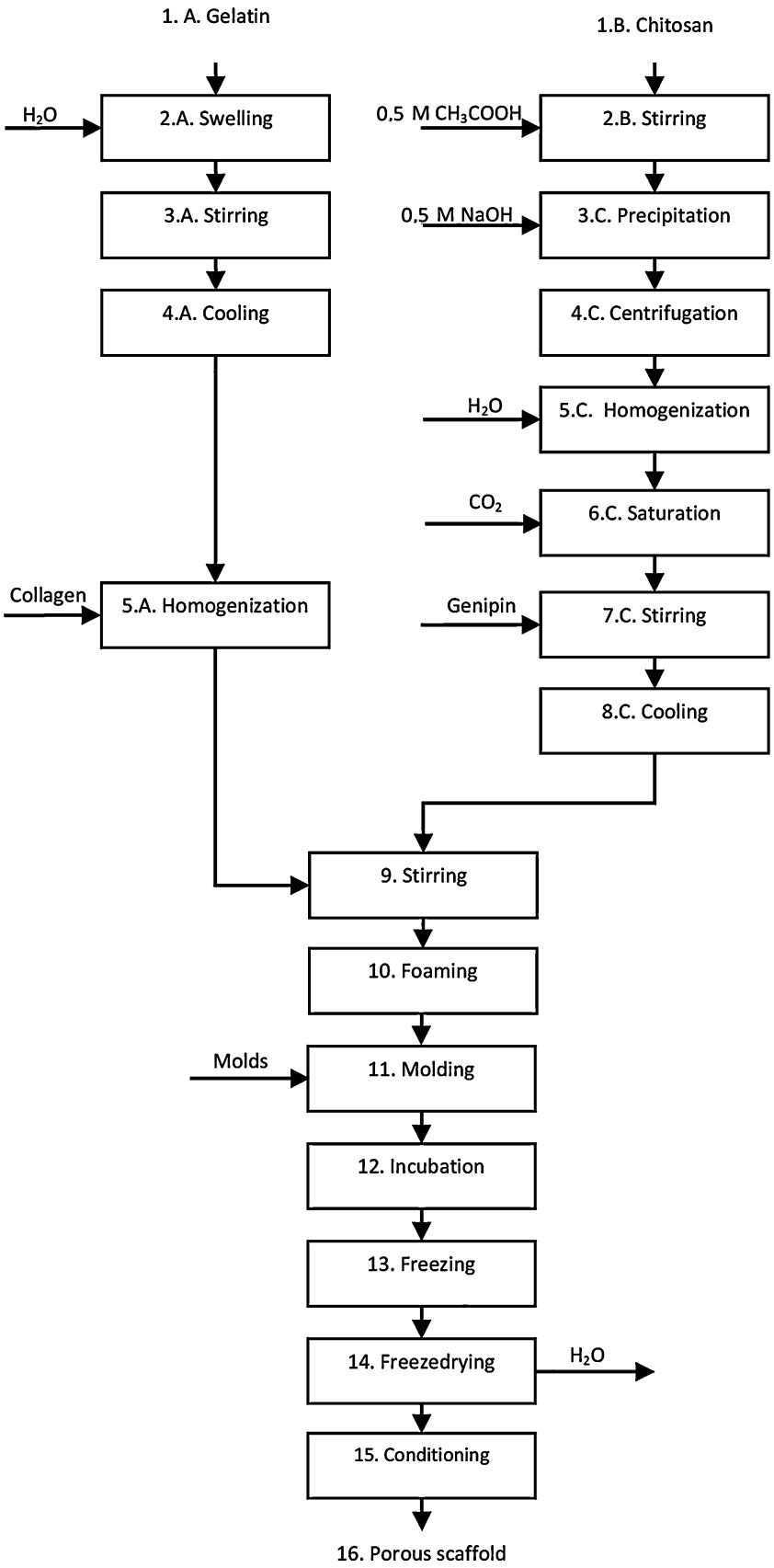

Fig. 1. Process flow chart for formulation of genipin crosslinked chitosan–protein scaffolds using CO_2 as the chitosan solvent.

placed in a desiccator that had been bottom-filled with a saturated lithium chloride solution at room temperature, which equilibrated the desiccator to a relative humidity (RH) of approximately 20%. A digital hygrometer was used to monitor the chamber conditions. WVTR values were calculated at pre-determined time periods by the following equation:

$$\mathrm{WVTR} = \frac{m}{A \times \Delta t},$$

where m is the mass (g) of the water loss at the specified time period, Δt is the time period (h), and A is the effective transfer area (m^2). Each determination was conducted in triplicate.

2.3.1. Mechanical properties

The mechanical properties of the scaffolds were characterized using a universal testing machine (Instron model 5543) according to previously described methods (Hoyer et al., 2012; Bi et al., 2011). Five cylindrical samples of the test scaffolds (Ø 25 mm × 4 mm) were compressed up to 50% deformation at a test speed of 0.5 mm/s, and the compression load (kPa) was measured. The test was performed for both dry and wet samples (after 24 h incubation in deionised water). Wet scaffolds were compressed for 25 cycles to perform fatigue tests.

2.4. Biodegradation studies with lysozyme

Chitosan–protein scaffolds (Ø 25 mm × 4 mm) were placed in 50 ml of phosphate buffered saline (PBS), pH 7.4, containing lysozyme (4000 U/ml final activity) and incubated at 37 °C with gentle agitation for the period of the study. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. To prevent microbial colonization, chloramphenicol was added to the medium at a concentration of 34 ng/ml. Samples were removed from the medium every 24 h for 7 days and rinsed with distilled water, dried, and weighed. The degree of in vitro degradation was calculated according to the following formula:

$$\text{in vitro degradation} = \left(\frac{W_0 - W_t}{W_0}\right)_{\text{lysozyme}} - \left(\frac{W_0 - W_t}{W_0}\right)_{\text{control}} \times 100,$$

where W_0 is the dry weight before the degradation test and W_t is the dry weight at time t. To discriminate between degradation and dissolution, control samples were stored for 7 days under the same conditions as the test samples, without the addition of lysozyme. Each determination was performed in triplicate.

2.4.1. Antioxidant activity

2.4.1.1. DPPH test. The ability of the scaffolds to react with oxygen-containing free radicals was assessed by the diphenylpicryl hydrazyl (DPPH) test adapted from Cullen et al. (2002). Briefly, different amounts of the materials tested were placed in 5 ml aliquots of a methanolic solution containing DPPH (the solutions were prepared fresh, and the absorbance at 540 nm was adjusted to 1.0), and the samples were shaken in the dark at 37 °C. The change in colour was monitored spectrophotometrically at 540 nm over a 6 h period. Measurements were performed hourly, and the scavenging effect was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = \left[1 - \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}}\right] \times 100.$$

The EC_{50} value is calculated by linear regression and is defined as the mass of sample material needed to reduce the initial absorbance at 540 nm of 1 ml of the DPPH solution by 50% after 2 h of incubation. The control consisted of the reagent solution without the material tested. Two commonly used medical dressings (gauze dressing and hydrocolloid alginate dressing) were used for comparison. Lower EC_{50} values indicate better ability to remove oxygen-containing free radicals from the test material. Each determination was conducted in triplicate.

2.4.1.2. ABTS assay. The total antioxidant capacity was evaluated according to the modified ABTS assay (Re et al., 1999). The $ABTS^{\bullet+}$ radical cation solution [(2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid))] was generated from ABTS salt (potassium persulfate (2.45 mmol) was reacted with 7 mmol of ABTS salt in 0.01 M PBS, pH 7.4, for 15 h at room temperature in the dark). The solution containing the resultant ABTS radical cations was diluted with methanol to give an absorbance of 1.0 at 734 nm. Different amounts of the material tested were suspended in 5 ml aliquots of the $ABTS^{\bullet+}$ solution, and the samples were shaken in the dark at 37 °C. The change in colour was monitored spectrophotometrically at 734 nm over a 6 h period. The scavenging effect and the EC_{50} value were calculated as described above.

2.4.1.3. Fe(II)-chelating ability. The Fe(II)-chelating activity of the chitosan–protein scaffolds was measured according to the modified method by Yen, Yang, and Mau (2008), where the Fe(II)-chelating ability was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, different amounts of the material tested were suspended in 4 ml aliquots of a methanolic solution containing $FeCl_2$ (2 mM) and ferrozine [5 mM, the absorbance (562 nm) of which had been adjusted to 1.0]. The samples were shaken at 37 °C for 60 min. The chelating effect and EC_{50} values were calculated as described above.

2.4.2. Biocompatibility tests

2.4.2.1. Cell culture. NIH-3T3 cells (a mouse embryonic fibroblast cell line) were obtained from the American Type Culture Collection (ATCC). The cells (mycoplasma free) were maintained in a monolayer culture at 37 °C in a humidified 5% CO_2 atmosphere in high-glucose Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich). The cell-doubling time under these conditions was 24 h.

2.4.2.2. Cell viability and morphology assessment. The cytotoxicity of the chitosan–protein scaffold was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The material tested (5 mm × 5 mm × 3 mm) was sterilized in 75% ethanol for 30 min, rinsed 5 times in sterile water for 5 min, and then placed into 24-well plates. Growth medium (2 ml) was added to each well. Next, the NIH-3T3 cells, at a density of 2×10^4 cells/well, were seeded in the 24-well plate with or without the material sample. Additionally, 2 negative controls were prepared: one with culture medium only and another with medium and material sample. After 72 h of incubation, the cell viability and morphology were assessed. Photographs of the control cells, pure material, and material with the cells were acquired using a light microscope (TELAVAL 3, Carl Zeiss, Jena, Germany). The attached and proliferated cells were quantified using the MTT assay. The experiments were repeated at least 3 times.

3. Results and discussion

3.1. Preparation of chitosan–protein scaffolds

Chitosan–collagen–gelatin scaffolds were fabricated according to the process illustrated in Fig. 1. Fish skin was chosen as a source of collagen and gelatin for several reasons: (1) lack of threat of prion

protein infection, (2) lack of alternative methods of fish skin processing in relation to bovine or porcine hides, which makes it easily accessible and relatively inexpensive, (3) desirable chemical properties, e.g. greater antioxidant potential. Gelatin is water soluble and its presence facilitates control of release of any active agents, that can be immobilized in the scaffolds (Kawadkar, Jain, Kishore, Pathak, & Chauhan, 2013).

The relative content of chitosan, collagen, and gelatin was optimized, and the best composition was selected for further characterization. The optimization process relied on the assessment of the chitosan–protein blend gelling process, mechanical properties, and the dissolution profile of the final dried material. When the protein component was present in excess, gel heterogeneity was observed after completion of the gelling process. Therefore, the protein content was maintained slightly higher than the chitosan concentration (at a level where no gel stratification occurred), due to the superior and well-documented benefits of collagen- and gelatin-containing materials on the wound healing process. The chitosan content was also limited by the viscosity of the blends prepared by CO_2 saturation. Aqueous solutions containing more than 2% chitosan were so viscous that mechanical stirring was impossible. At each stage of collagen processing, the temperature was maintained below 12 °C to prevent the loss of the collagen tertiary structure but not lower than 8 °C, which was efficient for cross-linking with genipin. During the incubation, the colour of the materials changed from white to dark blue (depending on the genipin concentration), which was associated with additional polymerization reactions between genipin molecules (Butler, Yiu-Fai, & Pudney, 2003). Freeze-drying of the previously frozen hydrogels lead to the formation of dry chitosan–protein scaffolds that did not contain acids (CO_2 was also removed during freeze-drying). This was confirmed in the swelling capacity test (no pH change was observed for the liquid).

3.2. Cross-linking degree

The cross-linking degree was 7.29 ± 1.43%, 13.29 ± 1.94%, and 27.64 ± 2.20% when cross-linking was conducted with 0.5%, 1.0%, and 2.0% (w/w) genipin, respectively. This observation is consistent with the results obtained by Wang et al., who tested collagen/chitosan materials obtained using acetic acid blends (Yan et al., 2010). Genipin is a natural compound obtained from

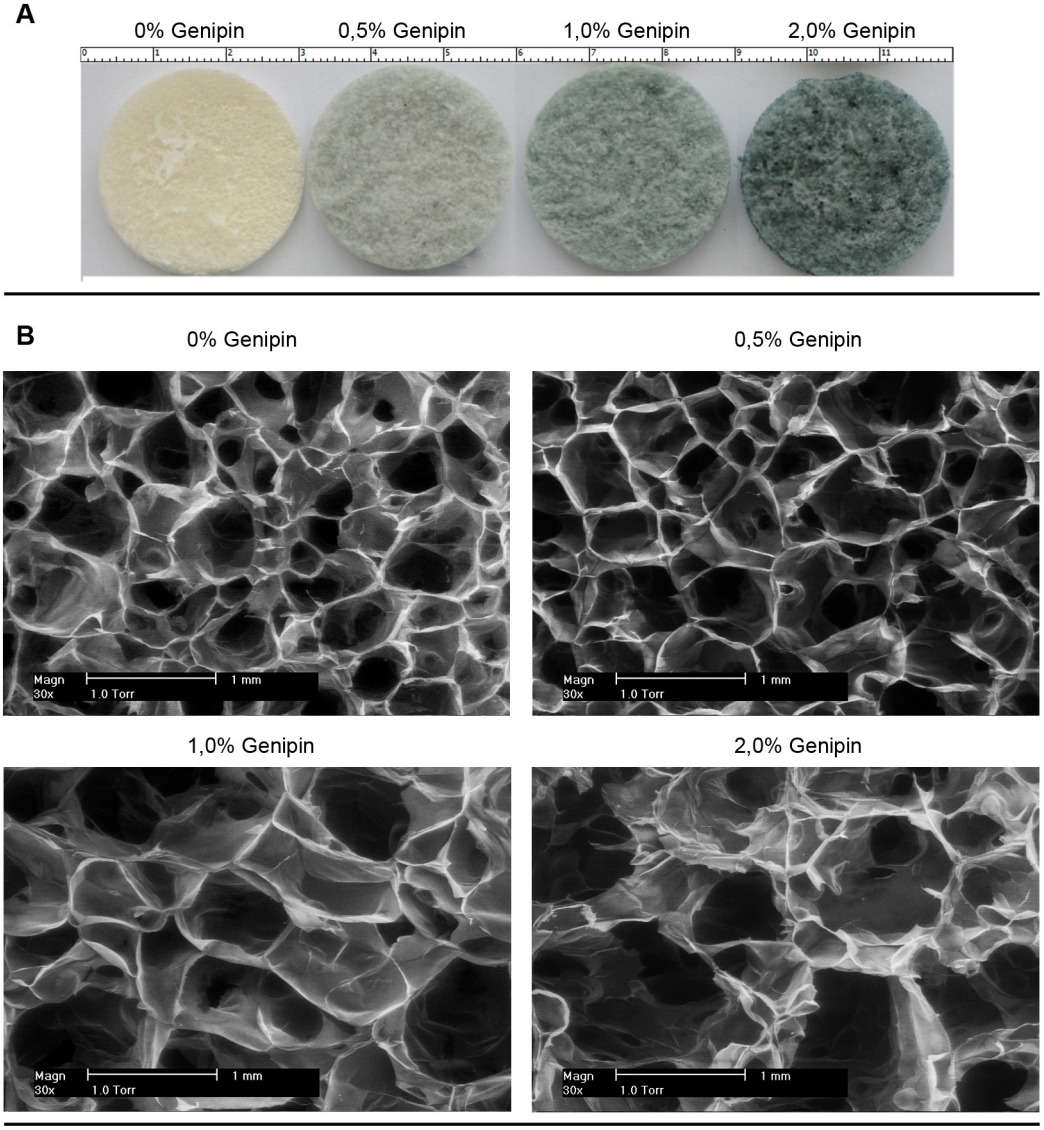

Fig. 2. Images of chitosan–protein scaffolds cross-linked with different concentrations of genipin (panel A). Cross-sectional SEM images of the chitosan–protein scaffolds (panel B).

Table 1
Average cross-sectional areas of the pores and porosity of the chitosan–protein scaffolds cross-linked with different concentrations of genipin.

Genipin concentration [%, w/w]	Cross-sectional area of the pores [μm^2]	Porosity [%]
0.0	187.9 ± 101.0	25.75 ± 1.47
0.5	274.6 ± 123.8	33.13 ± 1.30
1.0	533.9 ± 259.8	39.95 ± 1.25
2.0	1066.4 ± 396.7	44.75 ± 1.50

geniposide after enzymatic hydrolysis. Because of its lower cytotoxicity when compared with the commonly used glutaraldehyde, genipin is now being considered as a first of the choice cross-linking agent for biomaterials used as medical devices (Muzzarelli, 2009b). The mechanism of cross-linking has been extensively studied (Butler et al., 2003; Mu, Zhang, Lin, & Li, 2013) and a detail systematic study of genipin cross-linking effects on chitosan–gelatin scaffolds has been recently described (Sarem, Moztarzadeh, & Mozafari, 2013). The use of a cross-linking agent was necessary to maintain the 3D structure of the chitosan–protein materials obtained using the chitosan–CO_2 blend because the samples not subjected to cross-linking were flat due to low porosity.

3.3. Morphology of the chitosan–protein scaffolds

Fig. 2, panel A, shows the chitosan–protein scaffolds cross-linked with genipin at different concentrations. More bluish scaffolds were obtained as the genipin content increased. The formation of a blue pigment is the result of oxygen-radical-induced polymerization of genipin(Butler et al., 2003). SEM showed that pores inside the scaffolds were interconnected in irregular patterns, and the pore size was dependent on the genipin concentration (Fig. 2, panel B). Interestingly, as the genipin concentration increased from 0.5% to 2.0% (w/w), the pore cross-sectional area increased by 180 and 1100 μm^2 compared with the non-cross-linked scaffold (Table 1). To confirm this observation, the porosity of each scaffold was also estimated. The increased porosity with increased genipin concentrations showed a similar pattern. This is not a common result obtained by cross-linking treatment. In most cases, an increase in cross-linker concentration results in a material with smaller pores, similar to what occurs with glutaraldehyde and other types of genipin cross-linking (Chen et al., 2009; Yan et al., 2010). Such a phenomenon was also observed previously by the group of Han, when cross-linked chitosan–collagen scaffolds were prepared using an acetic acid blend (Bi et al., 2011). The mechanism of this unexpected observation is unknown, but we hypothesize that a genipin-dependent increase in the average cross-sectional area of the pores may be due to more favourable conditions for ring-opening polymerization of genipin and its long-range cross-linking effect on polymeric blends, which has been shown by the group of Li (Mu et al., 2013). The irregular structure of the scaffold walls, observed in the SEM images for the scaffold cross-linked with 2% (w/w) genipin, together with the mechanical properties, swelling, and dissolution tests, indicate that a genipin concentration greater than 2% can lead to the loss of the use-values of the chitosan–protein scaffold, which are needed in wound dressing applications (scaffolds cross-linked with 3% genipin are very fragile). Irregular porosity is mostly caused by the freeze-drying and sample preparation conditions. After ice crystals sublimate during freeze drying, the scaffold structure contains pores that correspond to the size and shape of the ice crystals (Hu et al., 2010). All scaffolds prepared in this study were freeze-dried at the same time and under the same conditions; thus, the difference in the pore structure is associated only with the cross-linking agent concentration.

3.4. Swelling/dissolution tests

The swelling capacity of the scaffolds cross-linked with different genipin concentrations is shown in Fig. 3, panel A. The cross-linked scaffolds had a higher swelling capacity compared with the corresponding non-cross-linked scaffold because of the weak mechanical properties of the non-cross-linked scaffold. A stable 3D structure was obtained when cross-linking with genipin, even at the lowest level (0.5%). Furthermore, it was shown that a further increase of the genipin concentration resulted in a reduced swelling capacity of the scaffold, which was associated with increased hydrophilicity of the material due to the increased cross-linking degree. The swelling capacity of natural polymer-based materials can be affected by the ionic strength of the test medium. Most research groups characterize their materials in water- or PBS-based media (Lin et al., 2009; Yang et al., 2010). These media do not contain multivalent cations, the presence of which is associated with “ionic cross-linking” at the surface of the material, causing an appreciable decrease in the swelling capacity of the wound exudate (Sadeghi, 2010). Fig. 4, panel B, shows a >2-fold difference in the swelling capacity of the chitosan–protein scaffold cross-linked with 0.5% genipin measured in distilled water vs. a medium with a salt composition similar to that of wound exudate (according to EN 13726-1). These results emphasize the importance of selecting swelling medium conditions that are similar to the conditions of the applied material. As the amount of entrapped water in the chitosan–protein scaffolds increased during the swelling tests, these materials changed texture from soft sponge to the elastic hydrocolloid, which mimicked the skin tissue texture. This texture change is a desired behaviour for wound healing because it may increase the contact area between the material and the

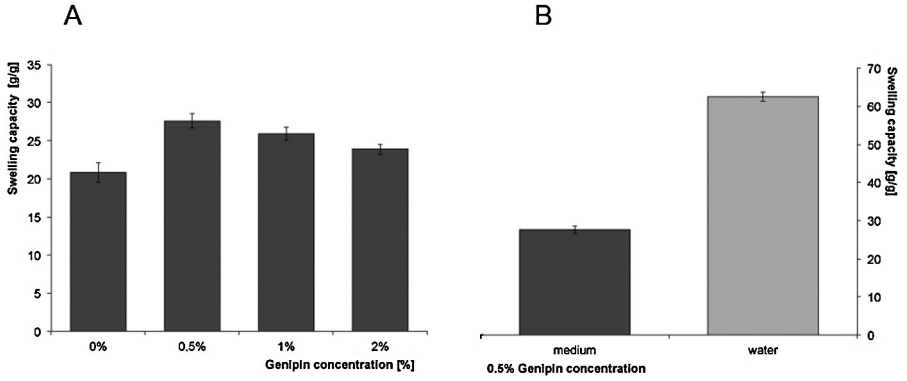

Fig. 3. (A) Swelling capacity of the non-cross-linked and cross-linked chitosan–protein scaffolds in a medium with a salt composition similar to that found in wound exudate. (B) Comparison of the swelling capacity in water and the test medium for a chitosan–protein scaffold cross-linked with 0.5% (w/w) genipin.

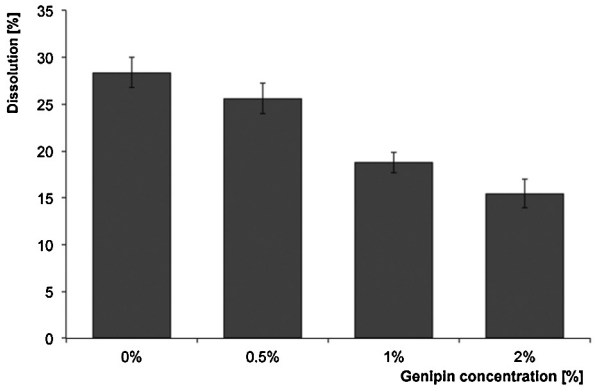

Fig. 4. Dissolution of the non-cross-linked and cross-linked chitosan–protein scaffolds in a medium with a salt composition similar to that found in wound exudate.

wounded tissue and may also provide a better barrier against secondary infections. This ability of wound dressings can also affect the economic aspects of wound healing management. Materials with better wound exudate binding capacity need to be replaced less frequently, which decreases the treatment cost. A comparison of our results with those reported for other chitosan-protein materials obtained from standard acidic acid blends (Tangsadthakun et al., 2007) shows that our technique results in a 2–3-fold increased swelling capacity. Other important aspect of the scaffold as a potential wound dressing is its ability to maintain the structure and integrity during the period of use. Dissolution degrees for the chitosan–protein scaffolds cross-linked with different genipin concentrations are presented in Fig. 4. An increased genipin concentration decreased the scaffold dissolution as the cross-linking degree increased. Dissolution profiles of the chitosan–protein scaffolds cross-linked with 0.5% and 2.0% genipin shows that samples with 0.5% genipin reached a maximum weight after 72 h, with a dissolution level of 32% (Fig. 5). After 72 h, a significant loss of weight was not observed. The sample cross-linked with 2% genipin dissolved slower, and after 120 h, this material had lost only 23.3% of its original weight. Because chitosan and collagen are insoluble in water, at the pH of the experiment, the weight loss is related mainly to the dissolution of gelatin. The equilibrium reached after a few days of incubation, with reference to the sample cross-linked with 0.5% genipin, is comparable to the total gelatin content in the formulated scaffolds [36% (w/w)]. Dissolution of the scaffold is likely mainly due to the presence of gelatin, but the temperature of the medium was sufficiently high for collagen denaturation. Under these conditions, non-cross-linked collagen chains could lose their tertiary structure and become more accessible for dissolution. Thus,

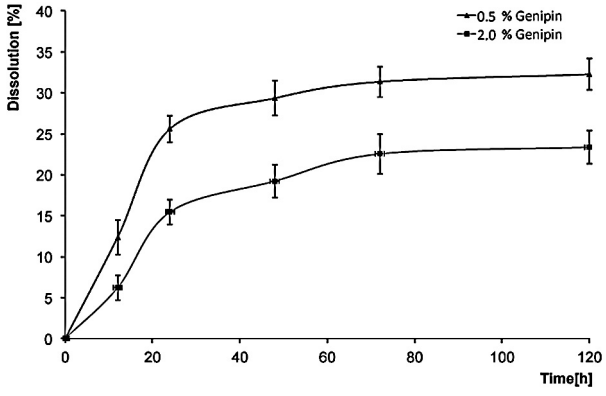

Fig. 5. Dissolution profiles for chitosan–protein scaffolds cross-linked with 0.5% and 2.0% genipin.

balance between the two effects likely determines the final dissolution degree. Regardless of the weight loss of each cross-linked scaffold, samples maintained its integrity, which is essential for wound healing.

3.5. WVTR

To ensure an appropriately moist environment for wound healing, the wound dressing should possess both good absorption capacity and a suitable WVTR to prevent excessive dehydration as well as build-up of exudate. The normal rate of water loss from the skin is approximately 200 g/m^2 per day, and the water loss increases when the skin is wounded, even to levels >5000 g/m^2 (Mu et al., 2013). The WVTR for samples crosslinked with genipin at concentration of 0.0%, 0.5%, 1.0%, and 2.0% was respectively: 17.52 ± 0.64; 17.83 ± 0.76; 16.95 ± 0.55; 17.02 ± 0.89 g/(h m^2). This means that cross-linking with genipin did not affect the WVTR. These materials can ensure a daily water vapour transmission rate at 400 g/m^2, which does not affect the normal rate of water loss from the skin.

3.6. Texture profile

Compression tests were performed using samples cross-linked with different genipin concentrations to assess the mechanical strength of the materials. The values of the compressive stress of the dry scaffold increased from 12.11 kPa to 29.17 kPa as a result of an increased genipin concentration from 0% to 2.0%, which indicated that the cross-linking reaction increased the mechanical strength of the scaffold due to the formation of chemical bonds (Fig. 6, panel A). These observations are in apparent contrast with those obtained from the morphology study, where it was shown that an increased genipin content increased the porosity and pore size of the scaffolds, which should therefore lead to a reduced mechanical strength of the scaffold. However mechanical strength of a porous scaffold is determined by the balance between (1) a decreased mechanical strength caused by a gradually increased pore size and (2) an increased mechanical strength due to the cross-linking reaction (Bi et al., 2011). For the cross-linked samples, the enhanced mechanical strength due to chemical cross-linking was stronger than the inhibition caused by the increasing pore size; thus, we observed an increased mechanical strength with increased genipin concentrations. When swollen scaffolds were analyzed after 24 h of incubation in water (Fig. 6, panel B), the compressive stress value was highest for the non-cross-linked sample, and decreased as the genipin concentration increased. These results indicate that the balance is influenced by the environment (i.e., filling of the pores). When the scaffolds were in a swollen state, pores were filled with water, and the pore size contributed more to the mechanical strength than the effect of the cross-linking reaction. To assess the durability of the chitosan–protein biomaterials, we also performed a fatigue test by continuous compression in a swollen state (25 times to 50% deformation) (Fig. 6, panel C). Each cross-linked scaffold retained its 3D structure, and the initial compressive stress value was maintained. These scaffolds also held most of the water within their structures. Cross-linked scaffolds lost comparable amounts of water (approximately 6%), while the non-cross-linked scaffold lost 7 times more. After the test, the non-cross-linked sample did not retain its 3D structure and had lost approximately 50% of its initial height. This result indicated that the cross-linked chitosan–protein scaffold should maintain appropriate mechanical properties during application in wound healing.

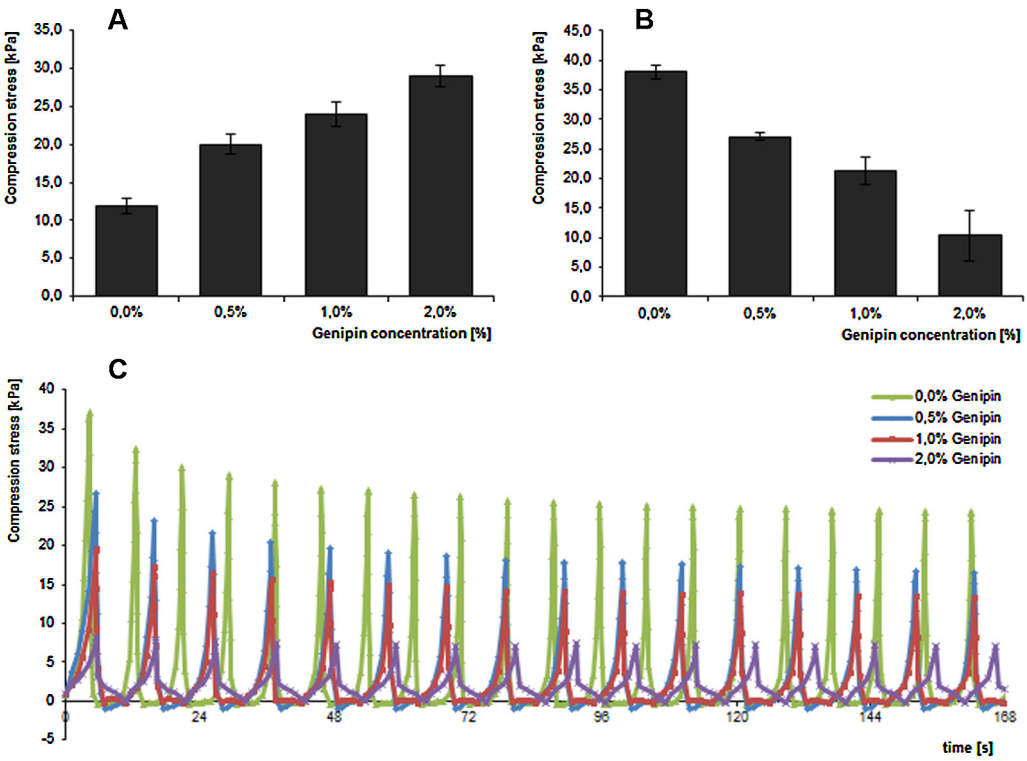

Fig. 6. Texture parameters of chitosan–protein scaffolds cross-linked with different genipin concentrations (A and B). Fatigue test (C).

3.7. *Biodegradation studies with lysozyme*

To evaluate the enzymatic degradation of the scaffolds, we selected the scaffold cross-linked with 0.5% genipin, which had the most promising material parameters, including swelling, dissolution, and texture. The weight loss [%] of the scaffolds during a 7-day test period is shown in Table 2. The values listed in the table have been corrected for the effect of dissolution of the materials in PBS and therefore represent the loss of weight caused by hydrolytic action of lysozyme. The final concentration of lysozyme

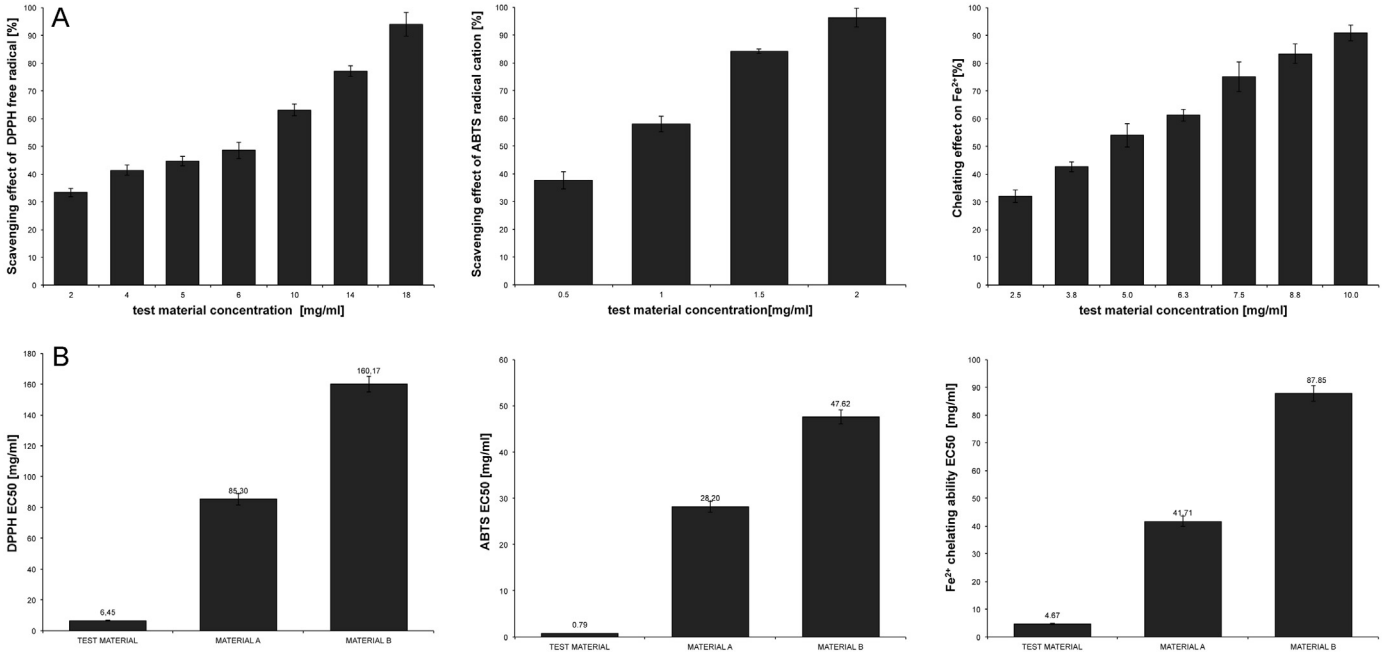

Fig. 7. Scavenging effect of the chitosan–protein scaffold cross-linked with 0.5% genipin on DPPH free radical, ABTS radical cation, and chelation of ferrous iron at different concentration of the test material (panel A). EC_{50} values (panel B) for a chitosan–protein scaffold compared with 2 commonly used wound dressings (Material A – alginate hydrocolloid dressing, Material B – cotton gauze dressing).

Table 2
Resistance of scaffolds cross-linked with 0.5% genipin to biodegradation by lysozyme.

Time [h]	Biodegradation [%]
24	1.05 ± 0.06
48	1.94 ± 0.36
72	2.37 ± 0.71
120	4.08 ± 1.01
168	5.78 ± 0.45

was selected to correspond to the enzyme activity of 4000 U/ml, similar to that found in wound exudate from a bacterial infection (Hasmann et al., 2011). A biodegradation test depicted that only a small percentage of weight loss is associated with lysozyme activity.

3.8. Antioxidant activity

The process of wound healing can be negatively affected by various factors: continuous inflammation, pathogen infection, and oxidative stress. Oxidative stress is caused by the imbalance between oxygen species and the antioxidant defence mechanisms of an organism (Wiseman & Halliwell, 1996) and is associated with the excessive activity of free radicals and extracellular metal ions (Waddington, Moseley, & Embery, 2000). To evaluate the antioxidant potential of our chitosan–protein scaffolds, 3 widely used tests were adapted, and the scaffold cross-linked with 0.5% genipin was chosen as a sample. The dose-response graphs for the various tests are shown in Fig. 7, panel A. In all 3 tests, the tested material revealed its ability to effectively remove free radicals or chelate ferrous iron. Graphs in panel B represent the calculated EC_{50}, which is the concentration required to the decrease the initial DPPH, ABTS, or Fe^{2+} concentration by 50% within the measured time. These graphs also show a comparison of the EC_{50} values of our scaffolds with those of two commonly used wound dressings (alginate hydrocolloid dressing–material A, cotton gauze dressing–material B). In each test, the chitosan–protein scaffold revealed significantly higher antioxidant activity than the control materials. These findings are the result of the cumulative effect of all 3 natural polymers used. The antioxidant properties of chitosans, gelatins, and collagens isolated from different sources have been reported in several papers (Alemán, Giménez, Pérez-Santín, Gómez-Guillén, & Montero, 2011; Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Nam, You, & Kim, 2008; Rajapakse, Mendis, Byun, & Kim, 2005). Although the antioxidant activity of the chitosan–protein scaffold is much lower than

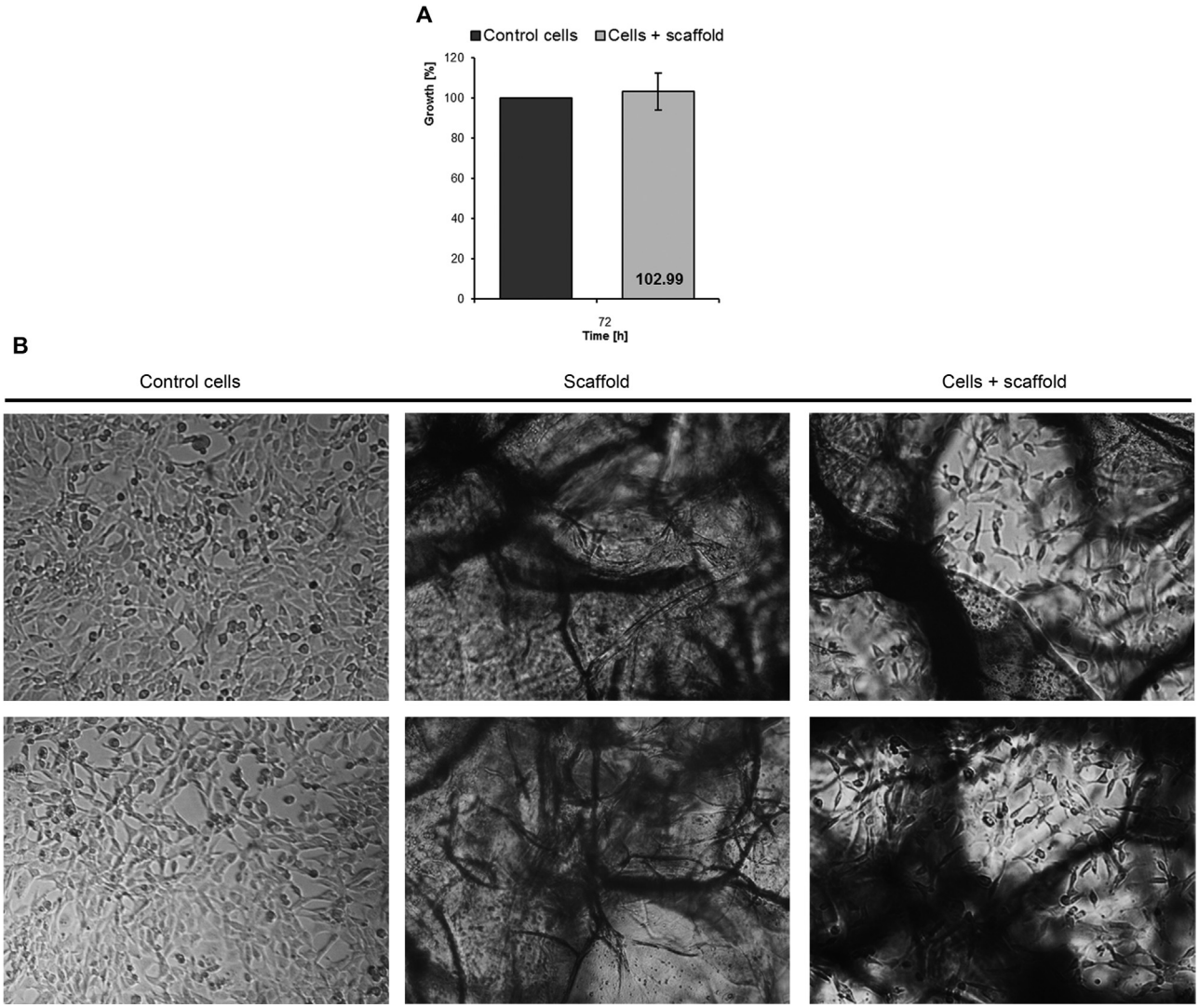

Fig. 8. MTT viability assay of fibroblast NIH-3T3 cells on the chitosan–protein scaffold cross-linked with 0.5% genipin after 72 h of incubation (panel A). Optical microscopy images (150x) of fibroblast NIH-3T3 cells (negative control) and fibroblasts cultured in the presence of the chitosan–protein scaffold (panel B).

that of low-molecular-weight antioxidants such as glutathione, vitamin E, vitamin C, and the flavonoids, our results indicate that the chitosan-protein materials prepared in this study can provide suitable conditions for wound healing. During the wound healing process, moderate antioxidant activity is desired because it has been shown that complete depletion of reactive oxygen species results in impaired healing, similar to when excessive amounts of reactive oxygen species are present (Roy, Khanna, Nallu, Hunt, & Sen, 2006; Sen, 2003).

4. Biocompatibility

The in vitro cytotoxicity of the chitosan–protein scaffold cross-linked with 0.5% genipin has shown that the introduction of the test material to the growth medium did not induce cytotoxicity against mouse NIH 3T3 fibroblasts, and the cells viability was at the level of the control (Fig. 8, panel A). Panel B, shows the morphology of fibroblasts cultured in the presence of the chitosan–protein scaffold after 3 days, compared with the control (scaffolds were sliced to obtain thin layers). The fibroblasts grew uniformly in the pores of the chitosan–protein structure. This finding may be explained by the excellent swelling ability of the scaffold as well as the even distribution of collagen, to which cells have high affinity. The results obtained showed that the cells could proliferate and maintain their fibroblast morphology while in contact with the chitosan–protein scaffold.

5. Conclusions

The use of CO_2 instead of organic or inorganic acids for chitosan blend preparation allowed us to obtain stable materials with properties relevant for wound healing application. Elimination of acid solvents at the beginning of the process allowed elimination of the time- and cost-consuming processes associated with residual acid removal and re-drying. The concentration of genipin most influenced the material properties, including colour. Based on the cross-linking degree, swelling capacity, morphology, and mechanic strength, the optimal concentration of genipin for cross-linking of the chitosan–protein scaffold was 0.5% (w/w). Using this concentration, the balance of all critical parameters appeared to be achieved, and the colour of the material was light blue-green (a dark blue colour formed during cross-linking with 2% genipin). Colours that are similar to that of skin are more acceptable to patients, adding to the value of the obtained material with 0.5% genipin. In summary, we demonstrated that the presented chitosan–protein scaffold has the basic desirable properties for wound healing application. Notably, this material should ensure effective exudate management, which is a key wound dressing requirement. The scaffold components endowed antioxidant properties and were not cytotoxic; therefore, the chitosan–protein scaffold should accelerate the healing process. Future research will be pursued to further evaluate the properties of the chitosan–protein scaffold.

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