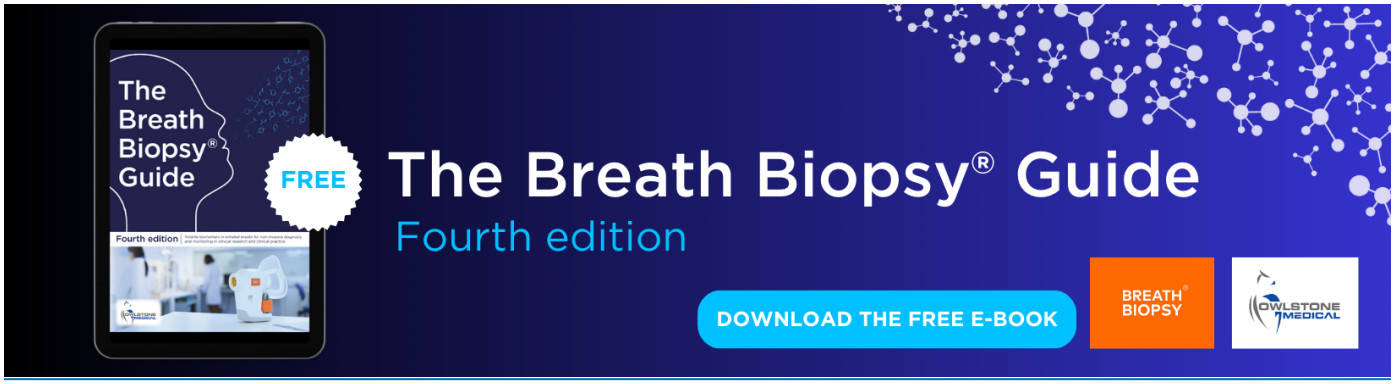
An innovative co-axial system to electrospin *in situ* crosslinked gelatin nanofibers



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An innovative co-axial system to electrospin *in situ* crosslinked gelatin nanofibers

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Abstract

Crosslinking of gelatin nanofibers maintaining a fibrous morphology after exposure to an aqueous solution is still a challenge. In this work, we developed an innovative method based on the use of an ad hoc designed co-axial needle to fabricate gelatin mats crosslinked with a very small amount of genipin and still able to retain their morphology when immersed in aqueous solution. Genipin-containing gelatin nanofibers are obtained by allowing mixing of the two solutions just within the needle. Genipin content of the electrospun mats can be modulated by varying feeding rates of the inner and outer solutions and their relative concentration. A subsequent thermal treatment of the mats, performed at 55 °C or 37 °C for 1 or 3 days and followed by rapid rinsing in ethanol and then in PB, allows one to obtain highly crosslinked gelatin nanofibers that perfectly maintain their morphology after immersion in an aqueous solution, display improved mechanical properties and enhanced stability. This new approach allows us to achieve gelatin mat stabilization using a very small amount of genipin with respect to other methods and to avoid post-treatment of the mats with the crosslinking agent, with a consequent significant reduction of the final cost of the materials. Moreover, *in vitro* tests demonstrate that the crosslinked mats support normal human primary chondrocyte culture, promoting their differentiation.

1. Introduction

Gelatin, a natural polymer obtained through collagen denaturation, is employed in a variety of different fields, including the food industry, cosmetics, pharmaceutical and medical applications [1–4]. Gelatin is indeed a low-cost material, and displays a number of desirable qualities, such as biocompatibility, biodegradability and non-immunogenicity. Moreover, it exhibits many integrin binding sites for cell adhesion, signalling and differentiation, which accounts for its wide employment in tissue engineering and wound dressing as well as in the drug delivery field [5–9].

In this sector, many applications rely on electrospun gelatin nanofibers. Due to the high solubility of gelatin, the fibrous morphology is easily lost when it comes into contact with water. To improve water resistance, gelatin nanofibers need to be crosslinked. To this end, the most common crosslinking agents are aldehydes, carbodiimide, hexamethylene diisocyanate and acyl azides, which however are often reported to give cytotoxicity problems [10–15]. Non-toxic agents, such as oxidate dextran, avoid solubilization although many fibers appear fused together after immersion in water [11]. Very good results have been obtained using genipin, a natural agent obtained from the gardenia fruit, which is far less toxic than glutaraldehyde [8, 16]. The reaction mechanism between gelatin and genipin has been reported in the literature [17]: it has been proposed that genipin reacts with primary amine groups of the lysine residues to form covalently crosslinked networks. As for most crosslinking agents, the method developed for genipin was based on incubation of a gelatin nanofibrous mat in a suitable solution of genipin for an appropriate period of time. In fact, just a very small amount of genipin can be introduced directly in the starting electrospinning solution in order to avoid gelling [8]. Therefore, a post-treatment through immersion of

the obtained electrospun mat in highly concentrated genipin solution is required to achieve a suitable degree of crosslinking. The previously optimized crosslinking conditions ensured fiber morphology maintenance upon water contact, but required a considerable amount of genipin and time [8].

In this study we demonstrate that it is possible to drastically reduce the relative amount of genipin, avoiding post-treatment with the crosslinking agent, and still get highly stable mats, able to retain their morphology when immersed in aqueous solution. This result was achieved thanks to the use of a specifically designed coaxial needle where genipin solution (in the inner needle) is separated from gelatin solution and mixes with gelatin just within the outer needle, thus preventing undesired gelling. With respect to previously proposed solutions [8, 18, 19], the cutting-edge strategy advanced in the present work not only uses a significantly reduced amount of the crosslinking agent, but is also a cheaper, faster and simpler process to fabricate mats that are able to retain their fibrous morphology after immersion in an aqueous solution. The electrospun mats have been characterized in terms of fiber morphology, crosslinking degree and gelatin release. The influence of crosslinking on the mechanical properties of the mats was investigated. A normal human primary chondrocyte cell culture was used to assess cell viability and to evaluate cell differentiation and metabolic activity.

2. Materials and methods

2.1. Materials

Type A gelatin from porcine skin was supplied by Sigma-Aldrich (300 Bloom), genipin was provided by Wako Chemicals USA and was used without further purification. Acetic acid (AcAc) was supplied by Sigma-Aldrich.

2.2. Preparation of electrospun scaffolds

The home-made electrospinning apparatus is composed of a high voltage power supply (Spellman SL 50 P 10/CE/230), two syringe pumps (KD Scientific 200 series), a glass syringe, a stainless steel coaxial needle connected to the power supply electrode and a grounded aluminum collector (10 cm $\times$10 cm). The coaxial needle used in the present work (figure 1(A)) is constituted by an inner needle (OD = 0.9 mm, ID = 0.6 mm) positioned concentrically to the outer needle (OD = 1.5 mm, ID = 1.2 mm). The tip of the outer needle protrudes 10 cm below that of the inner needle. In addition the tip of the inner needle wall has 20 circular holes (Ø = 0.5 mm) organized in four lines distributed along the needle circumference. Each line contains five small holes separated 2 mm from each other (figure 1(B)). The solutions were individually dispensed at a controlled flow rate by using syringe pumps through a Teflon tube to the outer and inner needles. The flow rate of the shell solution was set at 0.18 ml h^{-1} while that of the core solution was 0.09 ml h^{-1}. The coaxial needle was placed vertical to the collecting plate at a distance of 15 cm and the applied voltage was set at 21 kV. The shell solution was prepared by dissolving gelatin in AcAc:H_2O (60:40 v/v) at a concentration of 30% w/v. To fabricate gelatin mats containing 9% w/w of genipin (labeled G9), the core solution was prepared by dissolving genipin at a concentration of 6% w/v in AcAc, while gelatin mats containing 18% w/w of genipin (labeled G18) were obtained by using a 12% w/v core solution of genipin. A reference mat not containing genipin (labeled GEL) was prepared by using as core solution pure AcAc. Electrospun mats were kept under vacuum over P_2O_5 at room temperature (RT) overnight in order to remove residual solvents.

2.3. Activation of electrospun scaffolds crosslinking

Electrospun mats containing genipin were fixed to plastic rings (CellCrown™12, Scaffdex) and subjected to different crosslinking treatments that were carried out either at 37 °C or 55 °C for either 1 or 3 d. After this first thermal treatment, the mats were immersed in ethanol for 5 h at the same temperature. Samples were then washed either in MilliQ water or in phosphate buffer solution (PB, 0.1 M, pH = 7.4) for a few minutes and finally dried at RT. Crosslinked samples were labeled as follows: Sample_XdTY. Xd indicates the days of thermal treatment, *T* the temperature of thermal treatment and Y the washing media (W for water and B for PB). For instance, the gelatin mat containing 9% w/w of genipin that was crosslinked for 1 d at 37 °C and finally washed in PB was labeled: G9_1d37B.

2.4. Scaffold characterization techniques

Scanning electron microscopy (SEM) observations were carried out by using a Philips 515 SEM at an accelerating voltage of 15 kV, on samples sputter-coated with gold. The distribution of fiber diameters was determined through the measurement of about 200 fibers by means of an image acquisition and analysis software (EDAX Genesis) and the results were given as the average diameter $\pm$ standard deviation (SD).

The extent of crosslinking of gelatin mats was determined by an Ultraviolet (UV) assay of not-crosslinked ε-amino groups before and after crosslinking treatment [20]. Electrospun samples of about 5 mg were incubated with 1 ml of a 4% w/v $NaHCO_3$ solution and 1 ml of 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution at 0.5% w/v for 4 h at 40 °C. 3 ml of HCl 6 M were then added and the solution was maintained at 110 °C for 24 h. The absorbance of the diluted solution was measured at 346 nm in a Kontron Uvikon 931 spectrophotometer against a blank. Equation (1) relates the solution absorbance and moles of ε-amino groups per gram of gelatin:

$$\frac{\text{moles } \varepsilon \text{ ammino groups}}{\text{g GEL}} = 2 \cdot \frac{A \cdot 0.02}{1.46 \cdot 10^4 \cdot bx} \qquad (1)$$

where *A* is the measured absorbance, 0.02 l is the volume of the analyzed solution, 1.46×10^4 ($l \times mol^{-1} \times cm^{-1}$)

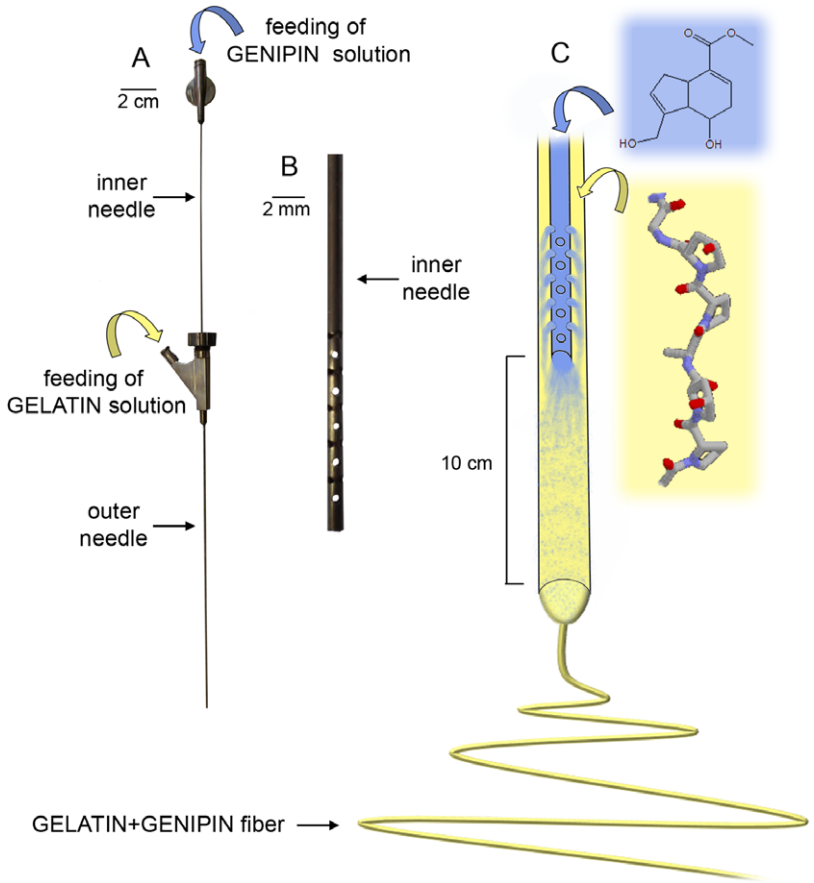

Figure 1. Innovative coaxial needle. (A) Picture of the coaxial needle, (B) enlarged picture of the tip of the inner needle characterized by the presence of 20 holes of 0.5 mm in diameter distributed along needle circumference, (C) sketch of the core and shell solution mixing process inside the outer needle (not drawn in scale).

is the molar absorptivity of 2,4,6-trinitrophenyl-lysine (TNP-lys), b is the cell path length in cm, and x is the mat weight in g. The crosslinking degree is the ratio between the moles of free ε-amino groups in crosslinked gelatin and the moles of ε-amino groups in not-crosslinked gelatin.

The amount of gelatin released by the crosslinked electrospun mats kept in PB at 37 °C for different periods of time was measured by using a spectrophotometric method. About 1 mg of electrospun sample was immersed in 1 ml of PB at 37 °C. At defined time intervals the PB was removed and substituted with a fresh one. 200 μl of the extracted solution was incubated at 40 °C for 30 min with 3 ml of PB and 2 ml of a solution containing 4% w/v of aqueous $CuSO_4$ and bicinconinic acid in a ratio of 1:50 v/v. The absorbance of the solution was measured at 562 nm in a Kontron Uvikon 931 spectrophotometer against a blank and the gelatin concentration was calculated by using a pre-determined calibration curve [21]. The amount of released gelatin was measured after 1, 24, 48 and 72 h and after 7 and 15 d of immersion in PB.

Tension tests were performed by recording the stress–strain curves of electrospun mats using an Instron Testing Machine 4465, with a crosshead speed of 1 mm min^{-1}, and the Series IX software package. Eight rectangular specimens for each mat (5 mm wide, gauge length = 20 mm, thickness around 20 μm measured by microcaliper) were analyzed. Mechanical data (tensile modulus (E), strain at break (ε_b) and stress at break (σ_b)) of the strips were given as the average value ± SD. Single factor analysis of variance (ANOVA) was employed to assess statistical significance of the results. $P < 0.001$ was considered statistically significant.

2.5. *In vitro* tests

2.5.1. Cell seeding and culture

G18_3d37B and G18_1d55B mats were fixed to plastic rings (CellCrowns™12, Scaffdex), and sterilized by immersion in 85% ethanol for 15 min, then 70% ethanol for 15 min, followed by three rinses with PB plus 2% penicillin/streptomycin (Euroclone), 0.2% amphotericin B (Sigma). Mats were kept in this solution overnight under UV irradiation (230 V at 50 Hz). The next day the PB solution was removed and the mats were pre-wetted in culture medium.

A normal human primary chondrocyte culture derived from human knee articular cartilage (NHAC-kn, Clonetics™ Cell System, Lonza Milano srl, BG, I) was used for the experiment. Cells were expanded in monolayer cultures, using a chondrocyte growth medium (CGM, containing FBS 5%, gentamicin sulfate-amphotericin B 0.1%, bFGF-B 0.5%, R3-IGF-1 0.2%, insulin 0.2%, transferrin 0.1%). When cells reached 70–80%

confluence they were detached from culture flasks by trypsinization, and centrifuged; cell number and viability were checked with the trypan blue dye exclusion test (Sigma, UK). At the first passage, a concentrated cell suspension of 1×10^5 cells in 50 μl was seeded dropwise on a 1 cm^2 surface of sterilized mats (six samples for each material) in 12-well plates. After 1 h, to allow cell attachment and scaffold colonization, 950 μl of chondrocyte differentiation medium (CDM: CGM supplemented with TGFβ-1 0.5%, R3-IGF-1 0.2%, insulin 0.2%, transferrin 0.2% and ascorbic acid 2.5%) was been added to each well to activate chondrocytes . Cultures were maintained at standard conditions at 37 °C ± 0.5 with 95% humidity and 5% CO_2 ± 0.2 up to 7 d. The same concentration of cells was also seeded on polystyrene of the culture plate as a control (CTR). A positive CTR (CTR+) with a 0.5% phenol solution in a medium for cytotoxicity tests was added.

2.5.2. Cytotoxicity

Cell viability was evaluated after 48 h of culture on crosslinked scaffolds and CTRs by WST1 reagent test (WST1, Roche, D): 100 μl of WST1 (tetrazolium salt) and 900 μl of fresh culture medium were added at every well and cultures were incubated at 37 °C for a further 4 h. Tetrazolium salt is transformed to formazan by reductase of mitochondrial respiratory chain, active in viable cells only. Supernatants were measured by a spectrophotometer at 450/625 nm. Results were reported as optical density (OD) and values directly correlated with cell number.

At 48 h LDH activity (Lactate dehydrogenase, Roche, D) was measured in the supernatant to assess cytotoxicity: 100 μl of reagent was added to 100 μl of cell supernatant in a 96-well plate; after 30 min of incubation at RT in the dark, samples were evaluated by spectrophotometer at 490/655 nm.

2.5.3. Cell bioactivity and cell morphology

After 7 d of culture, cell viability and most common markers of chondrocyte differentiation and activity were evaluated. Cell viability was measured by the WST1 reagent (see section 2.5.2). At the end of experimental time the supernatant was collected from all wells, centrifuged to remove particulates, and used to perform the following immunoenzymatic assays (Boster Biological Technology, Ca, USA): cathepsin B (CTSB, Boster Biological Technology, Ca, USA), collagen type II (COLL2, Cloud-Clone USCN Life Science, Wuhan, China).

To observe cell colonization onto G18_3d37B and G18_1d55B, at the end of experimental time, cultures were washed in PB and fixed in a solution of 4% formaldehyde in PB for 15 min at 37 °C. Then the samples were permeabilized in 0.5% Triton X-100 for 15 min, washed in PB, and a FITC-conjugate phalloidin solution (Sigma Aldrich, Steinheim, Germany) 1:100 in PB was added for 30 min at 37 °C, followed by DAPI (Invitrogen, Life Technologies Italia, Monza, I) for 5 min. After washing, samples were examined by fluorescence microscope and images from different wavelengths were merged.

SEM investigation of cell morphology was carried out on samples fixed in 2.5% glutaraldehyde, immersed in pH 7.4 PB 0.01 M for 1 h, dehydrated in a graded ethanol series and sputter-coated with Pt:Pd alloy.

2.5.4. Statistical analysis

Statistical evaluation of data was performed using the software package SPSS/PC+ Statistics TM 23.0 (SPSS Inc., Chicago, IL USA). Data are reported as mean ± SD of triplicate at a significance level of $p < 0.05$. After having verified normal distribution and homogeneity of variance, a one-way ANOVA was used for comparison between groups. Scheffé's post hoc multiple comparison tests were performed to detect significant differences between groups.

3. Results

3.1. Scaffold preparation and crosslinking

Genipin-containing gelatin mats were obtained by using an innovative specially designed coaxial needle, depicted in figure 1, which permits the electrospinning of different solutions simultaneously, at desired values of feeding rate, concentration and mixing time. The genipin solution flowing in the inner needle mixed with the gelatin solution inside the outer needle before the formation of the electrospun jet to obtain gelatin fibers with homogeneously dispersed genipin.

The SEM images of as-spun mats of G9, G18 and GEL, produced using this coaxial needle, are shown in figure 2. Mats containing two different concentrations of genipin were produced by changing the genipin concentration in the core solution. The obtained fibers have in all cases a bead-free morphology with nanometric diameters in the range 300–400 nm.

The crosslinking of gelatin promoted by the genipin contained in the fibers was subsequently activated by applying different steps, as sketched in figure 3(A) and documented by the photographs of figure 3(B) for the G18 sample: first, the white as-spun mats were subjected to thermal treatment and incubated at a certain temperature (either 37 °C or 55 °C) for a given time (either 1 or 3 d), in order to activate the crosslinking reaction [22]; subsequently the partially crosslinked brown-coloured mats were immersed in ethanol for 5 h (at the same temperature of the previous thermal treatment) and finally soaked either in water or in PB before SEM analysis. The increase of crosslinking degree after the different steps is qualitatively appreciated by the change of mat coloration that was white after electrospinning and acquired a blue colour after PB conditioning. Figure 3 also reports SEM images of the G18 mats after the different steps of the crosslinking procedure: it can be inferred that mat conditioning in PB better maintained fiber morphology with respect to conditioning in water

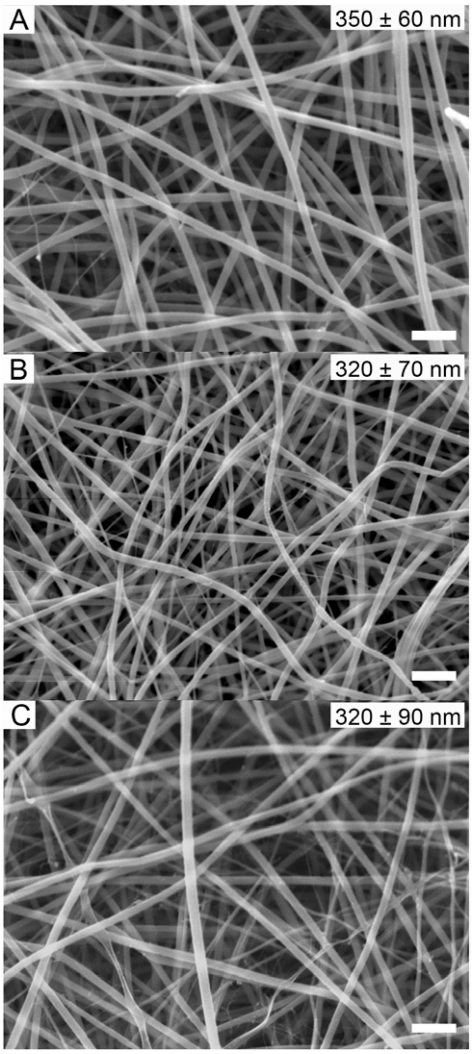

Figure 2. SEM micrographs of as-spun electrospun mats. (A) G9, (B) G18 and (C) GEL. Fiber mean diameter and standard deviation are reported for each sample. Scale bar = 2 μm.

(G18_1d37W and G18_3d37W turned into non-porous films while G18_1d55W only slightly preserved the fibrous pattern). Furthermore, it is evident that, when the thermal treatment was carried out at 37 °C, the prolongation of treatment time (3 d versus 1 d) significantly improved the final fiber morphology (compare figure 3(C4) with figure 3(D4)). Moreover, the increase of temperature from 37 °C to 55 °C further improved the final fiber morphology (compare figure 3(C4) with figure 3(E4)), even if the thermal treatment at 55 °C was carried out for a shorter period of time (compare figure 3(D4) with figure 3(E4)).

Mats obtained with a genipin concentration of 9% w/w did not give satisfactory results, as shown in figure 4, for G9_3d37B and G9_1d55B samples. Indeed, even if the fibrous morphology was maintained after the heat treatment step, the subsequent immersion in ethanol caused fusion of a considerable part of the fibers. Moreover, fiber morphology appeared quite affected after immersion in PB, whereas the mats turned into a non-porous film when immersed in water (images not shown).

3.2. Scaffold characterization

Further characterizations were carried out only on samples that maintained fibrous morphology after immersion in aqueous solution, i.e. G18_3d37B and G18_1d55B.

Figure 5 reports SEM images where the maintenance of the porous structure is evident for both samples, even if a more open porosity and less fiber fusion is displayed by the G18_1d55B sample compared to the G18_3d37B one. The extent of crosslinking was quantified by calculating the moles of free ε-amino groups per gram of gelatin (equation (1)). The results indicate that G18_3d37B was less crosslinked than G18_1d55B (55 $\pm$ 5% versus 79 $\pm$ 5%), in agreement with SEM observations.

The amount of gelatin released from the crosslinked mats when immersed in PB at 37 °C is reported in figure 6. The results shows that the release of gelatin is faster in G18_3d37B sample with respect to G18_1d55B one. Moreover, after about 20 d in PB the former sample is completely dissolved while the latter still remains solid. This behavior is perfectly in line with the different crosslinking degree of the two samples.

Tensile stress–strain analyses of the crosslinked samples were carried out and compared with that of GEL sample not containing genipin: mechanical data are reported in table 1 and representative curves in figure 7. Crosslinking greatly affects the mechanical properties of the mats that, in comparison with the not-crosslinked GEL sample, displayed an increase of one order of magnitude of elastic modulus and stress at break, as well as reduced deformation at break. By comparing the two crosslinked samples, G18_3d37B is more rigid and fragile compared to G18_1d55B, having a significantly higher stress at break. This apparent disagreement with the results of crosslinking degree can be easily justified by the lower porosity and greater fiber fusion of G18_3d37B, which contribute to increase the stress at break.

3.3. *In vitro* tests

Cell viability (figure 8(A)) was assessed by using normal human primary chondrocyte culture derived from human knee articular cartilage after 48 h of culture on the scaffolds. G18_3d37B and G18_1d55B values (77% and 85% of CTR—respectively) indicate absence of cytotoxicity. In fact, cytotoxicity implies a reduction of viability greater than 30% with respect to CTR: the values measured for the mats were higher and not statistically different from CTR. However, CTR+ was 11% and significantly lower than CTR- and experimental samples ($p < 0.005$), as expected.

LDH enzyme activity (figure 8(B)) is an indirect parameter of cytotoxicity as the enzyme is released in the culture medium when the number of dead cells, or cells with damaged plasma membranes, increases. Results of LDH dosage show significantly higher values in CTR+ ($p < 0.005$) when compared to experimental samples and CTR. LDH and viability data showed a highly significant inverse Pearson correlation ($p < 0.005$).

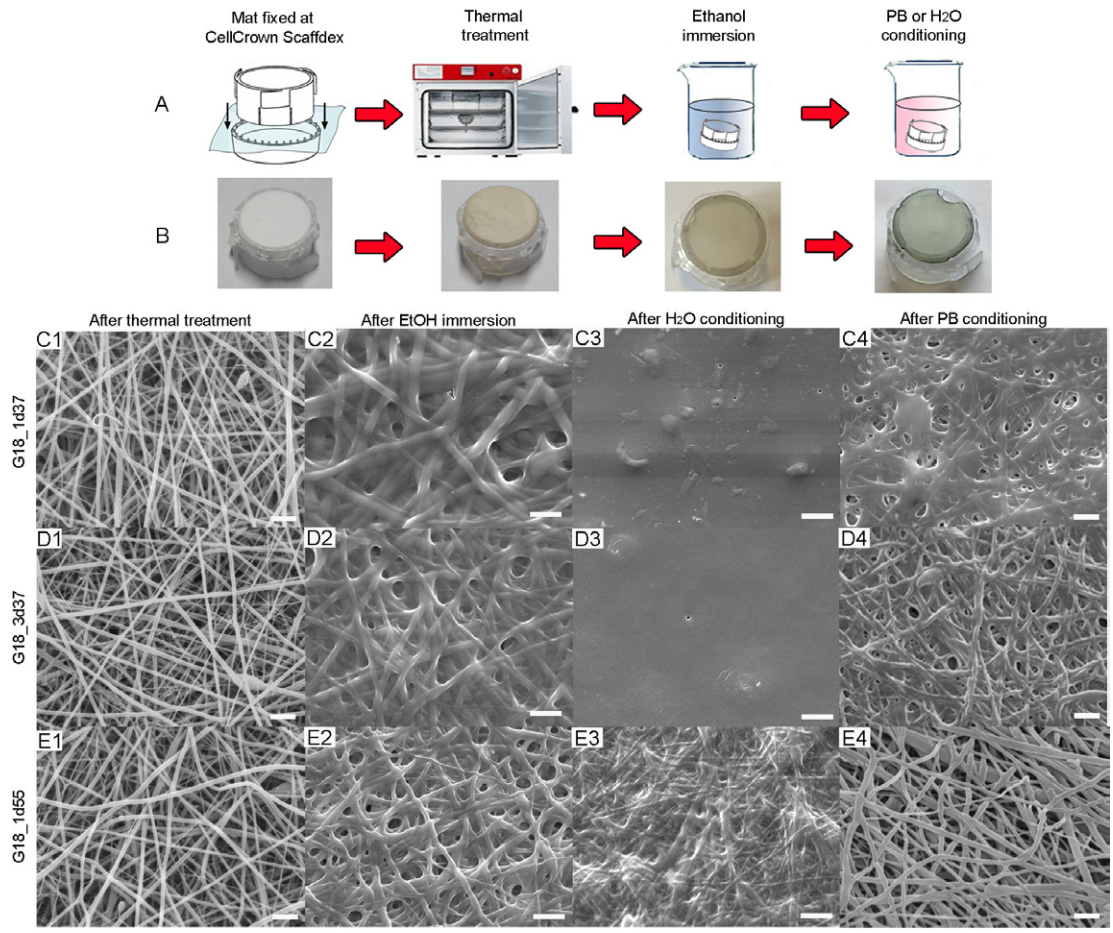

Figure 3. Activation of crosslinking of G18 sample. (A) Sketch describing the steps of crosslinking procedure applied to genipin-containing gelatin mats and (B) corresponding pictures of G18 sample fixed at CellCrown™12 plastic rings. SEM images of G18_1d37 (row C), G18_3d37 (row D) and G18_1d55 (row E) samples after different steps of the crosslinking procedure: thermal treatment (column 1); EtOH immersion (column 2); H_2O conditioning (column 3); PB conditioning (column 4). Scale bars: C1, C4, D1, D4, E1 and E4 = 2 μm; C2, C3, D2, D3, E2 and E3 = 5 μm.

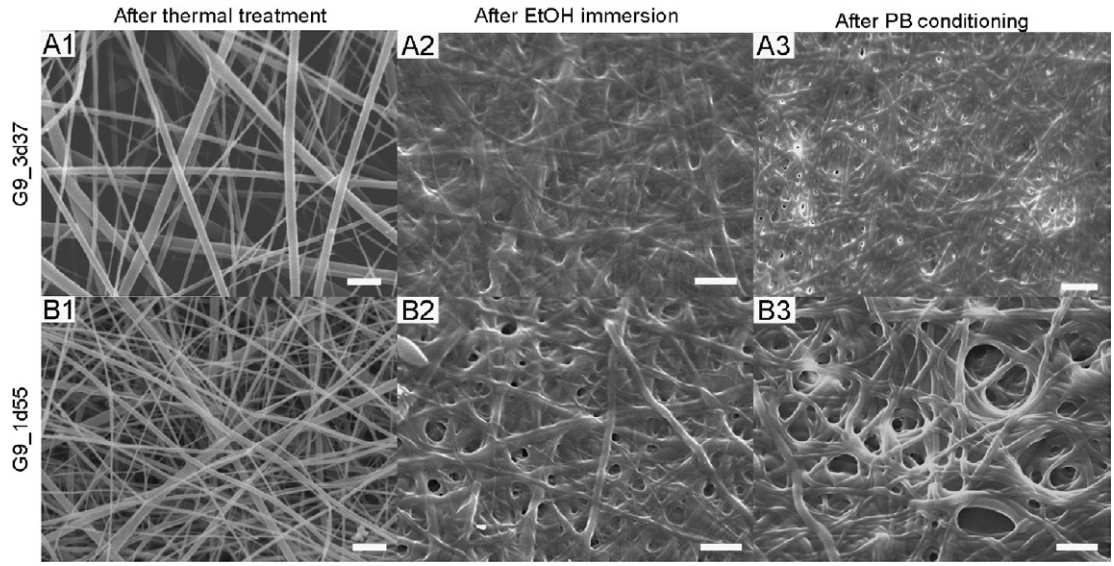

Figure 4. Crosslinking of G9 sample. SEM images of G9_3d37 ((A1)–(A3)) and G9_1d55 ((B1)–(B3)) samples after different steps of the crosslinking procedure. Scale bars: A1 and B1 = 2 μm; A2, A3, B2 and B3 = 5 μm.

NHACs were cultured for 7 d in direct contact with biomaterials to evaluate differentiation and metabolic activity. NHACs cultured on G18_3d37B and G18_1d55B samples showed a significant higher viability when compared to CTR (120% and 125% respectively versus 100%, $p < 0.05$). No significant differ-

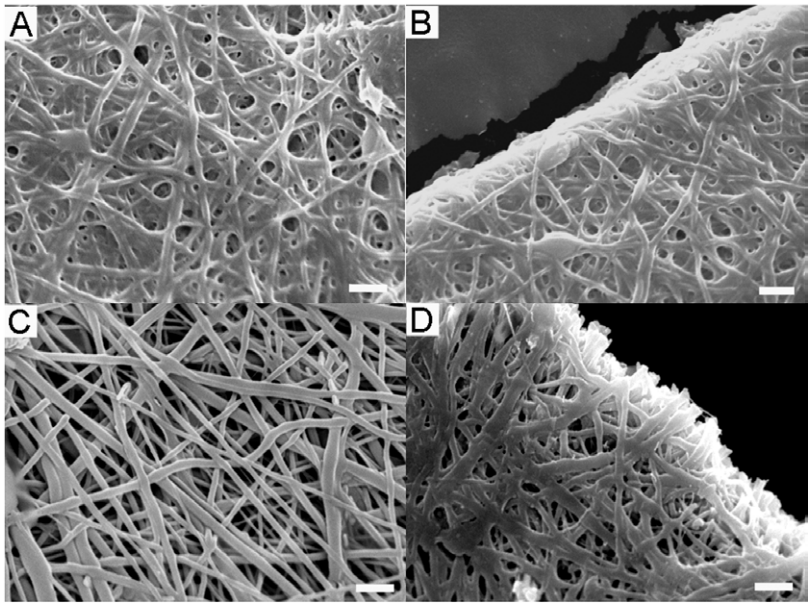

Figure 5. Optimized crosslinked samples. SEM images of G18_3d37B ((A) and (B)) and G18_1d55B ((C) and (D)). Scale bar = 2 μm.

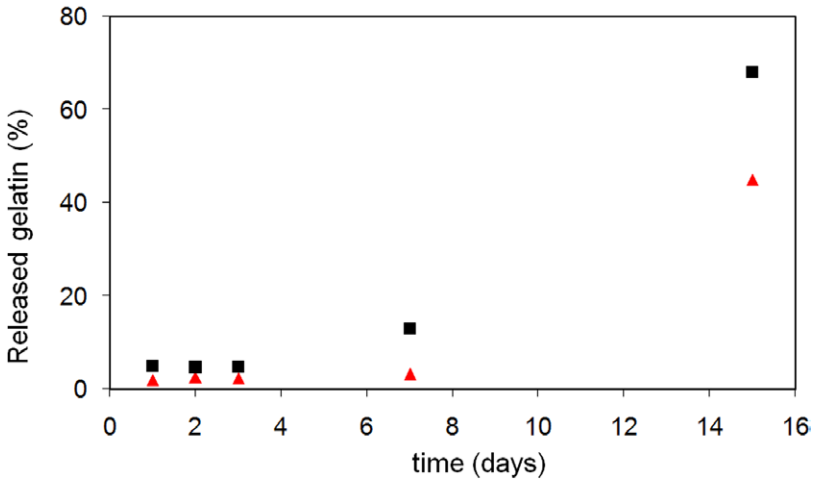

Figure 6. Gelatin release from optimized crosslinked samples. Gelatin released from G18_3d37B (square) and G18_1d55B (triangle) samples when kept in PB at 37 °C for different times.

Table 1. Mechanical properties of GEL, G18_3d37B and G18_1d55B samples: tensile modulus (E), stress at break (σ_b) and strain at break (ε_b).

Sample	E (MPa)	σ_b (MPa)	ε_b (%)
GEL	120 ± 20	3.1 ± 0.4	18 ± 3
G18_3d37B	1300 ± 250	52 ± 5	6.2 ± 0.4
G18_1d55B	1100 ± 150	29 ± 2	8 ± 2

ences were found between G18_3d37B and G18_1d55B (figure 9(A)).

The good viability on experimental samples was also demonstrated by the results of phalloidin staining: the images reported in figures 10(A) and (B), where cell nuclei are blue and actin filament are green, show that cells grow adherent to the whole surface of the crosslinked scaffolds. Moreover, at 7 d chondrocytes appeared attached and well spread, and rich in filopodia on all the samples, as shown in the SEM images reported in figures 10(C) and (E) for G18_3d37B, and in figures 10(D) and (F) for G18_1d55B. An interesting aspect, in view of the formation of tissue engineering constructs, was the evidence of in-depth cell penetration into both the G18_3d37B and the G18_1d55B scaffolds, with the presence of cells throughout and underneath the scaffold, as evidenced by the SEM micrographs reported in figures 10(G) and (H).

Chondrocyte differentiation was evaluated through measurement of some common parameters at 48 h and 7 d, namely CTSB cathepsin B (CTSB), which displays a down-regulated production in differentiated culture with respect to undifferentiated one, and collagen type II (COLL2) as one of the main components of articular cartilage extracellular matrix, synthesized

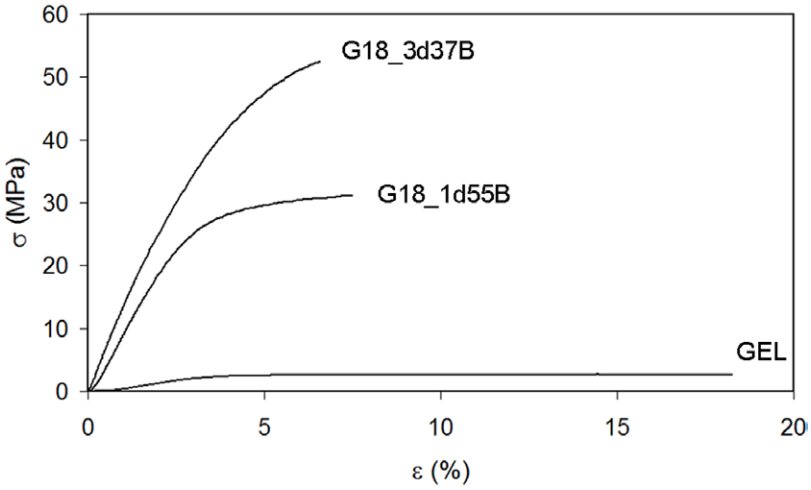

Figure 7. Tensile stress–strain curves of GEL, G18_3d37B and G18_1d55B samples.

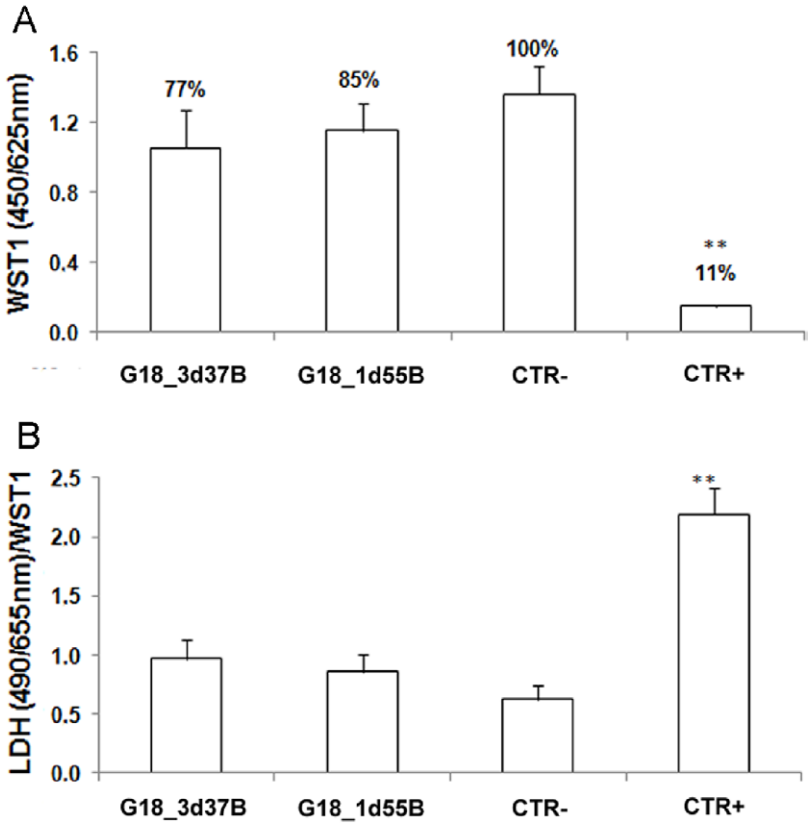

Figure 8. Cytotoxicity tests. Chondrocyte viability (A) and LDH production (B) at 48 h (**$p < 0.005$. A: **CTR+ versus G18_3d37B, G18_1d55B, CTR−; B: **CTR+ versus G18_3d37B, G18_1d55B, CTR−.

by differentiated chondrocytes. Chondrocytes cultured on G18_3d37B, G18_1d55B and CTR for 7 d showed significantly lower values of CTSB with respect to the values measured at 48 h ($p < 0.05$). In contrast, no variation in CTSB production with time was observed in control group treated with undifferentiating medium (CTRnd, figure 9(B)). Results obtained on COLL2 indicate that its production increases with time, from 48 h to 7 d ($p < 0.05$) both in the experimental groups and in CTR. Moreover, at 7 d COLL2 levels in G18_3d37B were significantly higher ($p < 0.05$) than in G18_1d55B and on CTR (figure 9(C)).

4. Discussion

The use of gelatin electrospun fibers in tissue engineering applications needs stabilization of the gelatin fibers against water dissolution by crosslinking treatments. These treatments require the use of chemical agents that, at high concentrations, might be toxic for cells and increase the cost and time of scaffold production. We previously focused on the use of genipin as low toxicity crosslinking agent for stabilizing gelatin electrospun fibers [8]. Conversely to previous literature reports, where crosslinking was always performed by exposing

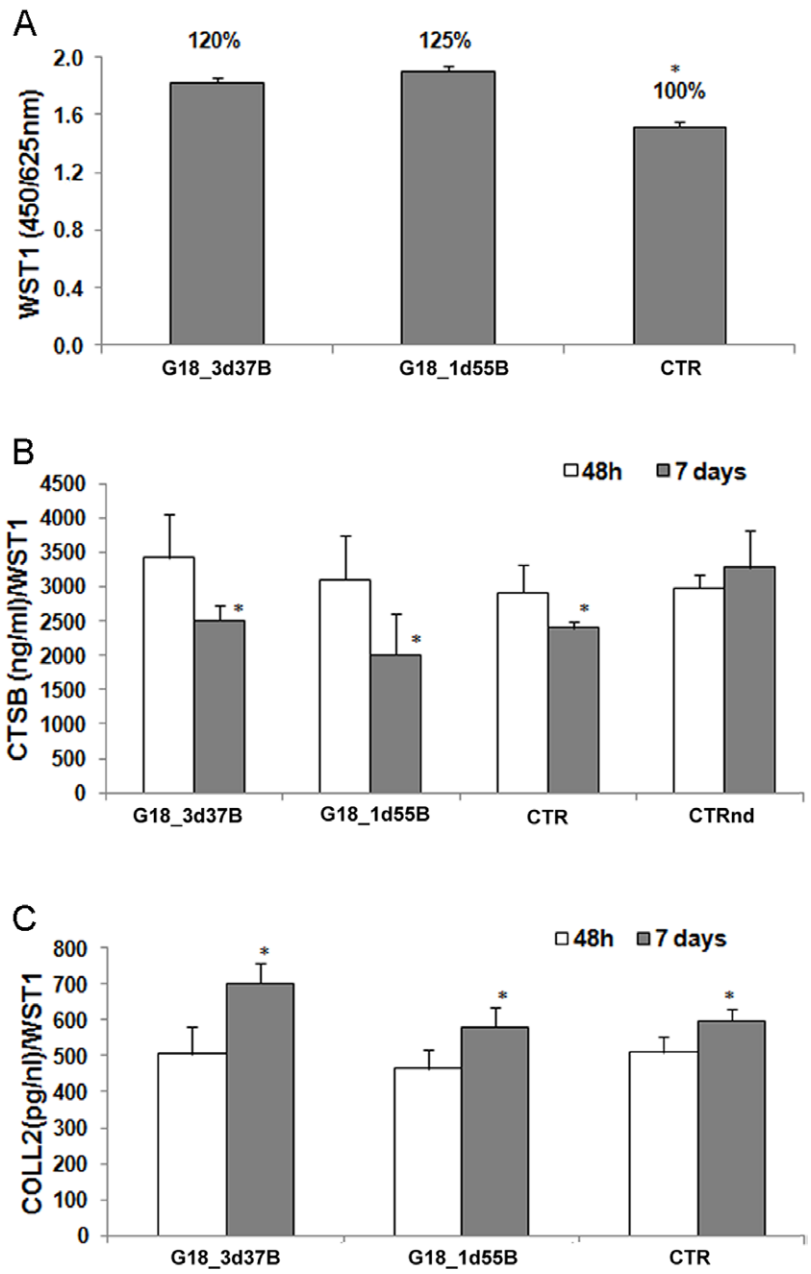

Figure 9. Viability and activity of chondrocytes cultured on G18_3d37B and G18_1d55B, as well as on CTR. (A) NHAC proliferation at 7 d. (B) CTSB and (C) COLL2 production at 48 h and at 7 d. (*$p < 0.05$). A: *CTR versus G18_3d37B, G18_1d55B; B: G18_3d37B, G18_1d55B, CTR: *7 d versus 48 h; C: G18_3d37B, G18_1d55B, CTR: *7 d versus 48 h; 7 d: *G18_3d37B versus G18_1d55B, CTR.

the as-electrospun gelatin fibers to the chemical crosslinker [13, 23, 24], in the authors' previous work it was demonstrated that the introduction of genipin in the gelatin solution during electrospinning significantly improved the maintenance of fiber morphology [8], even if only small amounts of genipin could be introduced in the starting solution in order to prevent gelification. A similar approach was later applied by Angarano *et al* [6] as well as by Meng *et al* [25] for crosslinking electrospun collagen. However, in our previous work [8] we demonstrated that a small amount of genipin added to the gelatin solution, even if it limited the dissolution of gelatin mat in water, was not enough to guarantee a satisfactory maintenance of fibrous structure, which required successive exposure of the mat to a highly concentrated genipin solution.

With the aim to simplify the crosslinking procedure and to decrease the overall amount of genipin used to achieve a high degree of gelatin crosslinking, in this work we modified the instrumental electrospinning apparatus by using for the first time an 'ad hoc' designed coaxial needle (figure 1).

This needle allowed us to keep the gelatin and the genipin solutions separated in two different syringes. In contrast to electrospinning a starting solution containing both genipin and gelatin with a conventional single needle, the separation of the two solutions avoids any possible change of fiber morphology during

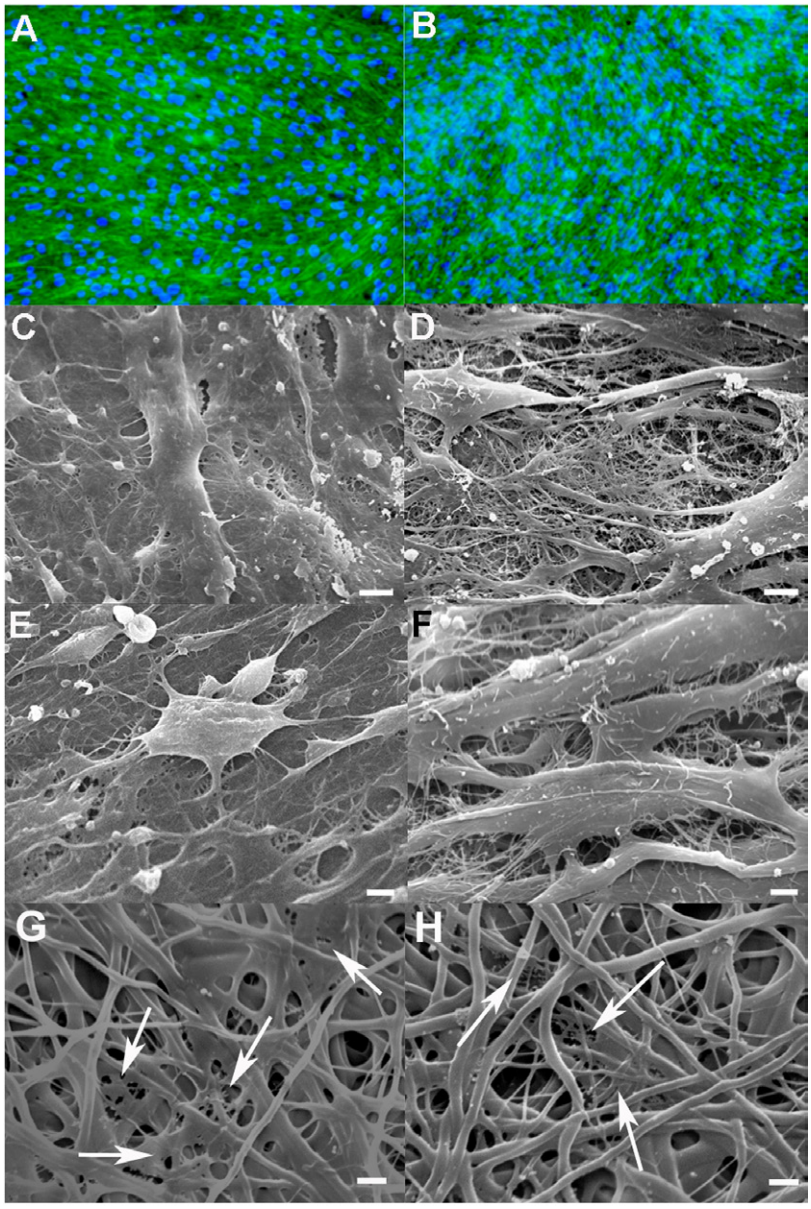

Figure 10. Fluorescence detection of phalloidin staining showing nuclei (blue) and actin filaments (green) of NHAC adhered to material surface and SEM images of chondrocytes after 7 d of culture. Fluorescence images of cells cultured on G18_3d37B (A) and G18_1d55B (B) (10×). SEM micrographs of cells cultured on G18_3d37B: top side ((C), (E)) and underside (G). SEM micrographs of cells cultured on G18_1d55B: top side ((D), (F)) and underside (H). The arrows in (G) and (H) indicate cells infiltrated within the scaffold. Scale bars: (C) and (D) = 5 μm; (E)–(H) = 2 μm.

time as a consequence of solution viscosity change (occurring when gelatin crosslinks in the presence of genipin) and, most of all, avoids solution gelification. The direct implication of this set-up is the possibility to increase the amount of genipin added to the fibers.

It is worth noting that our coaxial needle differs from those commonly employed to get core–shell fibers that are designed to limit the mixing of the two solutions and are often designed with the tip of the inner capillary protruding below the outer one by few decimals of millimeters for facilitating the entrainment of the inner solution by the outer one to gain core–shell fibers [26]. In our coaxial needle, the tip of the outer capillary protrudes several centimeters below that of the inner needle to permit the mixing of the two solutions inside the needle well before the formation of the Taylor cone. Solution mixing was also promoted by the specific design of the inner needle that enabled us to extrude the inner solution from several holes inside the outer needle. Moreover, the duration of solution mixing can be controlled by the protrusion of the outer needle with respect to the inner one and by the gelatin solution flow rate. By applying the electrospinning conditions described in section 2 we calculated that the gelatin and the genipin solutions mix together inside the coaxial needle for about 25 min before being ejected and electrospun. As-spun obtained mats of G9 and G18 were subsequently submitted to the procedure depicted in figure 3(A), in order to activate the crosslinking reaction promoted by genipin. SEM characterization of mats following the different steps,

reported in figures 3 and 4, showed that the different thermal treatments did not modify fibrous morphology, which was partially lost after immersion in ethanol. Both these steps were found to be crucial for stabilizing the fibers. Indeed, all electrospun mats immersed in aqueous solution just after thermal treatment, as well as those directly soaked in ethanol, completely lost their fibrous morphology. As far as the third step is concerned, the results of SEM investigation indicated that conditioning in PB is preferable in order to better maintain the fibers' morphology. This is not surprising since it has been shown that PB can contribute to the crosslinking reaction of genipin [27], and that genipin displays its maximum crosslinking activity at pH 7.4 [22].

Overall the results of figures 3 and 4 show that a satisfactory maintenance of fibrous morphology can be obtained when gelatin fibers are loaded with an amount of genipin of 18 wt% and the mat is kept either at 37 °C for 3 d or at 55 °C for 1 d, with successive immersion in EtOH and then in PB buffer (i.e. G18_3d37B in figure 3(D4) and G18_1d55B in figure 3(E4)). It is pointed out that the temperature of thermal treatment was not further increased beyond 55 °C to avoid fiber melting.

The crosslinking degree measured for G18_1d55B is somewhat higher than the one found for G18_3d37B and justifies its lower release of gelatin in solution.

The obtained results demonstrate that the crosslinking procedure developed in this work provides a facile and relatively fast method to prepare gelatin nanofibers that are able to maintain their morphology when immersed in water solution. It is worth noting that the amount of genipin required to achieve this result is much lower than that previously reported [8]. In fact, by taking into account the operative conditions, the new crosslinking approach requires a genipin amount of about 0.02 mg per mg of mat, whereas the previous one required about 2.5 mg of genipin per mg of mat. This means a reduction by more than two orders of magnitude of crosslinker costs. Furthermore, the time necessary to complete crosslinking is also significantly shorter than the one optimized in our previous work [8].

Results of *in vitro* tests performed by culturing NHAC on G18_3d37B and G18_1d55B samples showed absence of cytotoxicity and a significantly higher viability for both samples compared to CTR. This result was confirmed by phalloidin staining tests that demonstrated a good cell adherence to the scaffold surface as well as cell spreading and, more interestingly, in-depth cell infiltration. Moreover, by using CTSB and COLL2 as parameters to investigate cell differentiation, it was demonstrated that both types of investigated mats promoted NHAC differentiation.

5. Conclusions

The successful crosslinking method developed in this work exploits a new, specially designed, coaxial needle that permits the incorporation of genipin inside gelatin nanofibers during the electrospinning procedure. Successive activation of genipin-gelatin reaction is obtained by rapid rinsing into EtOH and buffered solution at a fixed temperature, and allows one to obtain highly crosslinked mats that retain their fibrous morphology when immersed in aqueous solution. Moreover, the mats show enhanced mechanical performance and higher stability to the degradation process. However, the amount of genipin used in the process is relatively very small when compared to previously reported methods [8]. *In vitro* tests performed using human primary chondrocytes demonstrated that the presence of genipin in the mats does not cause any cytotoxicity. On the contrary, crosslinked mats exhibit promotion of chondrocyte viability and differentiation.

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