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Mechanical and structural response of a hybrid hydrogel based on chitosan and poly(vinyl alcohol) cross-linked with epichlorohydrin for potential use in tissue engineering

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The development and characterization of a hybrid hydrogel based on chitosan (CS) and poly(vinyl alcohol) (PVA) chemically cross-linked with epichlorohydrin (ECH) is presented. The mechanical response of these hydrogels was evaluated by uniaxial tensile tests; in addition, their structural properties such as average molecular weight between cross-link points (M_{crl}), mesh size (D_N), and volume fraction (v_s) were determined. This was done using the equivalent polymer network theory in combination with the obtained results from tensile and swelling tests. The films showed Young's modulus values of 11 ± 2 MPa and 9 ± 1 MPa for none irradiated and ultraviolet (UV) irradiated hydrogels, respectively. The cell viability was assessed using Calcein AM and Ethidium homodimer-1 assay and environmental scanning electron microscopy. The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan thiazolyl blue formazan (MTT Formazan assay) results did not show cytotoxic effects; this was in good agreement with nuclear magnetic resonance and fourier transform infrared spectroscopies; their results did not show traces of ECH. This indicated that after the crosslinking process, there was no free ECH; furthermore, any possibility of ECH release in the construct during cell culture was discarded. The CS-PVA-ECH hybrid hydrogel allowed cell growth and extracellular matrix formation and showed adequate mechanical, structural, and biological properties for potential use in tissue engineering applications.

Keywords: chitosan hydrogel; mechanical properties; tissue engineering; scaffold

1. Introduction

'The need is real; 116,839 people are waiting for an organ and 18 people of them will die each day waiting for an organ.'[1] The need for tissue or organs according to the statistics, reported by Organ Procurement Transplant Network and Scientific Registry of Transplant Recipients, is increasing nowadays.[1] Although the number of donors has also increased, the host often presents some complications such as infections or even

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rejection of the tissue after transplantation.[2] To avoid this, tissue engineering (TE), a multi-disciplinary scientific field, emerged last few decades ago. TE has focused in developing 2D and 3D scaffolds for organ/tissues replacement. Considering a cell biology perspective, the scaffolds should be seen as extracellular matrix (ECM) biomimetic structures; furthermore, the main objectives of scaffolds are: (a) defining a space to mold a regenerative tissue, (b) to substitute tissue functions temporary, and (c) to guide the tissue in-growth; as it has been previously reported.[2–4] To produce conventional scaffolds, several techniques have been used such as particulate leaching, gas foaming, fiber networking, phase separation, melt molding, emulsion freeze drying, solution casting, freeze drying, and combination of those.[2,5] All of them have tried to satisfy the basic requirements of scaffolds which are: appropriate porosity – for optimal nutrient waste flow, pore size – ranged between 5 and 10 times the cell diameter, and also an adjustable biodegradation rate – to preserve mechanical stability – during the in-growth tissue; nevertheless, one disadvantage of these techniques involve low and inhomogeneous mechanical properties. That is why a continuous effort to enhance mechanical properties of scaffolds has been done in biomaterials. Hydrogels are not the exception; chitosan (CS) and poly(vinyl alcohol) are polymers that have been used separately or as a blend of them in biomedical applications.[6–9] In recent years, hydrogels have represented an excellent alternative as scaffolds in TE because they are similar to extracellular matrix of many tissues. This application implies that these materials must be biocompatible, permeable, and hydrophilic, and they must also have appropriate mechanical properties, which are required in medical applications.[10,11]

Some reports have studied the biocompatible character, and chemical and physical properties of hydrogels based on CS.[12–15] It is known that the CS provides excellent biocompatible, biodegradable, and antimicrobial properties; nevertheless, the CS is partially crystalline; it means that CS is a brittle material with a weak strain at break. To enhance the mechanical properties of CS, the poly(vinyl alcohol) (PVA) is added. Some authors have carried out research to understand the mechanical, thermal, and biological response of CS-PVA blend.[16–18] According to results reported in literature, it is clear that CS-PVA blend provides a material with potential for biomedical applications.[7,19]

An interesting application of CS-PVA blend was reported by Alhosseini et al. [20] They used the CS-PVA blend to develop neural tissue by electrospinnig process and they proved that the addition of small quantity of CS to PVA scaffolds improved the viability and proliferation of nerve cells.[20] Although the CS-PVA blend is commonly used in biomedical applications, some of them require better mechanical properties than what the CS-PVA blend can offer. Some efforts have been carried out to improve CS-PVA mechanical properties in order to render them useful in biomedical applications such as drug delivery vehicles or scaffolds in TE.[21–23] Physical and chemical cross-linking processes have shown to improve mechanical properties of CS-PVA materials [24,25]; particularly, some studies, related to mechanical and structural properties of CS-PVA hydrogels, report the use of glutaraldehyde (GA) as a cross-linker reagent to enhance their mechanical behavior.[21–23,26,27]

Genipin is another cross-linker reagent that has been recently used to improve the mechanical response of CS-PVA blend [28]; Bispo et al. showed that the CS-PVA blend cross-linked with Genipin had good biocompatible properties and also they found that increasing the content of CS relative to PVA, the swelling index of the blend decreased; reflecting the reduction on the mobility of polymer network and the hydrophilic behavior of the blend.[28]

Although there is significant knowledge about CS-PVA blends; nobody has evaluated the influence of chemical and physical cross-linker reagents on the structural parameters, global mechanical behavior, and the cell viability of CS-PVA-ECH hybrid hydrogel films.

The aim of this work is to provide a simple methodology to determine the structural and mechanical variations of a suitable hybrid hydrogel for elastic cartilage regeneration based on chemically (ECH) and physically (UV irradiation) cross-linked CS-PVA blend. We have evaluated the influence of chemical and physical cross-linker reagents on the global mechanical behavior and the cell viability of CS-PVA-ECH hydrated films. Our methodology considers the shear modulus obtained from a uniaxial tension test and then with the combination of well-established theories such as rubber elasticity, viscoelasticity, and polymer network concepts [29,30] it was possible to determine the influence of cross-linker reagents on the structural and mechanical parameters of CS-PVA-ECH hydrated films.

2. Materials and methods

Our studies were performed using a blend of synthetic and natural polymer based on CS and PVA. The Hydrogel was synthesized using PVA and CS; subsequently, it was cross-linked with epichlorohydrin (ECH). The PVA had an average molecular weight (M_w) of 89,000 Da and 99% hydrolysis (Fluka; Buch, Switzerland). The CS had a medium molecular weight ca. 350,000 Da and a deacetylation $\geqslant$ 75.0%. We also used an analytical grade ECH (99%). Both CS and ECH were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.1. Sample preparation and cross-linking process

A 7.8% (w/v) aqueous solution of PVA was prepared by dissolving the PVA in hot distilled water at 80 °C during three hours. The CS solution was prepared by dissolving the CS in acetic acid 0.4 M (about 2.5% w/v) by stirring for 24 h to obtain a homogeneous suspension. Then, this suspension was stirred at 1500 rpm during 30 min to dissolve the CS completely. A polymer blend of CS/PVA (2:1.5 ratio) was mixed and stirred for three hours at room temperature. Then, catalytic aqueous medium (NaOH 1 M, 1.0 mL) and the cross-linker reagent (ECH) were added to the polymer blend and stirred for one hour at 40 ± 2 °C under a nitrogen atmosphere. The final concentration of ECH in the polymer blend was about 0.25 vol %. Then, the cross-linking polymer solutions were poured onto plastic Petri dishes (5.0 g); they were subsequently dried at 37 °C for 72 h. Finally, the films were carefully detached from the dishes and stored in a desiccator until testing.

2.2. Physical cross-linking agent of CS-PVA-ECH films

Since these films are intended to serve as scaffolds in TE, part of the synthesized materials were exposed to UV irradiation to sterilize them. This process generates a photo-cross-linking effect in polymers that could significantly modify their structure and mechanical behavior.[31] The irradiation cycle took place in a CL-1000 (254 nm) cross-linker (UVP, Upland California, USA); each sample was irradiated for 40 min. All the experiments performed in this work included a group of irradiated hydrogels, in order to determine possible changes in mechanical and structural properties of the CS-PVA-ECH hydrogels during the sterilization cycle.

2.3. Swelling test

The swelling properties of CS-PVA-ECH hydrogels were studied by immersing the samples in phosphate buffer solution (PBS) (pH 7.4) at 37 °C, simulating a bodily fluid. The dried hydrogel films were cut into small squares with 10 mm by side. Ten samples were used (five samples without UV irradiation and five with UV irradiation, CS-PVA-ECH and CS-PVA-ECH UV, respectively). At predetermined intervals of time,[1] the specimens were removed from the PBS and carefully dried using filter paper. The free water was removed so that the interstitial water was trapped only in the polymer network [32]. Subsequently, the samples were weighted and then returned to the same container until the equilibrium was reached. This procedure was useful to register the water absorption capacity of the hydrogel as a function of time. The percentage of water uptake was calculated using Equation (1) [33]:

$$\text{Water uptake} = ((w_s - w_d)/w_d) \times 100 \quad (1)$$

where w_s is the weight of swollen hydrogel at different swelling time and w_d corresponds to the weight before hydration. The pH of the buffer solution was measured by a pH meter (Series Φ340, Beckman Coulter, USA) at the same time intervals once the hydrogels were immersed in the solution; hence the pH variations as function of time were also registered.

2.4. Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H NMR)

The hydrogels were obtained as films (100 μm of thickness) and they were characterized using the FTIR spectroscopy in order to find the polymer chemical groups and interactions (CS and PVA) and to evaluate the formation of cross-linked networks from the films with ECH. FTIR spectroscopy was performed on dry samples by attenuated total reflectance (ATR); to measure this, it used a Spectrum GX, Perkin–Elmer, USA. The measured FTIR spectra were normalized and major vibration bands were identified and associated with the main chemical groups. In order to detect unreacted ECH (free ECH) in the films, the distiller water used in the washed process was analyzed by ^{1}H NMR. The ^{1}H NMR spectra were acquired using an AVANCE 400 spectrometer (Bruker, Switzerland) operating at the Larmor frequency (400.1718 MHz); the parameters were as follows: the acquisition time 2.5 s, pulse width 13.05 s, pulse delay 5 s, the number of scans 128, and sweep width of 10 kHz.

2.5. Mechanical characterization

2.5.1. Sample preparation for mechanical testing

Several rectangular strips of each material, CS-PVA-ECH hydrogels and CS-PVA-ECH UV, were carefully inspected by optical microscopy using an inverted microscope (Carl Zeiss Micro-Imaging, Thornwood, NY, USA) to select the samples for the mechanical tests; hence five rectangular strips (replicates, $n = 5$) of each material were mechanically tested but only three representative are shown. Before these were performed, the synthesized polymer films of CS-PVA-ECH and CS-PVA-ECH UV patches were hydrated according the procedure described in Section 2.3. The samples were subsequently cut with a special jig; the dimensions for all the samples were 60×20 mm

(length and width, respectively). The thickness of each sample was measured, along three different positions within the sample, with a Mitutoyo digital gage in order to obtain an average measurement for the thickness of the samples.

2.5.2. Equipment and mechanical analysis

The tensile tests were carried out in a servo hydraulic loading device (MTS 858 MiniBionix axial) according to ASTM D882-02 and F2150-02 standards.[34,35] The MTS machine has a 100 N load cell to measure the applied force during the uniaxial tensile test. The extension was measured using an LVDT sensor that was previously calibrated using a digital readout (IFD150HE Mitutoyo). The grips were specially designed and manufactured using stainless steel to hold the soft specimens firmly; additionally, soft marks were machined between two plates of the grips to press the samples, thereby avoiding any sliding of the samples between plates.[36] To control the MiniBionix MTS, a 407 MTS controller was used. National Instruments PXI-1002 chassis supported the data acquisition with the 6281 and 8331 PXI-boards. A virtual instrument (VI) was programmed in LabVIEW in order to synchronize and record the data by a PC.

The tensile tests were done at 24 °C using a strain rate of 8.33 mm·s^{-1} and a gage length of 26 mm. With the recorded displacement and force data, the macroscopic stress as a function of strain ratio ($\lambda - \lambda^{-2}$) curve was obtained for each case; here $\lambda = \varepsilon + 1$ and ε is the engineering strain. From these curves, the shear modulus (G) was calculated in the linear region of the curves by linear square fitting; finally, taking into account that the hydrogels can be considered as uncompressible materials, the Young's modulus (E) was calculated as $E = 3G$.[26,37–39]

2.5.3. Structural properties

The mechanical behavior of the hydrogels can be defined by three parameters: the average molecular weight between cross-link points (M_{crl}), the correlation length (D_N) – also known as the network mesh or pore size – [40] and the equilibrium polymer volume fraction in the swollen state (v_s).[41]

According to Section 2.5.2, the shear modulus can be used to calculate the structural features of the polymeric network. The average molecular weight (M_{crl}) was calculated using statistical theory, according to Equation (2) [27,37]:

$$M_{crl} = cRT/G \tag{2}$$

where c is the concentration (g m^{-3}) of CS and PVA in the cross-linking solution, T is the temperature in *Kelvin* at which the shear modulus was determined, and R is the gas constant (8.3145 J mol^{-1} K^{-1}). Additionally, D'Errico et al. [40] showed that the network mesh size (D_N) can be calculated by Equation (3) 'equivalent network model'. [42] The network D_N is an indicative of the average distance between consecutive cross-link points. According to Equation (3), (D_N) must be easily determined if the concentration (c) and the number average molecular weight (M_{crl}) are known.

$$D_N = \sqrt[3]{6M_{crl}/\pi Ac} \tag{3}$$

where A is the Avogadro number ($6.023 \times 10^{23}\,\mathrm{mol}^{-1}$).

Finally, the polymer volume fraction in the swollen state (v_s) for CS-PVA-ECH and CS-PVA-ECH UV hydrogels were calculated once the equilibrium was reached. These were obtained using the results of the swelling tests carried out previously. The swelling ratio at equilibrium (Q) is inversely proportional to the polymer volume fraction, (v_s), hence [35,43]:

$$v_s = Q^{-1} \tag{4}$$

All the structural properties were analyzed in quintuplicate using the shear modulus values obtained for each sample used in Section 2.5.2.

2.6. Biocompatibility test

Before the films were seeded, they were sterilized by UV irradiation and then they were washed several times with distilled water, PBS, and Dulbecco's Modified Eagle Medium (DMEM-F12). After this, 10 film samples (1 cm by side) were seeded with cells of auricular cartilage ($2 \times 10^5\,\mathrm{cell\,cm}^{-2}$) that was obtained from ears of New Zealand rabbits. This step was performed according to the guidelines of the Institutional Animal Care and Use Committee of Mexican Health Department, NOM 062-ZOO-1994. The cartilage was mechanically and enzymatically digested with 0.3% of collagenase (AC-3, ICN biomedicals, Costa Mesa, CA) at 37 °C. Cell cultures were maintained in DMEM-F12 with 10% FBS and 1% antibiotic/antimycotic mixture, (Gibco, BRL/Life Technologies, Grand Island, NY) in monolayer culture at 37 °C and 5% CO_2. After 12 days of *in vitro* culture, constructs (films seeded with rabbit chondrocytes) were evaluated by environmental scanning electron microscopy (FEI/Philips XL-30, OR, USA) using vacuum and back-scattered electrons (BSE) with 15–20 kV. In addition, an energy dispersive spectroscopy (EDS) was carried out involving five replicates per study condition.

The cell viability was assessed using Calcein AM and Ethidium homodimer-1 (LIVE/DEAD viability/cytotoxicity Kit L-3224; Invitrogen Gibco, NY). This provides two-color fluorescence cell viability based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters, green-fluorescent Calcein-AM to indicate intracellular esterase activity meaning cell viability, and red-fluorescent Ethidium homodimer-1 to indicate loss of plasma membrane integrity. Five constructs were incubated for 45 min at 37 °C with 0.1 µL Calcein AM (Invitrogen Life Science, Carlsbad, CA). Cells were also seeded onto tissue culture polystyrene plate as positive control for comparative analysis (the polymer without cell was used as a negative control). The samples were rinsed gently with PBS for 5 min and then they were fixed with 4% paraformaldehyde solution and finally they were observed under optical microscopy (Carl Zeiss Imager A1, Gottingen, Germany).

2.6.1. MTT assay

The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT Formazan assay; M2003, Sigma) was used as a measure of relative cell proliferation. The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells.[44]

The sample preparation was conducted with the films previously washed with PBS and DMEM-F12 and adhered by casting technique into 96-well microplate. Films with-

out cells were used as blank and chondrocytes cultured on polystyrene plate was used as a positive control. Chondrocytes were seeded at a density of 1×10^4 cells/well and cultured for 12 days at 37 °C, 5% CO_2, and 95% relative humidity. MTT was then added (0.5 mg mL^{-1}) for 4 h and it was culture in same conditions above mentioned, the medium was removed; then the samples were washed once with PBS and with fresh culture media. Finally, the formazan crystals were dissolved in DMSO:isopropanol solution (1:1); after removal of solution in a clean well plate, the absorbance reading was performed; and the absorbance of solution, without hydrogels, was measured at 550–600 nm using a Beckman Coulter spectrophotometric microplate reader to estimate the formazan concentration for over 12 days culture. The intensity of the absorbance is proportional to the number of living cells (cell proliferation).

3. Results and discussion

3.1. *Swelling test*

The hydrogels usually present a high hydrophilic behavior. This is an important feature of this kind of materials because the amount of liquid that they can absorb is directly related with their mechanical behavior. Figure 1(A) shows the water uptake percentage of the CS-PVA-ECH and CS-PVA-ECH UV hydrogels as a function of time; in addition, the changes in pH of the solution as a function of time were also measured during the experiment (Figure 1(B)); it was found that the pH value was equal to 7.20 ± 0.07. The curve of water uptake as a function of time describes the fluid absorption rate of the hydrogel. From this plot, we can observe drastic changes that occurred during the first hour for both materials. The CS-PVA-ECH films reached the maximum absorption at 10 min and the average of water uptake was 214%. The films irradiated with UV showed a lower ratio reaching a maximum absorption (167%) in five minutes. After this critical period, the absorption of both polymers decreased until equilibrium was reached

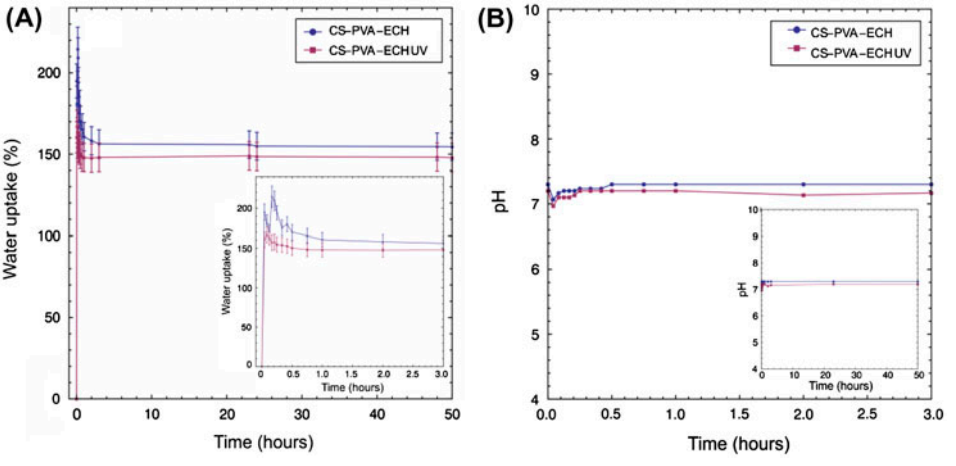

Figure 1. Swelling and pH behaviors of CS-PVA-ECH hydrogels. (A) Water uptake curves of the chemically crosslinked CS-PVA-ECH hydrogels (dots) and chemically–physically cross-linked CS-PVA-ECH UV hydrogels (squares); the water uptake percentages of CS-PVA-ECH and CS-PVA-ECH-UV hydrogels at equilibrium were 156% and 148%, respectively. (B) Curve of pH as function of time; in this case, changes in pH were registered during the experiment for both polymers and the average of pH was 7.20 with a standard deviation of ± 0.07.

at 156 and 148%, respectively. The difference between both groups of hydrogels can be explained by the double function of UV radiation (photo-crosslinking and sterilization). The UV radiation was used as a cross-linker reagent; therefore, the molecular chains were modified in length. This change affected the structural parameters of the polymer network, so that the entanglement of polymeric chains formed a more rigid network; as a consequence, the swelling percentage decreases.

The overshooting effect was present in the hydrophilic behavior of these hydrogels. This effect is characterized by a superabsorbent behavior at the initial stage of the swelling since in this moment the hydrogel present a maximum water uptake; then, a gradual deswelling is observed until the equilibrium is reached. Hence, the overshooting effect can be interpreted as the consequence of a swelling–deswelling process.[45]

The swelling–deswelling of a CS-PVA-ECH hydrogel can be well explained by Donnan swelling equilibrium. This is governed by the osmotic pressure gradient, inside and outside of the hydrogel, due to the electrostatic charges and counterion concentration built up inside the hydrogel. As the hydrogel swells and packed polymer coils expand, the osmotic pressure decreases; however, the elastic contraction forces, provided by the crosslinked network, increase. They eventually balance each other and the swelling equilibrium is reached.[46–48]

Also, the CS-PVA-ECH hydrogels have ionic groups (amino groups of CS) that can be protonated and deprotonated depending on the pH; these groups have a $pK_b \approx 6.4$. [47,49] In an aqueous medium of pH 7 ($>pK_b$) the CS presents a low charge density, so almost all $-NH_2$ groups are deprotonated and it can eventually form hydrogen bonds. Thus the swelling behavior of the polymer network is influenced only by an osmotic pressure gradient; however, as the fluid enters into the polymeric network, the groups $-NH_2$ of CS and $-OH$ groups of PVA and CS form hydrogen bonds internally and externally; thereby, increasing the crosslinking density of the polymer network is produced a contraction of itself; so part of water that had been previously absorbed is now expulsed until a new state equilibrium is reached. The pH as a function of time curve showed a decrement of pH due to the acetic acid liberation during the first 10 min (Figure 1(B)). This was also detected by ^{1}H NMR.

3.2. *FTIR and ^{1}H NMR*

Figure 2 shows FTIR and NMR spectra of pure and blended polymers, the Figure 2(A) shows the CS, PVA, and ECH spectra while the blends of CS-PVA, CS-PVA-ECH, and CS-PVA-ECH UV (treated with UV radiation) are shown in Figure 2(B). According to FTIR analysis, the CS-PVA-ECH films showed significant chemical changes such as: (a) hydrogen bonding formation and hydroxyl group's reactivity; the reaction of epoxy compounds with simple alcohols can lead to the expected ethers (changes in intensity at 3270, 2960–2830, 1410, 1230 cm^{-1}),[50,51] (b) shifts in bands corresponding to saccharide structure of CS were present (1150–910 cm^{-1}), and (c) higher reactivity of amines and amides during exposure under UV light and due ECH cross-linking made shifts in bands 1680–1480 cm^{-1}. Also intensity variations in bands correlated to the backbone of polymeric network (CH−, CH_2 reactivity, 1370 cm^{-1}, 835 cm^{-1}) were detected.

It is important to realize that there is no C–Cl peak (700–800 cm^{-1}) or any other peaks (epoxy groups at 915 cm^{-1}) that suggest the presence of remnants of ECH in the hydrogels. These results have demonstrated that chemical and physical cross-linking processes of CS-PVA-ECH were successfully applied and no remnants of ECH were

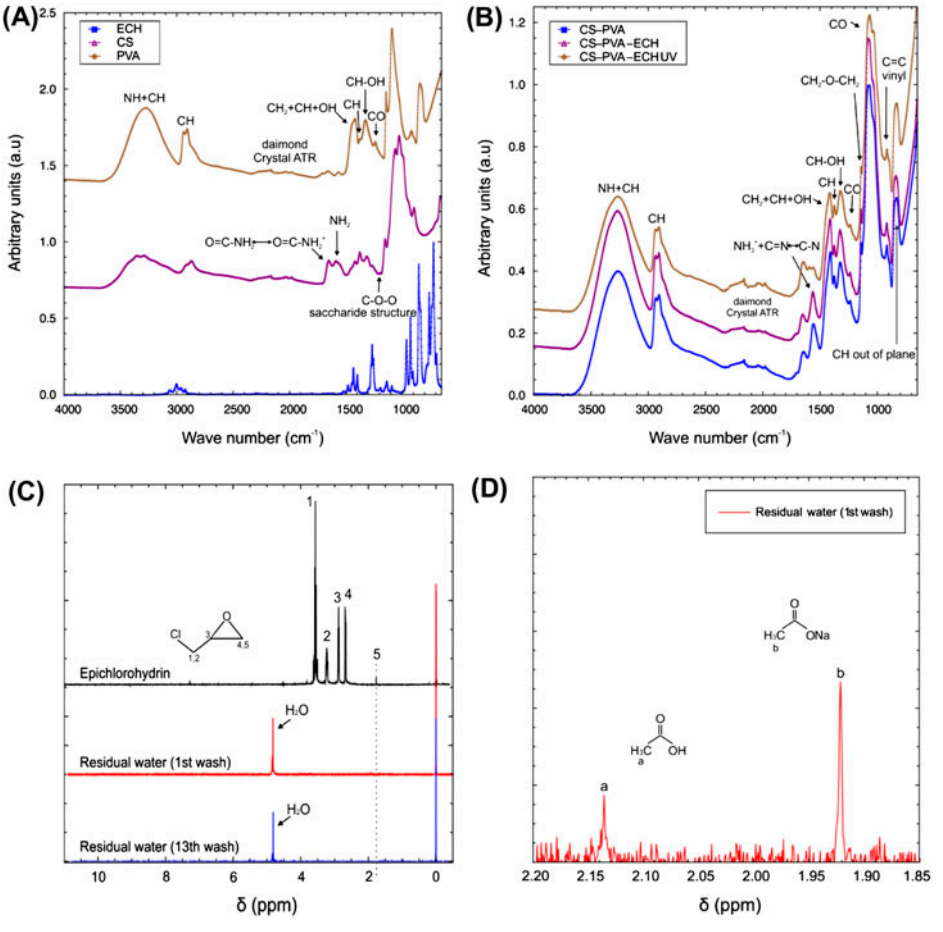

Figure 2. FTIR spectra of CS-PVA-ECH hydrogel. (A) FTIR spectra of pure polymers: CS, PVA, and crosslinking agent (ECH). (B) FTIR spectra of polymer blends: CS-PVA, CS-PVA-ECH, and CS-PVA-ECH UV. (C) ^{1}H NMR spectra of ECH and the used water in the first and last wash, the presence of unreacted ECH was not observed and (D) zoom of the residual water after the first wash; it was found that this peaks were not of ECH.

present in both hydrogels. To corroborate that there was no trace of unreacted or free ECH remnants, the water used in the previous washings was analyzed by ^{1}H NMR (proton NMR). Figure 2(C) shows a comparison between the spectra of the analytical grade ECH (ECH, 99%) – used as cross-linker agent – and the spectra of the water used in the first and last wash. Figure 2(C) shows a little peak close to the peak number five of ECH; in order to identify this peak, it was applied a zoom in Figure 2(C); therefore, it was possible to observe the exact position of the mentioned peak in Figure 2(D). In fact, two peaks were observed and they did not correspond to ECH peaks; the first (labeled with an "a" in Figure 2(D)) located at 2.13 corresponds to CH_3 of acetic acid; the second one labeled with a letter "b" corresponds to an CH_3 of a sodium acetate formed by the reaction between acetic acid and sodium hydroxide. This result confirms again the absence of remnant ECH in the hydrogels.

3.3. Mechanical characterization

As it was previously discussed, the swelling behavior is intimately related to the structural and mechanical properties of the hydrogel; therefore, its elastic behavior is highly dependent on the fluid quantity that they can absorb.[27,29,37] Therefore, in hydrated state, most hydrogels behave like elastomers, which means that their mechanical behavior is nonlinear, and this will mainly depend on the polymer network architecture.[26,27,52] Figure 3 shows this mechanical response for CS-PVA-ECH and CS-PVA-ECH UV hydrogels, which are curves of stress as a function of elongation ratio $\lambda - \lambda^{-2}$.

The obtained curves for all tested samples showed that the mechanical behavior of the hydrogels was not exactly the same even for samples of the same group. This may be due to changes in the cross-linking and polymerization processes, swelling conditions, or even the inherent mechanical anisotropy of these materials. For this reason, further analysis was performed for the region located between 0.01 and 0.05 in the elongation ratio ($\lambda - \lambda^{-2}$) axis. For this region, we determined the slope of the curve, which can be considered as an equivalent shear modulus ($G_{0.01-0.05}$).

Taking into account the mentioned range, and using the elastic modulus previously reported for pure CS and PVA films (6.15 ± 4.86 and 49.5 ± 3.2 MPa, respectively),[53,54] a comparative analysis between each group of samples was possible. The Young's modulus (E) for CS, PVA, CS-PVA, and CS-PVA-ECH (with and without UV) films are presented in Figure 4. The average for Young's modulus of the CS-PVA-ECH hydrogels was 9 ± 1 MPa, while the CS-PVA-ECH UV hydrogels showed an increase in the value of the Young's modulus, this being 11 ± 2 MPa. These values for the Yong's modulus are lower than those reported by Costa-Junior et al. and Bahrami et al. for CS-PVA hydrogels, they were around 17 MPa.[21,22,26] It is well known that an increment in strength of CS membranes under stress can, in general, be attributed to either the strain-dependent increase in hydrogen bonding and/or the strain-induced crystallinity.[28,55,56] In addition, it was also reported for the same authors that cross-linking of

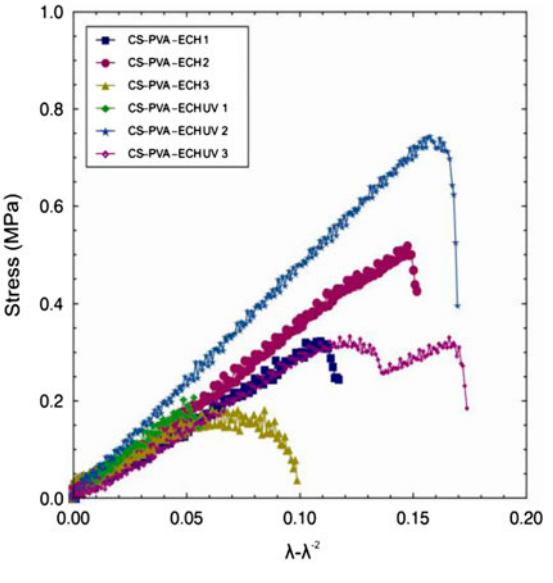

Figure 3. Stress as a function of $\lambda - \lambda^{-2}$ elongation ratio curves for CS-PVA-ECH and CS-PVA-ECH UV hydrogels. CS-PVA-ECH hydrogels had a lower elongation ratio than CSPVA-ECH-UV hydrogels. Both groups of curves showed a slightly non-linear behavior.

CS with a cross-link reagent up to certain degree may disrupt the interactions of its hydrogen bonds and reduce its crystallinity.[53] Hence, as a result of this, a decrease in ultimate tensile strength was observed for CS membranes cross-linked with a relatively high concentration of glutaraldehyde or genipin.[28,56] In our cross-linked membranes, we also observed a decay in ultimate tensile strength which may due to the cross-linking effect of ECH; however, the CS-PVA-ECH hydrogels have an/a elastic or Young's modulus similar to those reported for human elastic cartilage (around 5–12 MPa) by Park et al. [57,58]. This, therefore, suggests that the CS-PVA-ECH hydrogel can be potentially used as a substitute for this tissue.

Note that the elastic modulus (E) and the value of the ultimate tensile strength (UTS) for CS-PVA-ECH and CS-PVA-ECH-UV are very similar to those values reported for human elastic cartilage.[57,58] It is obvious that the CS-PVA hydrogel present a better elastic modulus in comparison with the others. We have to clarify that the cross-linker agents were used to get more stable polymers under the presence of a dissolvent; for example, when they are hydrated. Although CS-PVA blend exhibits better mechanical properties, its main disadvantage is that this blend tends to dissolve easily.

The mechanical responses of these materials are strongly related to their swelling behavior, because an increment in the cross-linking degree yields a decrement in swelling percentage; thus, the polymer network cannot absorb the same volume of interstitial water, and the polymer matrix reduces the chains mobility. As a result, the

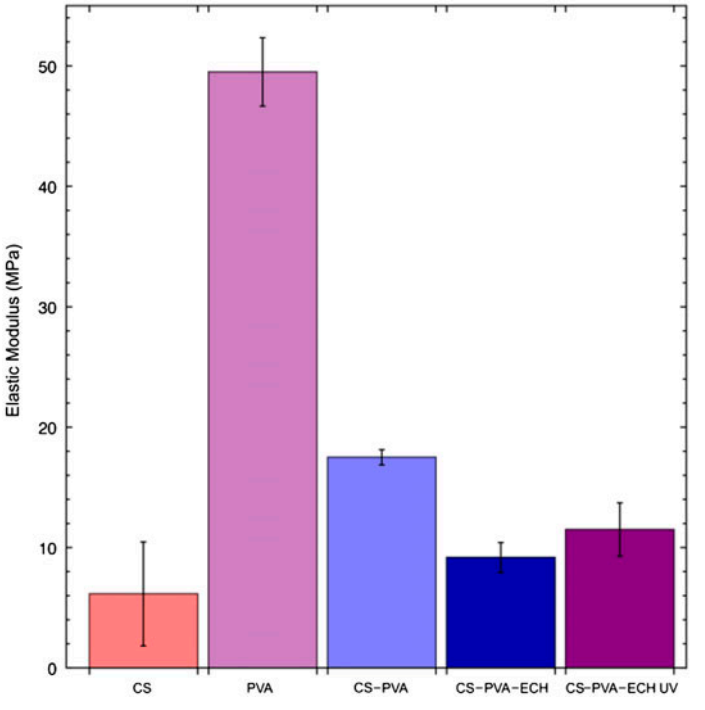

Figure 4. Comparative plot between the elastic modulus of CS, PVA, CS-PVA, CS-PVA-ECH, and CS-PVA-ECH UV. The reported elastic modulus for pure CS films was 6.15 ± 4.86 MPa [53]; and for pure PVA was 49.5 ± 3.2 MPa [54]; the elastic modulus for the blend CS-PVA is around 17.5 MPa according to literature.[22,26] Our results showed that the average elastic modulus of CS-PVA-ECH UV hydrogels were 11 ± 2 MPa; it was slightly higher than the CS-PVA-ECH hydrogels (9 ± 1 MPa).

hydrogels become less flexible and increase their UTS limit. The obtained results were in good agreement with previous reports on the literature.[22,29,58–61]

3.4. Structural properties

The effect of UV radiation in the polymer network structure of these hydrogels can be clearly seen from Table 1. As mentioned previously, the UV radiation causes an extra physical cross-linking contribution; if this is added to the chemical cross-linking contribution, caused by ECH during the synthesis of the hydrogels, the radiated polymer shall present a higher cross-link density than the reference sample. The cross-link density was theoretically calculated using 3.12×10^{-2} mol of CS-PVA-ECH dissolution. Thus, CS-PVA-ECH UV hydrogels showed a $778 \pm 172\,\mathrm{mol\,m^{-3}}$ cross-link density, meaning an increment around 25% respect to nonirradiated CS-PVA-ECH hydrogels ($620 \pm 94\,\mathrm{mol\,m^{-3}}$).

Similarly, the molecular weight between cross-linking points (M_{crl}) and the mesh size (D_N) decreased with increasing cross-link density, while the value of the volume fraction increased (Table 1), which is in agreement with the theory of rubber-elasticity. [27]

Understanding the polymer network structure is extremely important to get information about their mechanical properties. In other words, their mechanical behavior can be described in terms of polymer network structure using theories, which involve structural parameters such as number of elastically active chains and effective molecular weight between cross-link points.[62]

Figure 5 shows that the elastic modulus increases while the value of M_{crl} decreases. It means that a polymer with a greater pore or D_N shall present a lower elastic modulus (see Figure 6). According to these results, we can state that the mechanical properties of the hydrogel, such as the elastic modulus, are governed by their structure at microscopic level, or equivalently by the molecular weight between cross-linking points (M_{crl}), the cross-link density, and the D_N.

From our results, we can infer that the equilibrium degree of swelling is closely related to the hydrogel structure; a high degree of swelling equilibrium is generally associated with large pore or D_N of gel network and higher M_{crl}, as it was reported for CS-PVA hydrogels [43]. This same behavior was found in the hydrogels studied here; the hydrogels of CS-PVA-ECH had a higher swelling percentage and also had a higher pore size and greater M_{crl} values than the CS-PVA-ECH UV hydrogels (Figure 7).

3.5. Biocompatibility test

After 12 days of cell culture, the constructs (CS-PVA-ECH UV with cells) were analyzed. The chondrocytes formed confluent monolayers on the surface of the

Table 1. Hydrogel structural properties.

Hydrogels	Cross-link density ($\mathrm{mol\,m^{-3}}$)	M_{crl}[a] ($\mathrm{g\,mol^{-1}}$)	D_N[a] (nm)	v_s[a]
CS-PVA-ECH	620 ± 94	84 ± 12	1.37 ± 0.07	0.64 ± 0.01
CS-PVA-ECH UV	778 ± 172	68 ± 15	1.27 ± 0.09	0.68 ± 0.01

[a]M_{crl} represents the molecular weight between crosslinking points, D_N is the mesh size of the polymeric network and v_s is the polymer volume fraction in the swollen state.

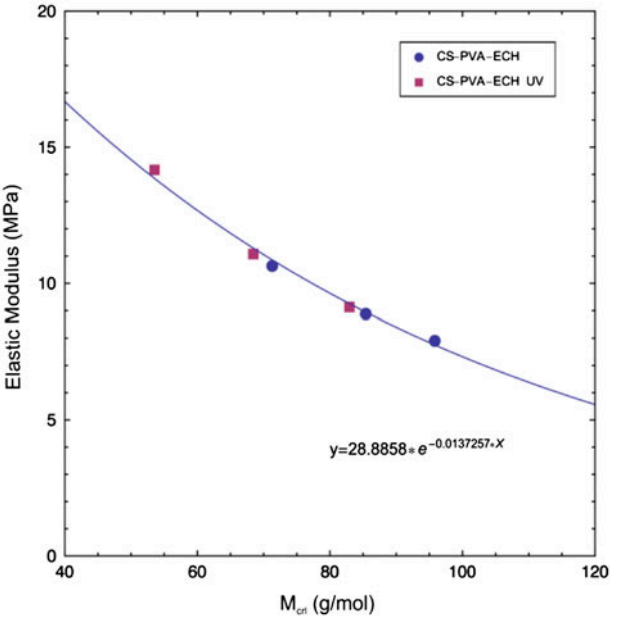

Figure 5. Elastic modulus as a function of M_{crl}. CS-PVA-ECH (dots) and CS-PVA-ECH UV (squares) hydrogels. For both hydrogels as the elastic modulus increases, the average molecular weight between cross-link points (M_{crl}) decreases.

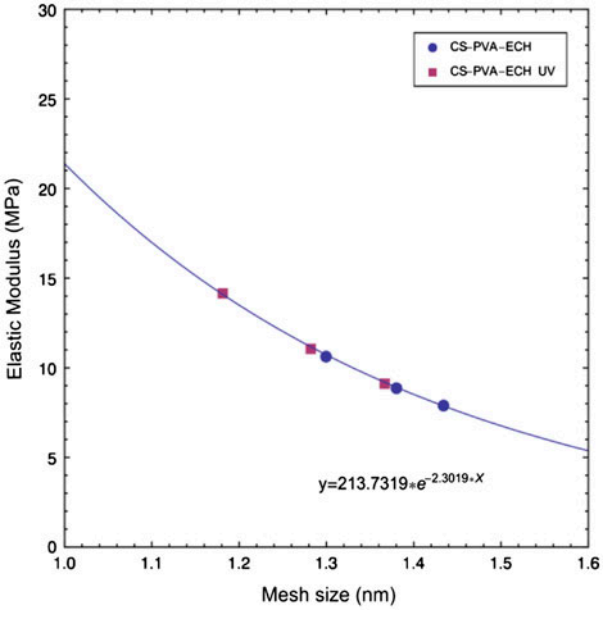

Figure 6. Elastic modulus as a function of mesh size (D_N). CS-PVA-ECH (dots) and CS-PVA-ECH UV (squares) hydrogels. CS-PVA-ECH hydrogels with larger pore size had a lower elastic modulus; this same behavior was shown by CS-PVA-ECH UV hydrogels.

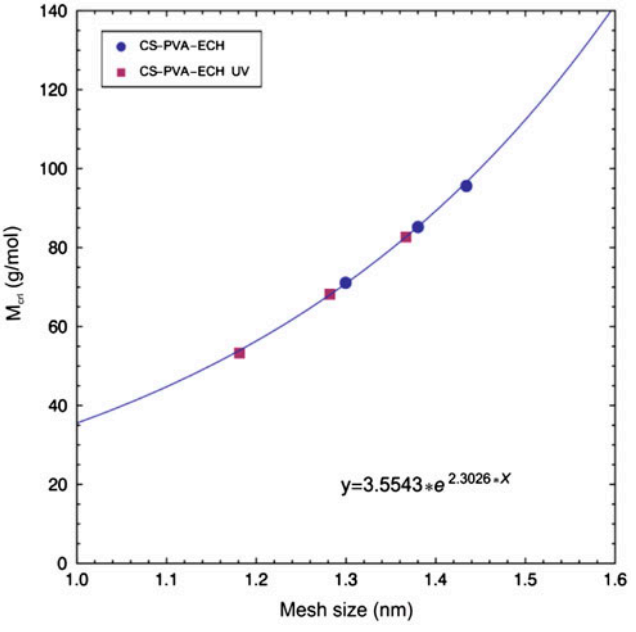

Figure 7. M_{crl} as a function of mesh size (D_N). CS-PVA-ECH (dots) and CS-PVA-ECH UV (squares). The plot shows an exponential growth of the molecular weight between cross-link points as a function of the mesh size for both hydrogels.

hydrogels. Figure 8 shows green fluorescent live cells on the polymer surface, over 99% of chondrocytes stained green indicating material's biocompatibility.

The films without cells showed mainly a smooth surface; in addition, no sign of erosion was observed (Figure 9(A)). Meanwhile, the film seeded with chondrocytes (Figure 9(B)) were distributed throughout the film, and some cell nodules could be seen at the surface of the CS films, suggesting the formation of ECM after 12 days of culture.

3.5.1. MTT assay

Biomaterials with potential use for TE applications are not only cytocompatible and supportive of cell adhesion but also promote cell proliferation. MTT assay involves a reduction reaction, which reduces MTT reagent to formazan when it is incubated with viable cells. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can, therefore, be used to measure cytotoxicity, proliferation, or activation. Thus, the absorbance of formazan indirectly reflected the level of cell metabolism.[63]

Figure 10(A) shows the absorbance intensity of formazan produced by cells in presence of CS-PVA-ECH, CS-PVA-ECH plus cells, and polystyrene plus cells (as control) after 12 days culture, respectively. The result shows that auricular chondrocytes had attachment and the proliferation rates were similar when they were cultured onto CS-PVA-ECH and polystyrene. Figure 10(B) represents the mean and standard deviation for the proliferation assay. According to Turkey HSD test, significant differences were not found between cells culture onto CS-PVA-ECH and the cells culture onto polystyrene well.

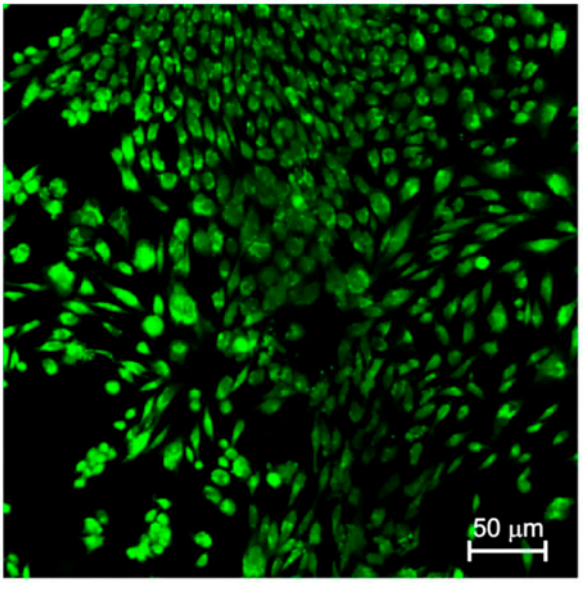

Figure 8. Calcein-AM staining; fluorescent micrograph showing the live auricular chondrocytes in green and dead cells in red (10×). About 99% of the chondrocytes on CS-PVA-ECH UV hydrogel were stained green, which indicated that the cells remain alive after 12 days of *in vitro* culture (please see the online article for the colour version of this figure: http://dx.doi.org/10.1080/09205063.2013.833441).

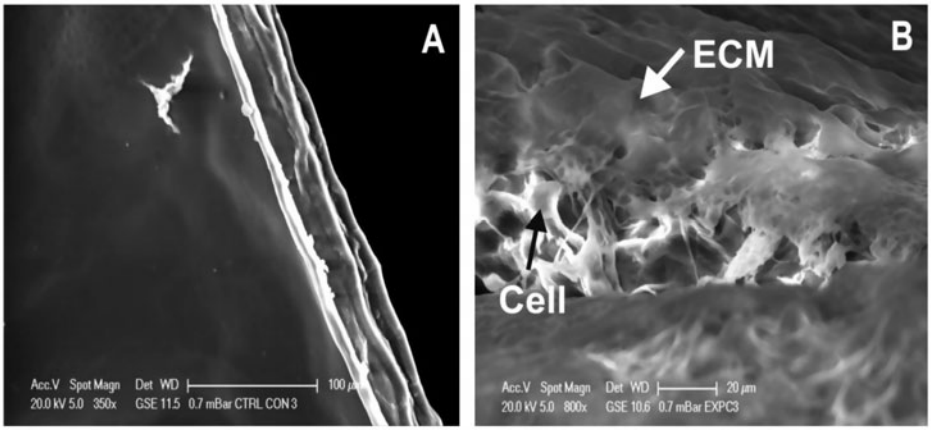

Figure 9. Polymer surface of CS-PVA-ECH UV hydrogel. (A) CS-PVA-ECH UV hydrogel surface before cell seeding (350×). (B) Cells attach and grown on CS-PVA-ECH UV hydrogel surface after 10 days of *in vitro* culture, the ECM formation also was observed (800×).

Our results showed that the use of ECH as a cross-link reagent was innocuous for cell culture under our methodology. The cell viability was supported by MTT results and the result of the indirect measurement for cell attachment obtained by calcein and ethidium also indicated cell viability and demonstrated no cytotoxic effect

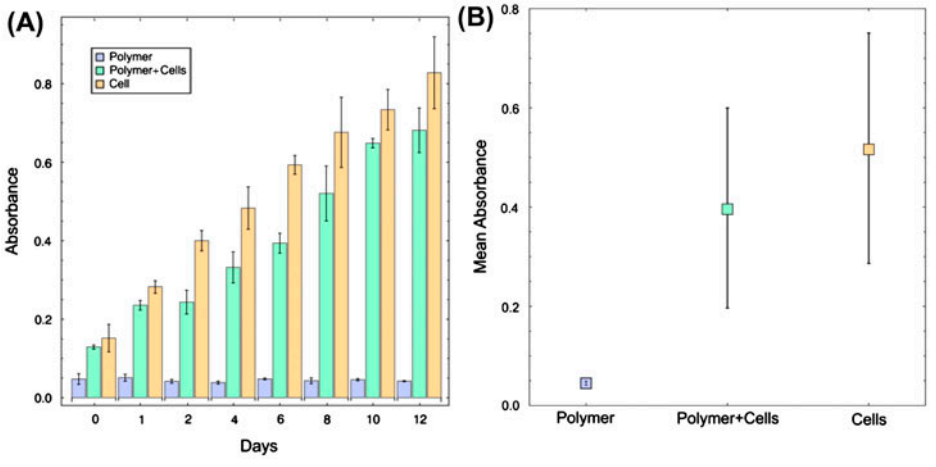

Figure 10. Absorbance as a function of cell proliferation during 12 days of *in vitro* culture; (A) in this figure, the blue bars represents the CS-PVA-ECH film without cells, the green bars represents the cell proliferation on the CS-PVA-ECH film, and the yellow bars correspond to the cell proliferation on polystyrene and (B) represents the mean and standard deviation for the proliferation assay where no statistical difference was found (between cells culture onto CS-PVA-ECH against cell culture onto polystyrene well) (please see the online article for the colour version of this figure: http://dx.doi.org/10.1080/09205063.2013.833441).

for over 12 days. The MTT results are in good agreement with NMR spectroscopy results (see Figure 2(C)) where no level traces of ECH were detected in film washes. This indicated that ECH was completely cross-linked with Chitosan-PVA polymer chains and any possibility of ECH release in the construct during cell culture was discarded.

4. Conclusions

In this study, it was possible to calculate the structural and mechanical properties of the CS-PVA-ECH hydrogels using the methodology proposed in this work. The combination of established theories such as rubber elasticity, viscoelasticity, and polymer network made possible the characterization of the hydrogels using a simple uniaxial tensile test. The mechanical properties, for example, elongation ratio and Young's modulus, showed a high dependence on their microscopic structure; which is defined by the average weight between cross-link points, D_N, and polymer volume fraction. The average Young's modulus of the CS-PVA-ECH hydrogels was 9 ± 1 MPa, while the CS-PVA-ECH UV hydrogels showed a small increment in this property. The CS-PVA-ECH hydrogels had a Young's modulus similar to those reported for human elastic cartilage range between 5 and 12 MPa. From biological results, it was possible to prove the biocompatibility of this material by calcein/ethidium and MTT assay. In addition, MTT results did not show cytotoxicity effects after 12 days in culture. In summary, the CS-PVA-ECH films have a significant potential for TE applications, such as scaffold for elastic cartilage in the reconstruction of the external ear, because their mechanical properties are similar to those of native tissue.

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Note

1. Time intervals in minutes were: 2.5, 5, 7.5, 10,12.5, 15, 20, 25, 30, 45, 60,120, 180, 240, 320, 1440 and 2880.

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