Torres-Giner, S.; Gimeno-Alcañiz, JV.; Ocio, MJ.; Lagaron, JM. (2009). Comparative Performance of Electrospun Collagen Nanofibers Cross-linked by Means of Different Methods. ACS Applied Materials & Interfaces. 1(1):218-223. https://doi.org/10.1021/am800063x

Comparative Performance of Electrospun Collagen Nanofibers Cross-linked by Means of Different Methods

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ABSTRACT. Collagen, as the major structural protein of the extracellular matrix in animals, is a versatile biomaterial of great interest in various engineering applications. Electrospun nanofibers of collagen are regarded as very promising materials for tissue engineering applications because they can reproduce the morphology of the natural bone but have as a drawback a poor structural consistency in wet conditions. In this paper, a comparative study between the performance of different cross-linking methods such as a milder enzymatic treatment procedure using transglutaminase, the use of N-[3-(dimethylamino)propyl]-N′-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide, and genipin, and the use of a physical method based on exposure to ultraviolet light was carried out. The chemical and enzymatic treatments provided, in this order, excellent consistency, morphology, cross-linking degree, and osteoblast viability for the collagen nanofibers. Interestingly, the enzymatically cross-linked collagen mats, which are considered to be a more biological treatment, promoted adequate cell adhesion, making the biomaterial biocompatible and with an adequate degree of porosity for cell seeding and in-growth.

KEYWORDS: electrospinning • collagen • cross-linking • bone tissue engineering

1. INTRODUCTION

Recently, the electrospinning technique has received a great deal of attention in the fabrication of ultrathin fiber networks from materials of diverse origins (1-4) and more lately as a well-known method to produce novel scaffolds for tissue engineering because of the nanoscaled dimensions of its physical structure (5, 6). Collagen has formerly been confirmed to affect the expression of bone cell phenotypes (7, 8) and has been recognized by undifferentiated bone-marrow stem cells, which become bone-forming osteoblasts after signals from bone-specific proteins in the matrix (9).

As a principal structural element of the native extracellular matrix (ECM) in many native tissues, neat collagen protein has emerged as an interesting polymer to electrospin for diverse bioclinical applications (10-14). Although electrospun nanofibers generally assess properties by which they would approximate the structural morphology of the natural protein found in the ordinary bone (15), any biomaterials based on pure collagen protein present insufficient resistance in water and collagenase environments and poor mechanical firmness to resist handling during implantation and in vivo loadings (16).

Some functional groups can bridge and link collagen to construct an interpenetrating and fully water-resistant network, which would maintain and define the shapes of tissue and organs, especially for connective tissue (17). Furthermore, cross-linking can also tailor the rate of biodegradation, providing collagen networks the specific rate to degrade into bioabsorbable components because cells produce their own natural ECM (18). Covalent cross-links can be created in a wide range of manners to increase the dimensional, mechanical, and biological stability of collagen biomaterials (19). Nevertheless, most traditional and conventional methods, based on old chemical or physical treatments, either can add potential cytotoxic effects (20) or can cause breakdown and proteolysis of the collagen protein helical structures, respectively (21).

In this context, many applications, including the biomedical field, have been limited for the common defects of electrospun collagen and their mats such as poor thermal stability, bad solvent stability, and low mechanical strength. So far, only potent chemical cross-linkers, such as glutaraldehyde (GTA) vapor, have introduced a high degree of cross-linking in the electrospun collagen-based proteins and afforded water-resistant fibers (22). For this, novel cross-linking methods, which match these conditions and introduce a low degree of cytotoxicity, are being pursued. As a new chemical cross-linker, N-[3-(dimethylamino)propyl]-N′-ethylcarbodiimide hydrochloride (EDC) is a relatively low cytotoxic compound, which facilitates the formation of amide bonds between carboxylic and amino groups on the collagen molecules with the advantage of not becoming part of the resultant linkage (23). EDC has then been currently used for enhancing the biostability of collagen scaffolds in the presence of N-hydroxysuccinimide (NHS), which helps to prevent the formation of side products and also increases the reaction rate (24).

On the other hand, physical methods are traditionally considered as good cross-linking alternatives, particularly when chemicals are not possible to use because they do not require that materials come into contact with solvents and, therefore, can be effective under solid-state conditions. For instance, short exposures to ultraviolet (UV) light are commonly known to affect terminal telopeptide molecules of collagen proteins with a high content of tyrosine, increasing the shrinkage temperature, the resistance to collagenolytic degradation, and the durability under load in collagenase (25).

Nowadays, natural substances are preferred because they avoid clinical uses of synthetic exogenous chemicals and give added value to biobased resources. Genipin (GP) is a current example of a naturally occurring relatively new cross-linking agent, which is obtained from its parent compound geniposide and may be isolated from gardenia fruits. GP cross-links primary amine groups of proteins, including collagen, with a high degree of stability and low acute toxicity and, therefore, in a better way than many commonly used synthetic cross-linkers (26-28).

Transglutaminases (TGs) are protein-glutamine γ-glutamyltransferases, which are calcium-dependent enzymes distributed intra- and extracellularly throughout the body of a large variety of organisms. As a member of the lysyl oxidize family of enzymes, TG is clearly identified to catalyze the oxidation of lysine to α-aminoadipic δ-semialdehyde in collagen matrixes (29). TG is also reportedly responsible for certain other biological events, such as epidermal keratinization, blood coagulation, and regulation of erythrocyte membranes (30). The use of this enzyme to improve the properties of biomaterials, such as collagen and food proteins, is

being broadly investigated because it catalyzes the formation of stable and resistant to proteolysis isopeptide bonds by transamidation of available lysyl and glutamyl residues (31, 32). The reaction is well-known to catalyze the formation of the amide cross-link from γ-carboxamide and primary amine functionalities, which results primarily in the formation of ϵ-(γ-glutamyl)lysine cross-links (33) and the incorporation of polyamines into suitable protein substrates also (34). Cross-linkings by TG can then produce large molecular weight aggregates with increased resistance to chemical, enzymatic, and mechanical disruption (35). Specifically, TG-treated native collagen type I from bovine skin has led to a compacted arrangement, which enhances cell attachment, spreading, and proliferation of human osteoblasts and fibroblasts when compared to a culture on native collagen (36).

In the present study, we analyzed and compared for the first time the resultant morphology, cross-linking extent, and cell-seeding capacity of electrospun nanofibers made of collagen protein cross-linked by different "fixing" methods. In spite of the fact that some of these methods have been applied before as cross-linking media in various biomaterials, they have never been used to study the performance of electrospun collagen fiber mats.

2. EXPERIMENTAL SECTION

2.1. Materials

Lyophilized collagen, Bornstein and Traub type I, from calf skin was purchased from Elastin Products Co. (Owensville, MO). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP), 1-ethyl-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS), sodium bicarbonate, and acetone were purchased from Sigma-Aldrich (Spain). Sodium dihydrogen phosphate was purchased from Fluka (Spain), while disodium hydrogen phosphate and hydrochloric acid was purchased from Panreac (Barcelona, Spain). Genipin (GP), methyl-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclonona-4,8-diene-5-carboxylate, was obtained from Challenge Bioproducts Co. Ltd. (Touliu, Taiwan), and Microbial Activa transglutaminase (TG) was kindly gifted by Impex Química (Barcelona, Spain).

2.2. Electrospinning Process

Particular details of the basic setup of our electrospinning apparatus can be found elsewhere (37). Nanofiber mats were prepared by electrospinning from solutions of 5 wt % collagen in 85 wt % HFP. Governing parameters were first optimized according to our previous research (38) and then fixed at 11 kV of power voltage, 13 cm of tip-to-collector distance, and 0.25 mL/h of volumetric flow rate. Environmental conditions were maintained stable at 24 °C and 60% relative humidity by having the equipment enclosed in a specific chamber with temperature and humidity control.

2.3. Cross-linking Reaction

The resultant collagen nanofiber mats were divided into five groups and then stabilized by means of the cross-linking treatments. The chemical solution was prepared by introducing 30 wt % EDC and NHS as 1:1 (w/w) in a 9:1 (v/v) acetone/water mixture as recently reported (39). Nanofibers were then fully soaked in the EDC/NHS-prepared solution. This was kept and dried at room temperature overnight, after which they were rinsed with deionized water several times to remove any residual chemicals. Irradiation of the nanofibers was performed in a cabinet of Biostar from Telstar (Spain) by placing the collagen nanofibers on a 15 cm sheet of aluminum foil under a 253.7 nm UV lamp of 30 W for 30 min, to prevent the onset of degradation (40). GP-cross-linked nanofibers were obtained when the selected mat was immersed into a cross-linking solution of 0.5 wt % GP at 25 °C, followed by drying overnight at room temperature and severe rinsing treatments with deionized water (41). Finally, the enzymatic cross-linking solution was prepared by dissolving TG in a phosphate buffer (1 UN/mL) of pH 6.0 at 37 °C, which are found to be the most favorable reaction conditions (42). The incorporation of TG into the collagen nanofiber network was easily completed by covering the prepared enzymatic solution with the electrospun mats, drying overnight at room temperature, and then also washing with deionized water. A concentration of TG over collagen of 5000:1 (w/w) was employed because previous literature established that this enzyme concentration is the most optimal for such conditions (43).

2.4. Morphology

Nanofibers were examined using scanning electron microscopy (SEM; Hitachi S-4100) at 8.0 kV, after having been sputtered with a gold−palladium mixture in vacuum. Fiber diameters were measured by means of Adobe Photoshop 7.0 software from the SEM micrographs. Cell images were taken using a phase-contrast

technique with an inverted optical microscope (Nikon Eclipse TE2000-S) coupled to a digital camera (Digital Sight DS-5M-L1).

2.5. Amine Group Content

TNBS chromophore is a common method used for end-group analysis to assay the extent of transamidation. When collagen is used as the substrate in a cross-linking reaction, lysine residues participate in the reaction to produce the amide bond, and therefore a change in the number of free amine groups occurs (44), which can help determine the extent of collagen cross-linking (45). Approximately 10 ± 1 mg of the electrospun collagen mat was dissolved in a glass solution containing 1 mL of 4% NaHCO3 and 1 mL of 0.5% TNBS and kept in the dark at 37 °C for 3 h with mild shaking. Subsequently, 3 mL of 6 M HCl was added, and the mixture was autoclaved at 121 °C for 20 min to hydrolyze and dissolve any insoluble material to ensure a neat absorption measurement (46). Resulting solutions were aliquoted into three wells, and the absorbance was measured using an S22 UV/vis spectrophotometer from Boeco (Hamburg, Germany) at 345 nm. The absorbance values exhibited graphically represent corrected optical density values, and the cross-linking degree (CD) in percent was calculated by eq 1, where cl is the cross-linked sample and ncl is the non-cross-linked sample (47). Results were calculated from the mean absorbance, and a value of 0% was associated with the control.

2.6. Thermal Analysis

Differential scanning calorimetry (DSC) of typically 2 mg was conducted on a Perkin-Elmer DSC 7 thermal analysis system (Waltham, MA) at a scanning speed of 10 °C/min. The thermal history applied was a first isothermal scan at −50 °C for 10 min and a subsequent heating scan from −50 °C up to about +100 °C. Before evaluation, the thermal runs were subtracted through analogous runs of an empty pan. The DSC equipment was calibrated using indium as a standard and fitted with intracoolers. The degradation temperature (Td) was taken as the maximum peak height of the heating endotherm.

2.7. Cell Culture

Osteoblast-like cells of the human osteosarcoma MG-63 cell line, obtained from European Collection of Cell Cultures, were cultured in a humidified atmosphere of 5% CO2 at 37 °C, in Minimum Essential Medium Eagle from Sigma-Aldrich (Spain) supplemented with 10% fetal bovine serum, 1% penicillin−streptomycin, 1% 200 Mm l-glutamine, and 1% amphotericin B solutions (volume %), from Hyclone (Logan, UT). A total amount of 3 × 104 cells/disk was plated in a 24-well culture plate with 1 mL of medium. Proliferation measurement was based on the cellular metabolic activity using the Alamar Blue colorimetric indicator dye from Invitrogen (Carlsbad, CA): cell growth results in a chemical reduction of the indicator, which changes from oxidized (blue) to reduced forms (red) and whose color intensity is directly related to the number of metabolically active cells. Cultured glass disks were incubated in 10% Alamar Blue in a complete growth medium for 4 h in the CO2 incubator, according to the manufacturer's instructions. Aliquots were transferred to a 96-well plate, and the absorbance was measured at 570 and 650 nm using a Multiskan spectrophotometer from Thermo (Waltham, MA).

3. RESULTS AND DISCUSSION

Electrospun collagen nanofibers presented a roundlike shape with no bead formation and with a fiber average diameter of 106.44 ± 21.74 nm. Figure 1 shows SEM images of the non-cross-linked biopolymer mat (Figure 1a) and of all cross-linked materials (Figure 1b−e). As a result of the cross-linking process, electrospun nanofibers became strongly interconnected, forming an apparently robust and stiffer network. Figure 1b shows the SEM image of the collagen mat cross-linked with EDC/NHS; this sample shows a network with a significant extent of interlinked fibers. This similar structural integrity has been observed for electrospun fibers of collagen type II using EDC in the presence of ethanol (48). After cross-linking, the sample became visibly yellowish and shrank slightly. This change is related to the creation of aldimine linkages (CH═N) between free amine groups of the collagen protein promoted by the chemical compound during cross-linking (49). The color was restored upon rinsing. Figure 1c exhibits the collagen electrospun fiber SEM image cross-linked by UV light, in which nanofibers are not seen to interlink to the same extent. Figure 1d shows the SEM image of the collagen mat cross-linked by GP. From this picture, it can be clearly seen that collagen nanofibers were not only extremely linked but also merged together, resulting in thicker fibers. GP-cross-linked mats were also dark blue in color and not water-soluble because this natural cross-linker produces strong blue pigments upon reaction with amino acids (50). Finally, Figure 1e exhibits the morphology of the nanofiber mat cross-linked by TG, in which it can be observed that the biocatalyst successfully cross-linked the collagen mat and did not result in significant fiber

thickening. No color changes were observed in UV- or TG-cross-linked materials. In general, it was observed that all samples, with the exception of the physical treatment by UV, formed fairly nice spiderweb-like cross-linked fiber mats, which can be considered as a result of inter- and intramolecular covalent bonds and bondings between the fiber junctions and are expected to favor the structural integrity of the resultant collagen biomaterial in the presence of high moisture levels.

With the aim of quantifying the above results, the so-called end-group analysis was carried out as an additional proof to determine the CD. This methodology determines the consumption of functional groups during cross-linking by a simple spectrophotometric analysis sensitive to the number of amine groups. Thus, TNBS absorbance decreased with the cross-linking extent, thereby signifying a decrease in the amine group presence with an increase in the cross-linking amount, as shown Figure 2. Collagen nanofibers cross-linked by means of EDC/NHS had the highest CD, i.e., 57.09%, while nanofibers treated by UV radiation only reached 25.38%. One of the most interesting observations is that for the TG cross-linker; though the CD was not higher than that of the chemical treatment, it was considerably high, i.e., 44.16%. Unfortunately, no data were recorded for samples treated with GP because of their intense blue color, which masked the absorbance measurement. Concerning the CD in collagen-based materials, such as gelatin, it is known that GP can provide values similar to those of highly efficient chemicals, for instance, GTA, which can be as high as 80−90% (41).

In order to verify recent results obtained by amine group quantification, in the assessment of collagen cross-linking, thermal analysis (DSC) was alternatively used. This method is based on the determination of the biopolymer degradation temperature (Td). The influence of cross-linking on thermal stability is based on the fact that, independent of the collagen nature, new covalent bonds associate with direct evidence of thermally improved collagen-based structures. Because protein thermal transitions are usually well-documented, Td changes can provide adequate reference for the cross-linking tissue strength. A rise in Td would indicate augmented network protein strength, and therefore an increase in the CD. For instance, neat collagen from turkey leg tendons was seen to degrade at about 68 °C (51), while epoxy- and carbodiimide-cross-linked collagens from dermal sheep exhibit a Td above 80 °C (52). Thermal analyses of the electrospun collagen nanofibers, carried out by DSC, are depicted in Figure 3 with Td values presented on curves. Thermal values, obtained directly from the thermal curves, were consistent with the above-mentioned literature values. Neat collagen presented a value of 67.1 ± 0.5 °C, while cross-linked mats by means of GP and EDC/NHS showed higher Td values, of 98.3 ± 1.8 and 86.5 ± 1.2 °C, respectively. For TG, Td raised to a value of 73.3 ± 0.7 °C, and not only was the value higher than the control, but also the degradation peak appeared to be more flat, indicating that this mat, at high temperatures, can be more thermally stable because it degraded much more progressively. However, Td for the UV cross-linking treatment resulted in a value of 58.3 ± 0.3 °C, a lower value than the non-cross-linked sample. This observation suggests that the physical treatment not only cross-links the polymer to a very low extent but also can be partially fragmented and denatured the collagen matrix, in agreement with recent previous literature (53).

To ascertain the projected biological properties of the biomaterials, cell proliferation of MG-63 osteoblasts on the electrospun cross-linked nanofiber mats of collagen was analyzed by the Alamar Blue assay. Confirmation of the cell viability in vitro on the cross-linked nanofibers would verify their biocompatibility, providing a preliminary confirmation of the utility of the collagen cross-linked biomaterial for in vivo applications. Nevertheless, neither the control nor the sample treated with UV resisted the contact with the water-based medium and underwent dissolution, making the current analysis infeasible. For EDC/NHS-, TG-, and GP-cross-linked collagen samples, as Figure 4 shows, the cell density increased, though in a different way, from day 4 to 21 by augmenting the Alamar Blue reduction (% AB). On the one hand, on samples treated with EDC/NHS and TG, cell proliferation progressively increased with time, suggesting that these cross-linked mats fully supported the growth of osteoblasts. Nonetheless, growth on EDC/NHS was faster during the initial days in comparison with the TG-treated sample because of, among other reasons, the potentially low cytotoxicity of the actual cross-linker and the higher cross-linking extent achieved for the former mat (23, 24). In any case, the high surface-to-volume ratios, due to the thin fiber diameters and the high porous morphology of such structures, are thought to enhance cell adhesion and proliferation. On the other hand, although the GP-cross-linked mat resisted the water environment and supported cell growth, it retarded and decreased the viability values. Regarding this, it is known that concentrations from 50 to 80 ppm of GP can significantly reduce the cell activity and the number of cells for 2 days (54). Nevertheless, uses of this natural cross-linker can still be attractive from a therapeutic viewpoint because it assesses interesting biological properties, such as suppressions of α-TN4 lens cell fibrogenic behaviors (55) or inflammatory reactions (56).

The cell morphology on the cross-linked collagen mat was studied by inverted optical microscopy. Top images in Figure 5 show the initial days of the cell culture in which a few osteoblasts are already seen to cling to the biomaterial surface. After 21 days of cell culture (see bottom images of Figure 5), a large number of cells can be seen covering the biomaterial surface in a clear visual inspection of the material bioactivity. Albeit some cross-linked fiber mat specimens were seen to detach to some extent from the bottom cover glass, and because the cells can also grow on the underneath glass, misleading the results, these particular specimens were removed from testing and only the biomaterial specimens that remained attached (a fact that was easily checked even with cells growing on top of the biomaterial by direct observation from underneath the cells) to the bottom glass were counted. Attached cells on cross-linked collagen mats showed flat and polygonal extensions, typical of a fibroblast-like morphology, as described by the cell-line supplier.

Chemically and enzymatically cross-linked nanofiber mats have been shown to be sufficiently strong to generate a collagen-based biomaterial, which is noncytotoxic and maintains an interpenetrating network during cell attachment. The above good results about the use of TG as a cross-linking agent are in agreement with more recent results that claimed the potential use of this biocatalyst to build cross-linked collagen−mimetic dendrimers with good cellular response (57).

4. CONCLUSION

Collagen-based biomaterials usually lack supporting integrity for tissue engineering applications. Conventional chemical stabilization via cross-linking, traditionally based on aldehydes, can strongly influence the material cytotoxicity by adverse reactions arising from residual and reversible fixation. In addition, reconstituted forms of collagen by means of physical methods, such as UV treatment, cannot ensure sufficient strength, can alter the polymer molecular weight and chemistry, and may disintegrate upon handling or collapse under the pressure from surrounding in vivo tissue. Thus, although most current methods are approved for clinical applications, they are no longer favored.

In this work, a range of various cross-linking agents from different sources were tried for the first time in electrospun collagen nanofibers to avoid the poor water resistance on natural collagen. From the results, it was observed that cross-linking collagens with the chemical EDC/NHS and the biocatalyst TG were the best treatments to obtain fully functional cross-linked biomaterials with enhanced osteoblast viability. Both materials proved to have a sufficient level of cross-linking degree and water-resistant morphology, with the chemical treatment exhibiting the best performance. Because osteoblasts are anchorage-dependent cells, the relatively good topographical resemblance of cross-linked nanofiber mats to ECM does provide sufficient physical support for cell attachment in both cases. These novel routes are therefore presented here as potential alternatives as cross-linking agents for electrospun biomaterials of interest in the biomedical field.

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6. FIGURES

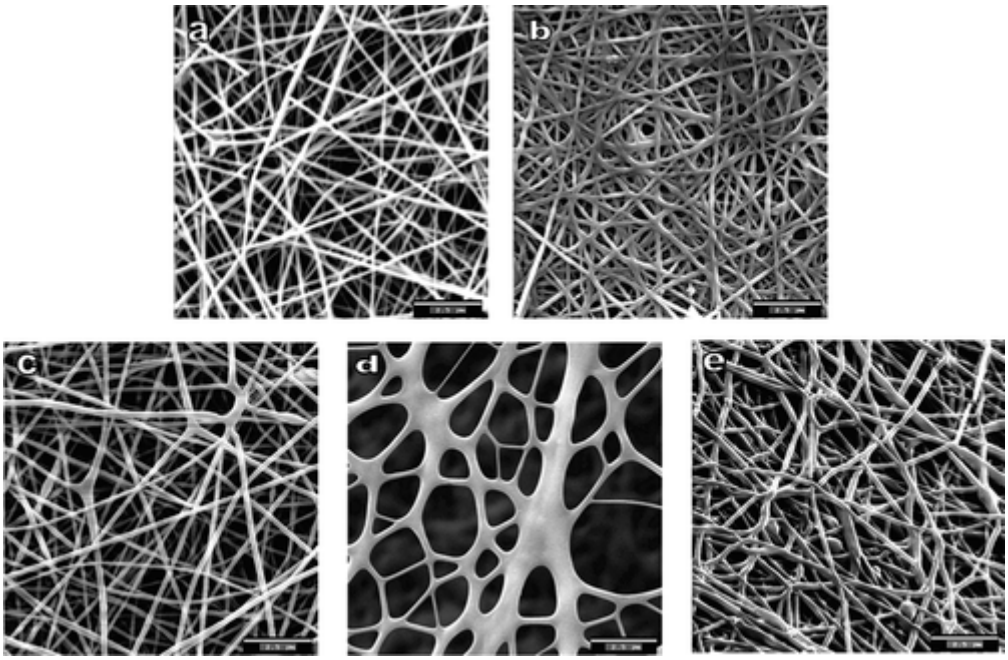

Figure 1. Typical SEM photographs of the collagen electrospun nanofibers after cross-linking with (a) control (none), (b) EDC/NHS, (c) UV, (d) GP, and (e) TG. Scale marker: 2.5 μm in all cases.

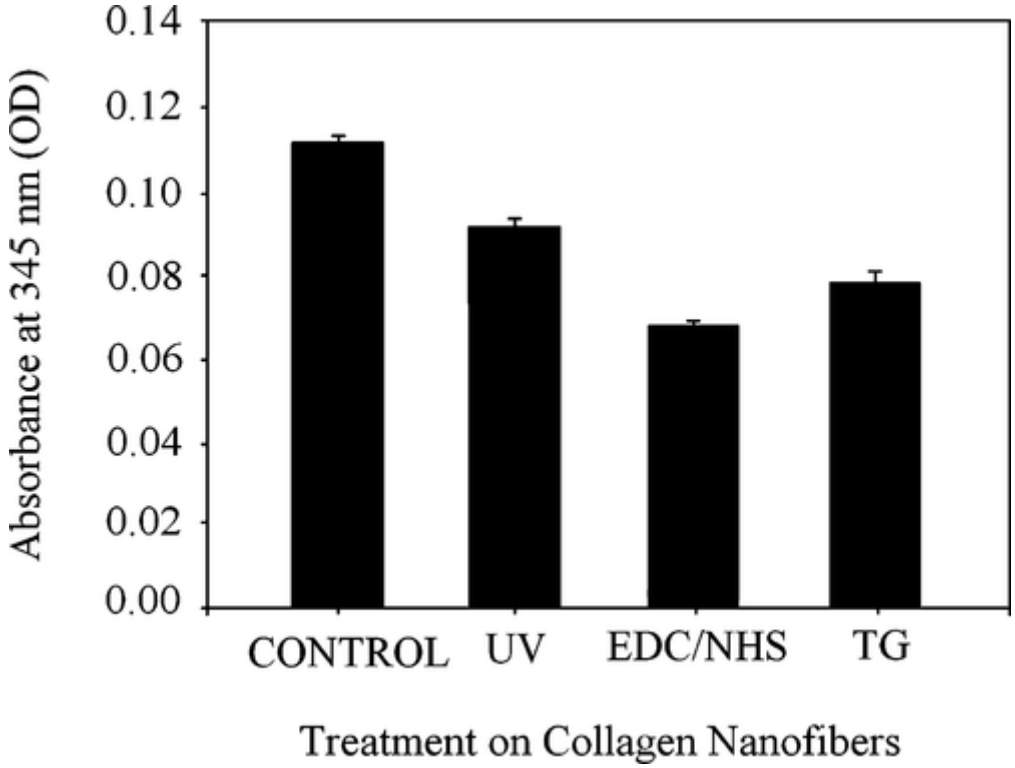

Figure 2. Amine group content of electrospun cross-linked collagen nanofibers.

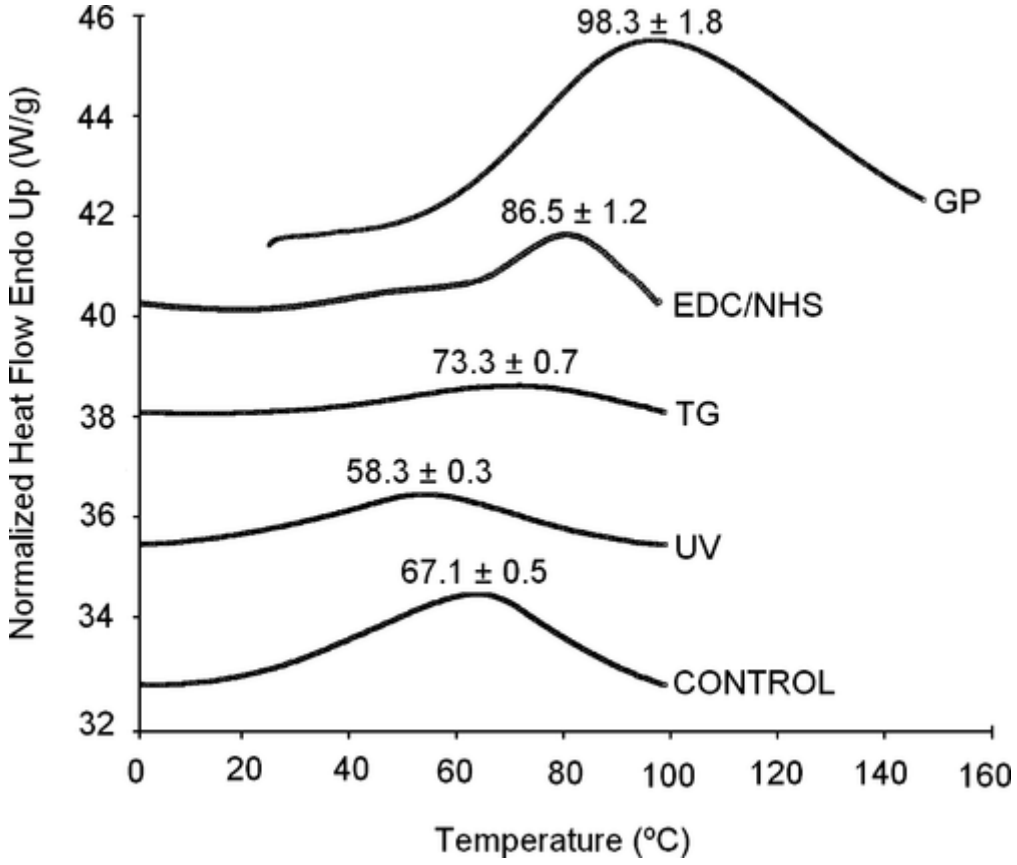

Figure 3. Thermal endotherms of electrospun collagen nanofibers cross-linked with, from bottom to top, control (no cross-linking), UV, TG, EDC/NHS, and GP.

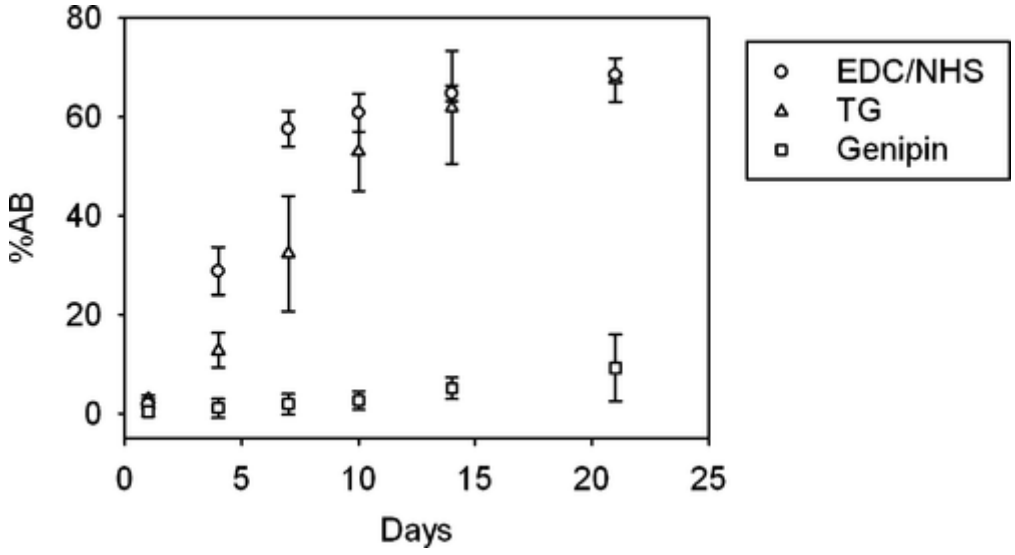

Figure 4. Osteoblast growth curve assays using four replicates over 21 days showing cell viability assessments of the electrospun collagen nanofiber mats cross-linked by different methods: EDC/NHS, TG, and GP.

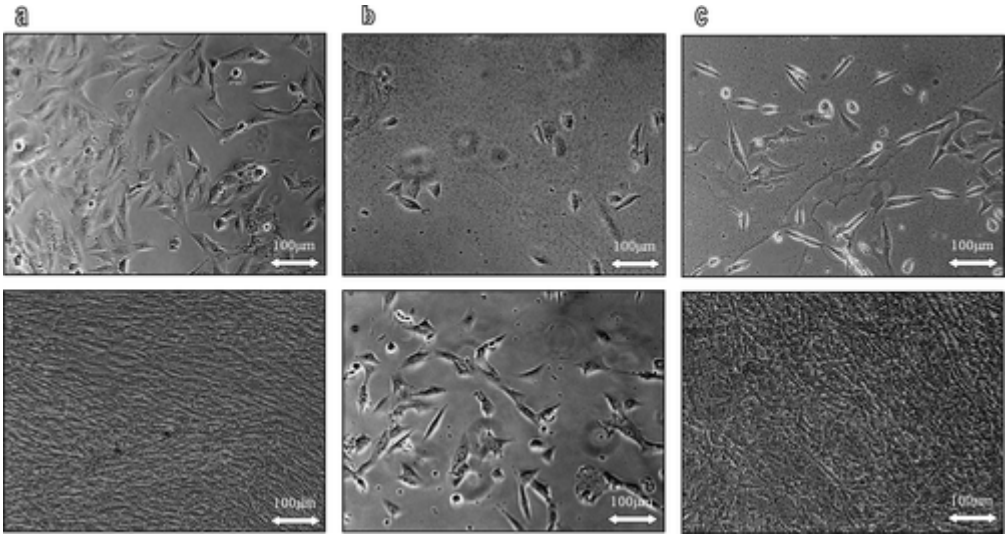

Figure 5. Typical optical images of osteoblasts cultured in a cell medium during the first week (top) and last week (bottom) on collagen nanofibers cross-linked by (a) EDC/NHS, (b) GP, and (c) TG. Scale marker: 100 μm in all cases.