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Biocompatible scaffolds composed of chemically crosslinked chitosan and gelatin for tissue engineering

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ABSTRACT: Chitosan-based scaffolds are widely studied in tissue regeneration because of their biocompatibility and biodegradability. Scaffolds are obtained by different techniques and can be modified with other polymers allowing controlling their properties. This article discusses the assembling of three-dimensional chitosan porous scaffolds blended with gelatin. Gelatin was used to enhance cells attachment due to the presence of cell adhesion motifs, while improving mechanical strength. 2,5-dimethoxy-2,5-dihydrofurane (DHF) was used as the crosslinking agent, because it allowed to control the reaction kinetics through temperature, time and DHF concentration. The results indicate that scaffolds morphology, pore sizes and distribution, compressive moduli and biodegradation *in vitro* with lysozyme, can be customized with variations of gelatin content and crosslinking degree. Scaffolds were neither cytotoxic nor genotoxic for human keratinocytes, exhibiting cell–substrate interactions. Our findings demonstrated that chitosan–gelatin scaffolds crosslinked with DHF, as a new crosslinking agent, are suitable in tissue engineering applications. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43814.

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INTRODUCTION

Tissue engineering aims to repair and replace affected tissues. One of the strategies is the development of three-dimensional (3D) porous scaffolds which are temporary templates that imitate the extracellular matrix (ECM), as implantable pieces that will lead to the formation of a new organized and functional tissue.[1,2] Scaffolds provide structural support and a proper environment for the cells, enabling the diffusion of nutrients, oxygen, and waste metabolites. Thus, cells can effectively accomplish their adherence, proliferation, and differentiation.

Imitating the ECM has become challenging for tissue engineering due to its inherent physical, chemical and biological complexity; therefore, it is crucial to choose the right biomaterial. Natural polymers such as chitosan,[3] collagen,[4] gelatin,[5] alginate,[6] chondroitin sulfate,[7] and hyaluronic acid,[8] among many others, are being studied because their similarity with the ECM. One of the most promising of these biomaterials is chitosan. It is obtained by the partial deacetylation of chitin, which is the second most abundant polysaccharide, found in crustacean carapaces, insects and fungi.[9] Chitosan is chemically alike to ECM's glycosaminoglycans (GAG), it is biocompatible, biodegradable, nontoxic, and undergoes enzymatic degradation by lysozyme.[1] These features make chitosan suitable for tissue engineering applications, such as the reparation of cartilage, blood vessels and nerves, wound healing and bone regeneration.[2]

Many techniques have been reported for the preparation of chitosan scaffolds. Each one generates scaffolds with different geometries, forms and pore size distribution. These characteristics influence the mechanical properties of the scaffolds and therefore their final performance.[3] Chitosan is commonly crosslinked enhancing its chemical and mechanical stability, consequently improving scaffolds' biological and mechanical properties. Chitosan is also chemically modified and blended with other polymers or proteins[10,11] such as collagen. Collagen has been used for two main reasons. First, because it is the most abundant protein in the human body, accounting for tissue strength and stability.[12] Second, because it owns cell adhesion motifs which enable scaffold—integrin interactions, which should improve

Additional Supporting Information may be found in the online version of this article.

cell proliferation, migration and differentiation.[13] However, the use of collagen has some disadvantages, mainly because of its high cost, mild immunogenicity and variable physicochemical and degradation properties.[14] Gelatin has been used instead, as it is obtained by the partial hydrolysis of collagen. Gelatin is far less expensive than collagen, is nonimmunogenic and is considered an appropriate biomaterial to be used in diverse biomedical applications. But what can be more significant, is that gelatin may also keep the collagen cell binding motifs or peptide sequences, which will mediate and enhance cell adhesion to scaffolds.[3]

Therefore blending gelatin and chitosan may promote cellular functioning of scaffolds. There are several reports in the literature about these blends, describing a wide variety of engineering applications such as cartilage tissue engineering,[15] supporting stem cells,[7] hepatocyte culture,[16] bone,[17] and human meniscus tissue engineering,[18] skin tissue regeneration[19] and nerve regeneration.[20] The main limitation of gelatin arises from its dissolution in aqueous environments, so other studies specify the use of crosslinking agents for these blends. Glutaraldehyde is the most well-known and characterized of all crosslinking agents.[21] Despite its many advantages, much recent works have been directed to the development of alternative crosslinking treatments which include carbodiimide[22] and genipin,[18] to avoid glutaraldehyde potential cytotoxic reactions.

One crosslinking agent which has not been addressed in the literature for chitosan—gelatin blends is 2,5-dimethoxy-2,5-dihydrofuran (DHF). DHF allows slowing down the kinetics of gelation by controlling the reaction temperature, time and DHF concentration. The furan ring breaks up in acidic media and temperature, forming cis-2-butene-1,4-dial which has two free aldehyde groups that will readily form imino bonds with primary amine groups.[23]

The possibility to control the reaction kinetics lead us to choose DHF as the crosslinking agent for chitosan based porous scaffolds, blended with gelatin and manufactured by the freeze drying technique. Therefore, this paper describes the preparation and the physical, mechanical and biological characterization of these hybrid scaffolds. Gelatin was used to improve cell—scaffold interaction, while the crosslinking agent prevented dissolution and degradation of water soluble gelatin as well as increasing mechanical strength and chemical stability.[24,25] Control of crosslinking reaction and gelatin content enabled the modulation of size, shape, and morphology of the scaffolds. These features were in turn associated with their mechanical properties, cytotoxicity and genotoxicity. Our findings demonstrate that chitosan/gelatin based scaffolds are suitable for tissue engineering applications. It is worth noticing that DHF turn out to be a biocompatible crosslinker which has not been previously employed for this blend.

EXPERIMENTAL

Materials

Chitosan from shrimp shells was purchased from Sigma-Aldrich (medium molecular weight, batch SLBF6034V). The viscosity-average molar mass of 188 kDa was measured using a 0.3 *M* acetic acid/0.2M sodium acetate mixture as solvent, at 25.0 ± 0.1 °C, using the following Mark–Houwink parameters: $a = 0.76$ and $K = 74 \times 10^{-5}$ dL/g.[26] The degree of deacetylation (DD) of 72% was determined by potentiometric titration following the procedure described by Jiang *et al.*[27] Bovine gelatin of 250 Bloom was purchased from Gelnex. This gelatin has a wide molecular weight distribution (40–250 kDa) as was seen by SDS-PAGE assay. This assay also showed the presence of α1 and α2 bands, indicating that this gelatin derives from collagen type I. This assay was conducted as described by Laemmli[28] (method and results are provided in Supporting Information).

Analytical grade 2,5-dimethoxy-2,5-dihydrofurane (DHF), 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), glutaraldehyde, acetic acid, absolute ethanol, agarose (Sigma A9414), NaOH purity 98%, ethidium bromide HPLC grade 99%, TRIS reagent grade and DMSO spectrophotometric grade, were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM) from Invitrogen, Thermo Fisher (USA), fetal bovine serum (FBS), and antibiotic Penicillin/Streptomycin were acquired from Gibco, ThermoFisher. Phosphate buffered saline (PBS), egg white lysozyme grade ultrapure, TRITON X-100 reagent grade, and EDTA N2 proteomic grade were purchased from Amresco and NaCl 99.5% pure from MERK. All chemicals were used without further purification.

Fabrication and Crosslinking of Chitosan–Gelatin Scaffolds

Chitosan solutions were prepared by dissolving 1.5 g of chitosan in 1% (v/v) acetic acid solution, and stirred for 4 h and then centrifuged at 9000 RPM for 15 min. Gelatin solutions were prepared in deionized water at 60 °C for 10 min at a concentration of 4% (w/v). These two solutions were mixed together at different volumetric ratios: 100/0, 75/25, 50/50, and 0/100. Then DHF was added at three different concentrations: 25, 33, and 41 m*M*, to achieve three crosslinking degrees.

To perform the crosslinking reaction, solutions were held at 60 °C for 2 h, followed by slow freezing until −20 °C during 12 h and freeze dried for 24 h at −80 °C, to obtain the porous scaffolds. Uncrosslinked scaffolds were prepared by the same method. Before characterization, all scaffolds were thoroughly washed in absolute alcohol and in sterile PBS and lastly, freeze-dried under the same conditions described above.

Morphology and Porosity

The morphology and porous structure of scaffolds were observed by scanning electron microscopy (SEM), using a JEOL JSM 6490 LV at high vacuum and 20 kV for accelerating voltage. Samples cross-sections were coated with sputtered gold before observation. Mean pore sizes were estimated using the software Image J. Thirty pores were selected from each sample to perform this calculation. Pores were approximated to ellipses, measuring and averaging the largest and shortest diameter.

For density measurements, nonporous and porous scaffolds were obtained. These scaffolds were dried until constant weight at 25 °C in vacuum. Densities were calculated by dry weight to volume ratio. The volume was calculated using expression 2, where *d* is the scaffold diameter, and l is the thickness. Three measurements were performed for each sample. Porous fraction

Table I. Experimental Design for Statistical Analysis

Nomenclature	Chitosan/gelatin (Vol/Vol)	DHF concentration (mM)
C1	100/0	—
C2	75/25	—
C3	50/50	—
C4	0/100	—
C1N1	100/0	25
C2N1	75/25	25
C3N1	50/50	25
C4N1	0/100	25
C1N2	100/0	33
C2N2	75/25	33
C3N2	50/50	33
C4N2	0/100	33
C1N3	100/0	41
C2N3	75/25	41
C3N3	50/50	41
C4N3	0/100	41

(ε) was determined through the approximation given by expression 1, where ρ_D is the density of the nonporous scaffold and ρ_P the density of the porous one.[10]

$$\varepsilon = \left(1 - \frac{\rho_P}{\rho_D}\right) \times 100 \quad (1)$$

$$V = \frac{\pi}{4}(d)^2 l \quad (2)$$

Mechanical Properties

Compressive strengths measurements were carried out in a universal testing machine (INSTRON 3345) equipped with a 10 N load cell and the crosshead speed set at 5 mm/min. Analyses were performed on dry scaffolds at room temperature and 60% relative humidity. Cylindrical shaped samples of 8 mm diameter and 3 mm length were cut. Compressive moduli were calculated from the slopes of the initial elastic section of the stress-strain curves at 2% strain. Ten samples were tested for each treatment.

In Vitro Enzymatic Degradation

The enzymatic degradation of chitosan/gelatin scaffolds was evaluated *in vitro* through lysozyme digestion tests. All scaffolds were incubated in PBS under continuous stirring and sterile conditions at 37 °C, handling two simultaneous treatments: one without lysozyme and the other one using 10 μg/mL egg white lysozyme. This concentration was chosen because it corresponds to the concentration of lysozyme in human serum.[29] The enzymatic solution was renewed every 5 days, to ensure continuous enzyme activity. Scaffolds were removed at different periods: 7, 14, and 21 days, washed with PBS twice and with deionized water for three times. Finally, scaffolds were frozen (−20 °C for 12 h) and lyophilized (24 h). The biodegradation was expressed as percentage of weight loss of the dried scaffolds after lysozyme treatment.

MTT Assay

Cell viability was determined by the standard MTT reduction assay, based on ISO standard 10993-1:2003.[30] HaCat cell line (human keratinocyte cell line) were seeded at confluence of 8×10^3 per well in 96-well plates and cultured at 37 °C in 5% CO_2 for 24 h. Then scaffolds were placed inside the wells at 24, 48, and 72 h. A latex piece of the same weight as the scaffolds was used as a positive control (dying of cells). After each incubation time, the scaffolds were removed and 10 μL of stock solution (5 mg/mL MTT in PBS) were added to each well. Cells were incubated for 4 h in the dark at 37 °C. After incubation, 100 μL of acid isopropanol was added. The absorbance of each sample was measured using an ELISA multiplate reader at 570 nm. Differences in absorbance show the metabolic activity of the cells, and are proportional to the number of living cells. Each experiment was repeated three times, with internal triplicates for each treatment.

Alkaline Comet Assay

DNA cell damage was evaluated by Alkaline Comet Assay. HaCat cells were seeded in 96 plate wells at 8×10^3 cells/well and incubated for 24 h at 37 °C and 5% CO_2. Next day chitosan scaffolds were placed inside the wells for 72 h to allow cells to interact with the culture media and cells. After the incubation period, scaffolds were removed. Cell samples were diluted in PBS, and 20 μL of cell suspension were mixed with 80 μL of 0.4% low melting point agarose at 37 °C, layered on to a glass slide and placed overnight in cold lysis solution containing 2.5*M* NaCl, 100 m*M* Na_2 EDTA, 10 m*M* Tris, 10% DMSO, and 1% Triton X-100. After rinsing twice in PBS, slides were treated for 20 min with a cold alkaline mixture of 300 m*M* NaOH and 1 m*M* Na_2 EDTA, pH > 13, followed by 20 min of electrophoresis at 300 mA. After electrophoresis, slides were neutralized and stained with 0.01 mg/mL ethidium bromide; 50 cells were scored per sample. Each experiment was repeated twice with internal duplicates for each treatment. After cell lysis, nuclei were stained with ethidium bromide and visualized under a fluorescence microscopy with a 20× objective. Images were acquired with a SONY digital camera using 2× magnification zoom. The DNA damage/nucleus was quantified using the endpoint measurement of % DNA tails by the Comet Assay Software Project (CASP) program.

Seeding and Culture of Keratinocytes on Chitosan–Gelatin Scaffolds

In order to determine the behavior of living cells on the scaffolds, HaCat cells were cultured on selected scaffolds. The scaffolds were washed with PBS and DMEM and placed in 24-well plates for incubation at 37 °C in 5% CO_2 for 24 h with 50×10^3 cells/well. After this time, cells were fixed in 2.5% glutaraldehyde solution at 4 °C for 1 h. Finally, scaffolds were thoroughly washed with PBS and dehydrated in hydroalcoholic solutions at increasing concentrations (10, 30, 50, 60, 70, 80, 90, and 100%). Cells attachment was assessed by SEM (JEOL JSM 6490 LV at high vacuum and 20 kV). Scaffolds cross-section, where cells were seeded, was coated with sputtered gold before observation.

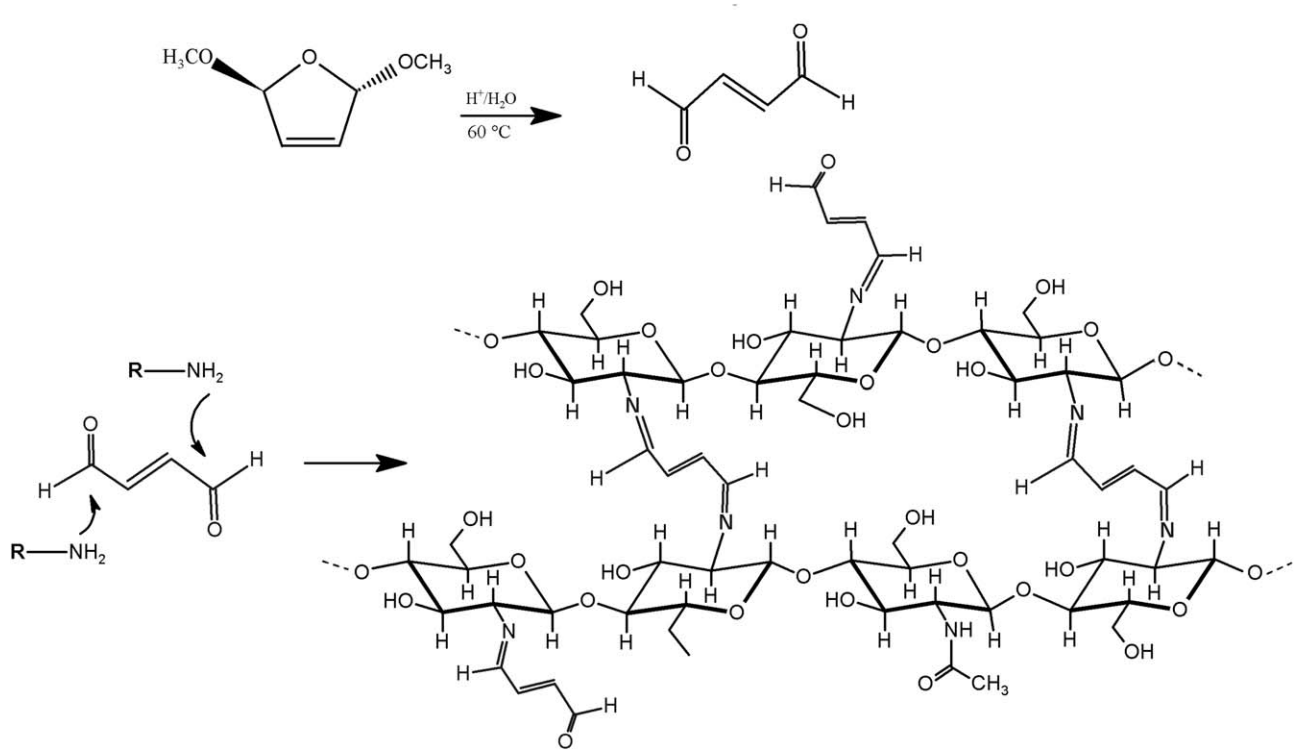

Figure 1. Crosslinking reaction scheme: activation of DHF furan ring and formation of Schiff bases through primary amino groups of chitosan and DHF aldehyde groups.

Statistical Analysis

Statistical analyses were carried out using Statgraphics Centurion Version 2007. Data were studied by analysis of variance (ANOVA) and the differences between the means were determined by the LSD Fisher multiple test. For data of test pairs that did not meet the assumptions for ANOVA, were performed a Kruskal–Wallis (K–W) test by factors. A value of $P \leq 0.05$ was considered statistically significant.

Scaffolds were separated by groups for subsequent statistical analysis. C group (C1, C2, C3, and C4) include the scaffolds with different volumetric ratio of gelatin (0, 25, 50, and 100%). N group (N, N1, N2, and N3) include the scaffolds with an equal volumetric ratio of gelatin but different DHF concentration (0, 25, 33, and 41 m*M*, respectively). Table I shows the experimental design.

RESULTS AND DISCUSSION

Crosslinking with DHF

Crosslinking mechanism of DHF resembles to that of glutaraldehyde, since the reaction occurs through Schiff bases formation. The main difference between both mechanisms is that DHF has to be activated with temperature (in acidic media), which triggers the oxidation of the furan ring producing a linear dialdehyde molecule, *cis*-2-butene- 1,4 dial (Figure 1). Thus the temperature to carry out the crosslinking reactions was fixed at 60 °C, which was optimized in the setting up of experimental conditions.

The reaction between the dialdehyde and chitosan was straightforward, because chitosan linear chains have many available primary amine groups (DD of 72%). But that is not the case for gelatin whose chains are sequences of different amino acids, and the crosslinker will only react with those that have primary amino groups such as lysine or hydroxylysine. Consequently, functional groups of gelatin to be crosslinked are less in number than those of chitosan and therefore gelatin had a lower crosslinking degree. This is why the concentration of gelatin solutions was 4% (w/v), higher than that of chitosan solutions of 1.5% (w/v). The value of 4% was found to be the lowest concentration of gelatin that crosslinked with DHF.

It is noteworthy to mention that dialdehyde is classified as a mutagenic molecule, when formed *in vivo* as a metabolite of furan degradation.[31] Other studies on furan and its metabolite conclude that the genotoxicity depends on the dosage and that toxicity is not yet demonstrated for humans.[32] Because of these reasons, the concentration of DHF had to be cautiously calculated. The three amounts of DHF used in this study were lower than the stoichiometric concentration necessary to completely crosslink all chitosan amino groups (72 groups per monomol). There were two main reasons for selecting these low DHF concentrations. The first one was to guarantee the complete reaction of the crosslinker with the primary amine groups of chitosan, and with those of gelatin. Thus all the dialdehyde was likely to be chemically bonded through imino links within the chitosan–gelatin network. Therefore, it was expected that no remaining DHF was left, which was also ensured by the thorough wash of the scaffolds. The other reason arose from the

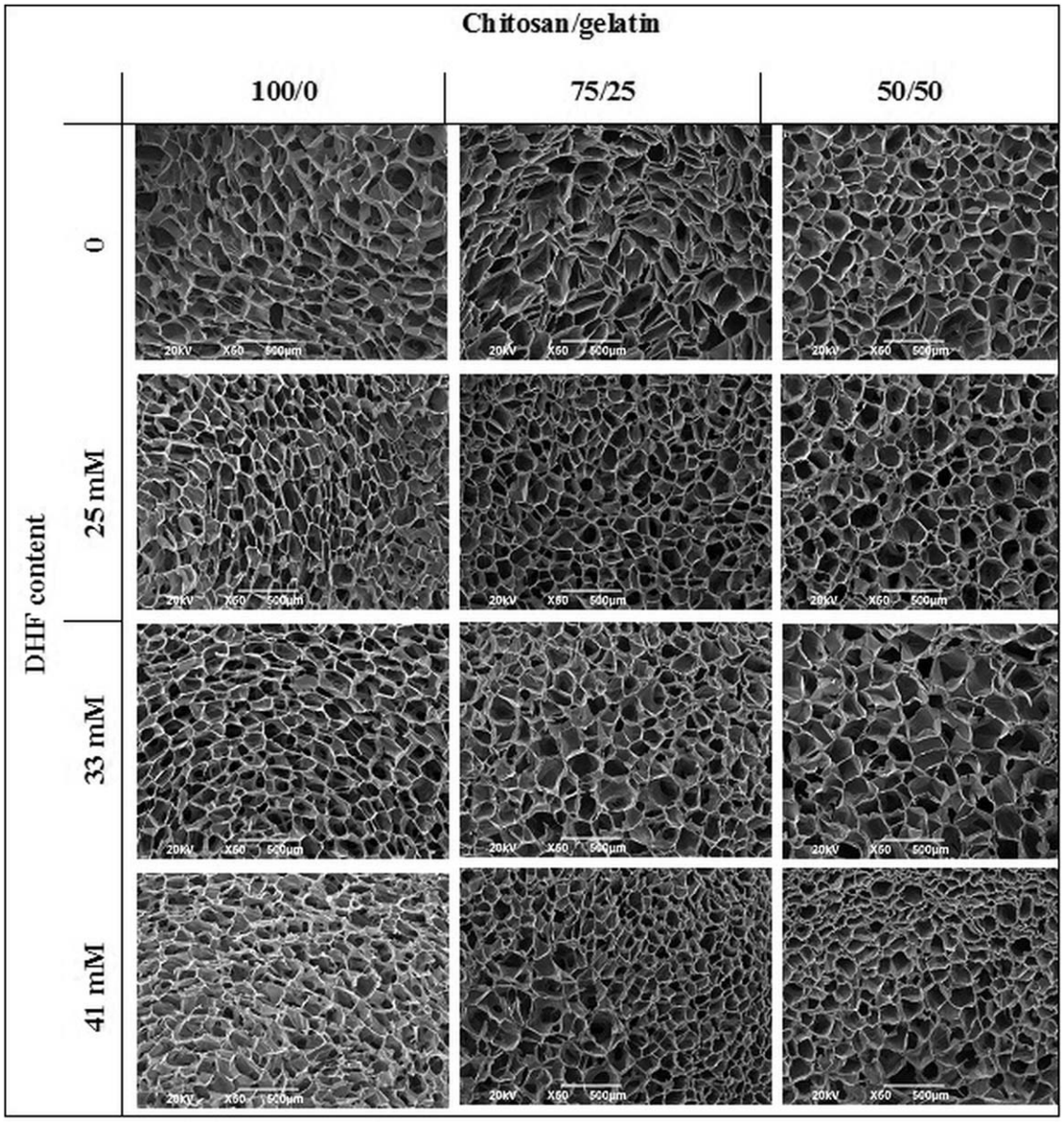

Figure 2. SEM micrographs at 50× of the cross-section of chitosan/gelatin scaffolds.

experimental finding that very hard scaffolds were obtained at high crosslinking degrees that later shrank and deformed. Nonetheless, the crosslinking degree is not reported in this work due to the uncertainty of the groups to be measured for its quantification and therefore, the concentrations of crosslinking agent were used instead.

Scaffolds crosslinking reaction was performed by two methods. One consisted on the immersion of scaffolds in a solution of the crosslinker, for subsequent heating at 60 °C while controlling the reaction time. This method was neither effective nor reproducible, due to the heterogeneous diffusion of the solution into the complex geometry of scaffolds. Therefore it was more convenient to add the precise amount of crosslinker into the polymers solution and then let the reaction take place. This last method was highly reproducible, besides the concentration of crosslinker could be exactly quantified, avoiding toxic excesses. Scaffolds obtained by immersion yielded unsatisfactory results for cytotoxicity *in vitro* as well as random physical properties, so these results are not included.

Morphology and Measurement of Porosity of Scaffolds

Scaffolds with high porosity content and interconnectivity between pores were obtained by the freeze drying process. Pore sizes were found in the range of 71–317 μm and the average porosity was higher than 90%. Figure 2 shows SEM micrographs of scaffolds. This average of pore sizes, along with the 3D shape and pore interconnectivity, provide a porous network which was demonstrated to be appropriate for human keratinocytes ingrowth, as it will be further shown.

Scaffolds had a wide distribution of pore sizes, marking an increasing tendency of the smaller pores frequency with the increase in gelatin content and the higher the crosslinking

Table II. Maximum and Minimum Values of Pore Sizes, Frequency, and Total Porosity of Chitosan/Gelatin Scaffolds

Nomenclature[a]	Pore size (μm) Min–Max	Pore size most frequently (μm)	Total porosity ± SD (%)
C1	117.232–275.993	220–260	96.657 ± 0.020
C2	118.643–293.518	180–220	96.018 ± 0.333
C3	84.433–239.234	140–200	96.505 ± 0.088
C4	68.446–206.387	140–160	91.366 ± 1.585[b]
C1N1	82.561–221.034	160–200	97.256 ± 0.256
C2N1	86.533–201.320	140–180	96.517 ± 0.162
C3N1	75.358–213.264	120–160	96.536 ± 0.099
C4N1	71.893–226.207	100–160	92.569 ± 1.382[b]
C1N2	97.628–231.370	180–200	96.991 ± 0.174
C2N2	94.185–243.215	140–160	94.022 ± 1.209
C3N2	81.154–235.453	120–180	95.520 ± 0.548
C4N2	115.030–317.680	100–180	93.672 ± 0.851[b]
C1N3	89.746–198.970	160–180	97.392 ± 0.268
C2N3	79.157–242.569	120–160	95.337 ± 1.225
C3N3	85.970–196.040	120–140	95.508 ± 0.763
C4N3	87.986–238.513	100–160	92.846 ± 0.933[b]

[a] C: Volumetric ratio chitosan/gelatin. N: crosslinking degree.
[b] Significant difference ($P < 0.05$). Data analyzed by K-W test. Mean ± SD. $n = 3$.

degree. The former case is explained because of the high Bloom value of the gelatin, which means a higher strength of the gel by increasing the intermolecular entanglement and interactions.[22] The latter case is due to the higher number of covalent bonds binding together chitosan and gelatin molecules. Both cases lead to the decrease of pore sizes. It was also observed that crosslinking improved the mechanical integrity, allowing the scaffolds to keep their shape without deformations. Conversely, total porosity percentages were not influenced by the increasing concentration of gelatin or crosslinking degree. Results are shown in Table II.

Mechanical Properties

Scaffolds mechanical properties were tested in dry conditions under uniaxial compression. The compressive moduli were in the range of 0.284–1.167 MPa for the uncrosslinked scaffolds and 0.416–2.216 MPa for the crosslinked scaffolds. These values may not be easily compared with reports from other authors, since moduli values are influenced by many variables such as chemical composition of the scaffolds and crosslinking which lead to multiple possibilities for chemical and physical interactions. Moduli values are also changed by the processing conditions which yield structures in a wide variety of morphologies; these values may also change by the measurement conditions. So, the most important issue to be remarked is that the mechanical strength was the adequate to maintain the structural integrity throughout the characterizations and the mechanical requirements of cells *in vitro*.

Compressive moduli for all scaffolds increased with gelatin content and crosslinking degree. This result was expected, due to the low strength of chitosan,[1] which confirms the necessity of crosslinking or modification. In this occasion, blending with gelatin improved the mechanical behavior of chitosan-based scaffolds. Results of the one factor variance analysis are shown in Figure 3. They indicate that there are statistically significant differences between the means of the compressive moduli for both groups: amounts of DHF (crosslinking degree or N group) and chitosan/gelatin volumetric ratios (C group). It is worth mentioning that even though the compressive modulus increased at higher crosslinking degrees, the lowest value of crosslinking did not differ statistically with respect to the uncrosslinked scaffolds.

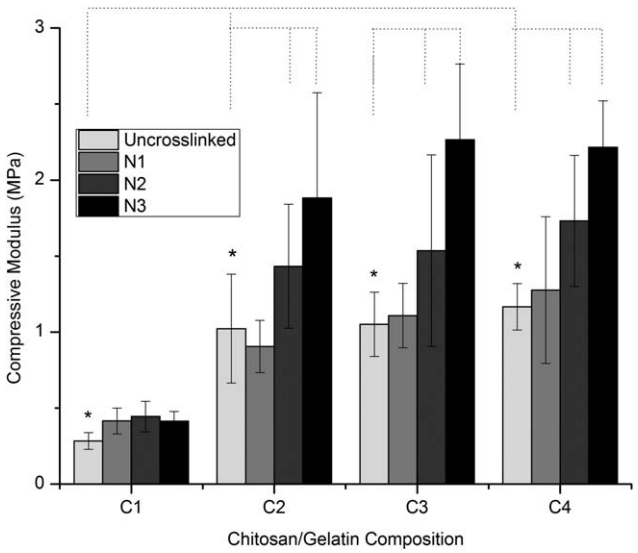

Figure 3. Mechanical behavior of chitosan/gelatin scaffolds. Compressive modulus as a function of gelatin content for different crosslinking degrees.

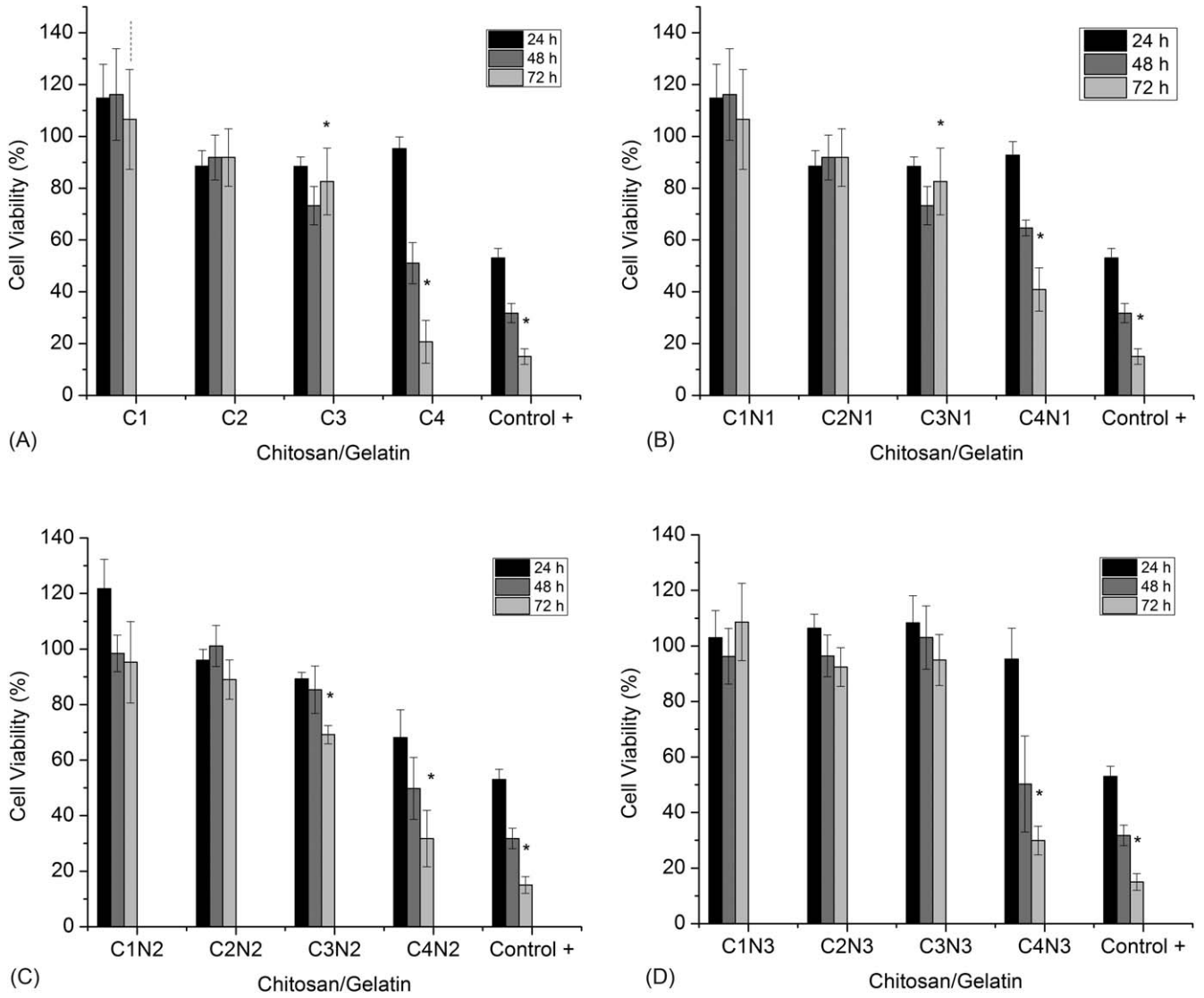

Figure 4. MTT assay results for human keratinocytes cells after 72 h cultured in chitosan/gelatin scaffolds for 24, 48, and 72 h. (A) uncrosslinked scaffolds, (B) crosslinked scaffolds with 25 m*M* of DHF, (C) crosslinked scaffolds with 33 m*M* of DHF, and (D) crosslinked scaffolds with 41 m*M* of DHF. *Significant difference ($P < 0.05$) with a 95% confidence level. Data were analyzed by analysis of Kruskal–Wallis.

Increasing compressive strength was also associated with a smaller pore size distribution (higher frequency of smaller pores). This result means that smaller pores provides larger strength to elastic strain versus the applied load.

Biological Studies

***In Vitro* Biodegradation.** Scaffolds were incubated in PBS with and without lysozyme to evaluate their resistance for bearing enzymatic hydrolysis. To examine the biodegradation, enzyme concentrations were similar to those found in the human body[29] and tested at physiological pH and temperature, in order to simulate *in vivo* conditions. Biodegradation, measured as scaffolds weight lost, increased with the tested times: 7, 14, and 21 days (data shown as supporting information). Enzymatic effect was clearly visible since all scaffolds incubated in lysozyme showed higher weight loss when compared to those incubated in PBS. They also lost their shape and strength. It was also observed that crosslinking confers chemical stability since uncrosslinked scaffolds were degraded faster than the crosslinked ones. Uncrosslinked chitosan/gelatin scaffolds showed a higher weight lost, probably due to the solubility of gelatin in an aqueous media. Both, weight lost and crosslinking, presented statistical significant differences.

The results indicate that lysozyme hydrolyzes chitosan, even when it is crosslinked. The specific degradation of chitosan by lyzozyme has implications in tissue engineering applications since this enzyme is present in certain human fluids or released from phagocytic cells.[33]

Cell Cytoxicity and Genotoxicity Results. Scaffolds were tested for cells cytotoxicity and genotoxicity by MTT and alkaline comet assay respectively. MTT results are shown in Figure 4. They can be summarized as follows: All scaffolds, uncrosslinked and crosslinked at three levels (N1, N2, N3), with a gelatin content below 25% showed a high cell viability, up to 90%, with

Table III. DNA Damage (% Tail DNA) in Chitosan/Gelatin Scaffolds after 72 h on Incubation with HaCat Cells

Sample	DNA damage (% tail DNA) ± SD
C1	2.621 ± 0.917
C1N1	2.527 ± 0.448
C1N2	1.976 ± 0.601
C1N3	2.476 ± 1.996
C2	2.441 ± 0.475
C2N1	2.507 ± 0.433
C2N2	3.878 ± 0.400[a]
C2N3	1.794 ± 0.390
Control+	42.447 ± 28.748

C: volumetric ratio chitosan/gelatin. N: crosslinking degree.
[a] Significant difference ($P < 0.05$). Data analyzed by K-W test. Mean ± SD. $n = 3$.

no significant statistical differences between samples after 24, 48, and 72 h. Higher gelatin content (50 and 100%) decreases cell viability of scaffolds at all the tested timepoints, showing significant statistical differences. Cell viability values were lower than 41% for uncrosslinked gelatin scaffolds and for the lower levels of crosslinking. Nevertheless, scaffolds with the highest level of crosslinking (N3) displayed high cell viability up to 50% of gelatin content. This behavior may be due to solubilization of the gelatin, with exception of N3, where it is chemically stabilized by reticulation. The solubilization may generate gelatin high concentrations and accumulation in the cell media, which can block cell membrane receptors thus inducing impaired cell function. This hypothesis is based on immunostaining techniques, wherein the gelatin is used as a blocking agent in Southern blot.[34]

These results demonstrate that crosslinking enhances cell viability due to the improved chemical stability and mechanical strength. It was also revealed that DHF is not toxic for cells within the measured timepoints and concentrations. These results also indicate that the amount of gelatin must not exceed 25%. MTT results let us conclude that crosslinked chitosan scaffolds with the right amounts of DHF and gelatin are not

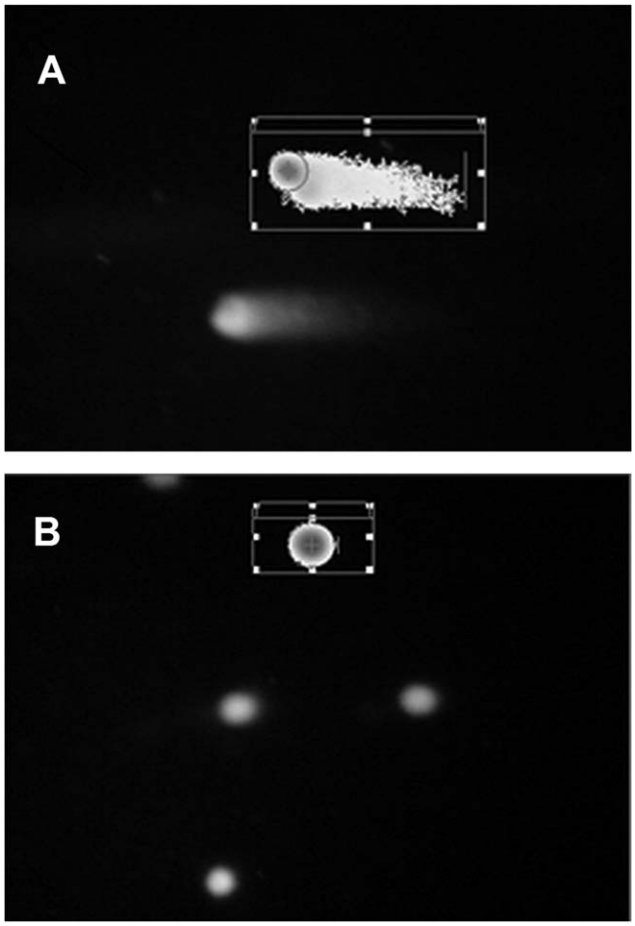

Figure 5. Micrographs of the genotoxic damage on HaCaT keratinocytes seeded on chitosan/gelatin scaffolds, evaluated by comet assay. (A) Latex as a positive control displaying the tail, (B) cells with no tails of C2N3 sample (scaffold with 25% gelatin and highest crosslinking degree).

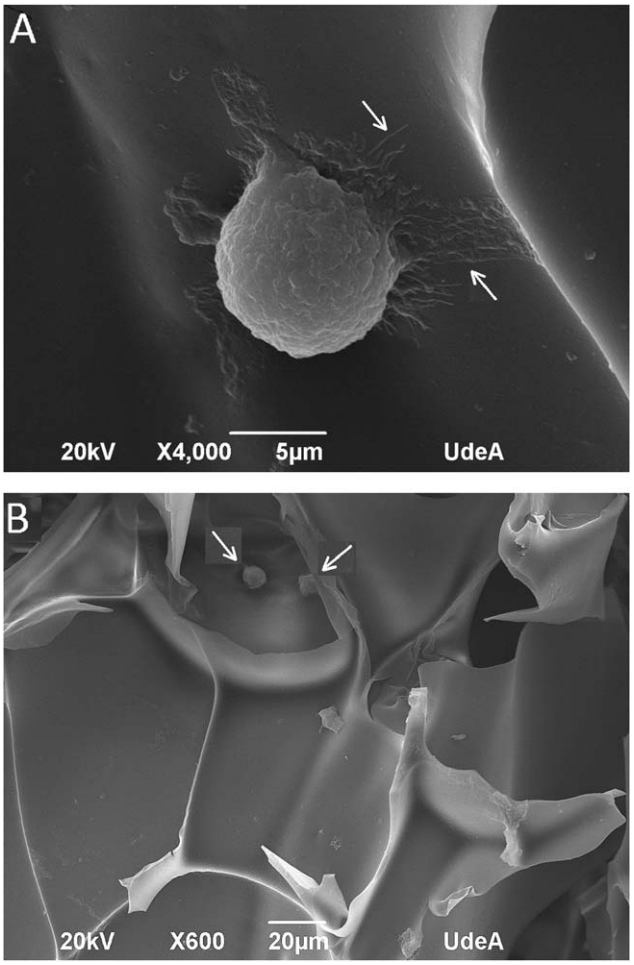

Figure 6. SEM imaging of HaCaT keratinocytes attached to chitosan–gelatin scaffolds showing *in vitro* cell-scaffold interactions. (A) C1N2 (scaffold with 25% gelatin and medium crosslinking degree) at 600×. Arrows indicate filopodias and lamellipodias; (B) C1N2 at 4000× showing cells adherence.

cytotoxic because they did not affect cell viability *in vitro*, proving that the selected biomaterials, as well as the manufacturing processes, are suitable.

Scaffolds with 25% gelatin, both cross and uncrosslinked were evaluated for genotoxicity, as they showed the highest cell viability from the MTT results. Genotoxicity of these scaffolds was tested in a keratinocyte cell line by *in vitro* comet assay after 72 h. This test can detect single-strand breaks of fragmented DNA that are able to migrate, since the lack of free ends and large size of undamaged DNA prevents migration. So the comet head will contain the high-molecular weight DNA and the comet tail the leading ends of migrating fragments. The relative tail to head intensities will reflect the number of DNA breaks.

The scaffolds did not show genotoxic damage as evidenced by the alkaline comet assay. Results showed in Table III indicate that the tested scaffolds did not generate DNA fragmentation; therefore they do not induce genetic damage. There are not significant differences between tail to head intensity ratio, when compared with the negative control (not treated cells).

Cells nuclei morphology differences are clearly seen in Figure 5. Figure 5(A) shows how latex (a positive control) generates cellular DNA fragmentation and a kite-shaped pattern, reflected in the size of the tail, while C2N3 scaffold, as a selected sample, shows that the cell nucleus remains intact [Figure 5(B)]. The absence of comet DNA tail in scaffold-associated cultured keratinocyte cells indicates that the developed scaffolds do not induce genotoxicity.

Keratinocytes Scaffolds Adhesion. Chitosan/gelatin scaffolds, both cross and uncrosslinked, exhibited cell adhesion after 24 h incubation. SEM image of Figure 6(A), shows how cells spread and adhere on the scaffolds surface through the formation of lamellipodias and filopodias (pointed out with white arrows), which are meant to help cell movement based on cell–substrate interaction. From these results it might be inferred that these 3-D scaffolds will provide a spatial framework for an adequate cell–cell and cell-matrix adhesion. Figure 6(B) shows the cells migration into the scaffold pores. Scaffolds with interconnected porosity and pore sizes in the range of 71–317 μm, which are bigger than cell dimensions (10–40 μm), may well allow cells adherence and migration.

CONCLUSIONS

Biodegradable highly porous 3D scaffolds based on chitosan, blended with gelatin and crosslinked with DHF showed interconnected pores and suitable mechanical properties, which allowed cells to adhere and grow.

Morphology, mechanical properties, and biodegradability relied on scaffolds composition which in turn determined their biological behavior. The relative comparison of biological response involving cell proliferation and viability on the scaffolds, suggests that blending of gelatin in chitosan and crosslinking, improved cellular functioning. It was demonstrated that DHF is biocompatible when used at appropriate concentrations. Thus cell viability was the highest for the upper crosslinking degree that was studied, and up to 25% gelatin content. Altogether these results demonstrate that these biocompatible hybrid scaffolds are potential templates to be used in tissue engineering, because they provide a proper framework for cells, which may well induce the growth of a healthy tissue.

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