Guiding Mesenchymal Stem Cells Differentiation into Chondrocytes using Sulfated Alginate / Cold Atmospheric Plasma Modified Polycaprolactone Nanofibrous Scaffold

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Abstract

Using tissue engineering approaches is one of interesting strategies to repair cartilage injuries. This study reports the preparation of surface-modified electrospun polycaprolactone nanofibrous scaffolds with highly negatively-charged sulfated alginate as functional support for chondrogenic differentiation of mesenchymal stem cells. In this regard, polycaprolactone (PCL) nanofibers were fabricated by electrospinning, surface-activated by cold atmospheric plasma and further surface-modified with aqueous solutions of sulfated alginate. The scanning electron microscopy (SEM) images showed the nanofibrous structure of electrospun PCL mats. The successful surface modification of PCL nanofibrous scaffolds with sulfated alginate was confirmed by the appearance of fingerprint region of mannuronic acid at 812 cm^{-1} in attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) spectra. The cytocompatibility of the nanofibrous scaffolds for mesenchymal stem cells (MSCs) was confirmed using MTT assay. Reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry for type 2 collagen marker were conducted to confirm the chondrogenic differentiation of seeded MSCs on the surface of scaffolds. The expression of type 2 collagen by RT-PCR and immunocytochemistry analyses confirmed the chondrogenic differentiation of MSCs. Our results showed that sulfated alginate surface-modified PCL (SM-PCL) nanofibrous scaffold as an appropriate substrate for cell attachment and growth could promote the MSCs differentiate to chondrocytes.

1. Introduction

Due to lack of access to blood-source supply and high cell density, cartilage tissue has a poor ability to repair [1]. Designing artificial cartilage and joints, and replacing them with damaged ones somehow overcomes this problem; however, the drawback of this method is the possibility of the transplanted cartilage rejection [2]. In recent years, new methods of cell therapy and tissue engineering (TE) have been developed to provide a clear vision to treat various types of injuries and diseases [3]. In tissue engineering, nanofibrous structures are constructed in the form of scaffolds in order to provide an appropriate surface for cells attachment, growth and differentiation [4]. The various types of scaffolds in terms of materials and properties have been developed to be used in cartilage tissue engineering [5,6].

Polycaprolactone (PCL) is a biodegradable polyester commonly employed for engineering of soft tissues due to its appropriate biocompatibility, biodegradability and physicochemical properties which makes it possible to be fabricated as scaffold [7,8]. Despite the desirable characteristics of synthetic polymers such as PCL, due to their hydrophobicity and low surface energy, these materials reduce cell adhesion and also decrease cell growth and proliferation[9]. Therefore, it is crucial to modify their surface or bulk properties before using them for TE applications[10–12]. The surface modification of PCL is an efficient method for improving the bioactivity of PCL-based materials[13].

Plasma surface modification is a very simple, green and widely-used technique that creates active functional groups on the surface of material without interfering its bulk properties [14,15]. In addition to the

biological functions, these active groups can also be employed for protein or other biomacromolecules immobilization on the scaffold surface[16,17]. Three-dimensional nanofibrous PCL scaffolds have been widely-used for growth and differentiation of mesenchymal stem cells (MSCs) in cartilage tissue engineering[18]. Li et al. showed that PCL nanofibers are a desirable substrate for MSC cultivation. Therefore, a PCL-based scaffold can support differentiation of MSCs into chondrocytes and cartilage repair in the presence of TGF-β1 [19].

Chondrogenic differentiation is better archived when biomaterials which can create an environment similar to the cartilage extracellular matrix (ECM) are employed [20,21]. Recent studies have suggested that sulfated glycosaminoglycans (GAGs) such as chondroitin sulfate, a major component of cartilage ECM, can promote regeneration of damaged cartilage[22]. In this context, sulfated GAGs have shown therapeutic benefits for knee osteoarthritis. Both chondroitin sulfate and heparin sulfate have a high affinity to bind the growth factors essential for cartilage homeostasis, for example, transforming growth factor beta-1 (TGFβ-1) [23,24]. However, the challenges associated with physiologically important GAGs such as the degree of sulfation, impurities, side effects, and high cost have encouraged the researchers to develop more defined GAG-mimicking biomaterials[25].

Alginate is an FDA-approved naturally-occurring polysaccharide which extracted from brown algae. Alginate is a linear binary copolymer of 1–4-linked regions of β-D-mannuronic acid and α-L-guluronic acid[26,27]. Alginate has been widely used in tissue engineering of bone and cartilage due to its inherent biocompatibility, non-immunogenicity, easy processability, and relatively low cost [28,29]. However, it shows limited interactions with cells and biomolecules of ECM[30]. In this regard, sulfated alginate has shown anti-inflammatory and anticoagulation properties, possessing high affinity to heparin-binding proteins and ability to form strong ionic interactions with cationic materials[31–34].

Sulfated alginate as a mimic of sulfated GAGs, exhibits a high negative charge density and could demonstrate diverse biological properties. Mhanna et al., studied on sulfated alginate hydrogels for the cultivation of cartilage and showed that these hydrogels promote proliferation and prolonged viability of the chondrocytes[35]. Furthermore, the sulfated alginates expressed a high degree of type 2 collagen that were able to sustain the cartilaginous phenotype.

Since the capability of sulfated alginate as hydrogel form on differentiation of MSCs to chondrocytes has previously been proven, we hypothesized that the sodium sulfated alginate as a coating may also afford the similar biological effects. To the best of our knowledge, there is no report that shows the sulfated alginate ability to chondrogenic differentiation where used as the coating. Therefore, the main aim of this study is coating of the sulfated alginate as a biologically-active carbohydrate polymer on PCL nanofibrous scaffolds and probing its ability for guiding chondrogenic differentiation of mesenchymal stem cells. Therefore, we first fabricated PCL nanofibers using electrospinning technique and then, activated their surface by helium cold atmospheric plasma. After that, we further modified the activated surface of nanofibrous PCL mat using an aqueous solution of sulfated alginate and found that its

appropriate cytocompatibility and ability to chondrogenic differentiate of MSCs can idealize it as an effective biomaterial for cartilage tissue engineering.

2. Materials And Methods

2.1. Materials

Sodium alginate (SA) with a number-average molecular weight of 32,000 and polydispersity index of 1.8, and the M/G ratio of 0.82 (details in supplementary information), polycaprolactone (PCL) with a molecular weight of 80,000 g mol^{-1}, 3-[4,5-dimethylthianzol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) compounds were purchased from Sigma-Aldrich. Sodium bisulfite and sodium nitrite as the sulfating agents were purchased from Merck, Germany. Merck or Sigma-Aldrich supplied all other chemicals.

2.2. General procedure for preparation of surface-modified electrospun PCL scaffolds

2.2.1. Synthesis of sodium sulfated alginate (SSA)

In brief, sodium sulfated alginate was synthesized through one-step substitution reaction of sodium alginate and the sulfating agent solution. For this aim, sodium alginate (5 g) was added to the aqueous solution of sodium bisulfite (11.10 g) and sodium nitrite (1.75) to obtain a solution with final solid content of 9 wt.%, and the reaction was performed under vigorous stirring at 90 °C for 1.5 h. After that, the solution was dialyzed using a dialysis bag (MWCO, 3500) against distilled water for 48 h and lyophilized to obtain the purified powder of sodium sulfated alginate[36].

2.2.2. Preparation of electrospun solutions and their electrospinning

First, 0.6 g PCL was dissolved in an acetic acid/formic acid solvent system with a volume ratio of 1:9 to obtain the solution with solid content of 5%, and stirred 2 h before use. For the electrospinning process, a 5-mL syringe with a 19-gauge needle was filled with the polymer solution. The values of flow rate and applied voltage were 1 mL h^{-1} and 25 kV, respectively. Also, the tip-to-collector distance, and collector rates were 120 mm and 250 rpm, respectively. The electrospinning process (Co881007 NYI, ANSTCO, Iran) was carried out using a horizontal system with a cylindrical collector covered by aluminum foil at room temperature.

2.2.3. Surface activation of nanofibrous scaffolds using cold atmospheric plasma

To improve the hydrophilic properties of electrospun nanofiber surfaces and creation of active functional groups for subsequent interactions, the surface of the nanofibrous mat was activated using helium cold atmospheric plasma (HCAP). Plasma was applied to the nanofibrous mat for 3 min at a pressure of 1 mbar.

2.2.4. Surface modification of nanofibrous mat using sulfated alginate

HCAP activated scaffolds were cut at 0.5×0.5 cm^2 sheets. After that, they were washed with distilled water and placed in sulfated alginate aqueous solutions with different concentrations of 3, 5 and 7 mg mL^{-1} for 24 h. The surface-modified scaffolds were placed in distilled water for another 24 h and dried in vacuo.

2.3. Measurements

2.3.1. Physicochemical characterization of electrospun scaffolds

The chemical structure of neat and sulfated alginate was studied using attenuated total reflectance-Fourier transform infrared (ATR-FTIR) analysis (RX I Spectrum, PerkinElmer, USA) equipped by ATR accessories. The infrared spectra of the samples were measured with 32 scans at the 8 cm^{-1} resolution and over a wavelength range of 4000-400 cm^{-1}. The proton nuclear magnetic resonance (^1H NMR) spectroscopy was performed to further characterize the chemical structure of sulfated alginate using Bruker DRX-500 Avance spectrometer (Germany). The spectra were recorded in D_2O as the solvent.

The degree of sulfation (DS), the average number of sulfate groups per uronic acid residue, was measured using elemental analysis method (Costech 4010, Italy). The experimental DS was calculated using the following equation:

$$DS = \frac{١٩٨[S]}{(٣٢٠٠ - ١٠٢[S])}$$

Where [S] is sulfur content (%) of sulfated alginate. The molecular weight (MW) of sodium sulfated alginate was measured using GPC 1100 Agilent equipped with PL gel column and water as the eluent.

For investigation of PCL nanofibers, morphology and the impact of surface modification using HCAP and sulfated alginate on nanofibrous structure, the scanning electron microscopy (SEM) (Model Vega, Tescan Co., Czech Republic) was employed. ImageJ software was used for measuring the fiber diameters. Besides, the ATR-FTIR spectra of neat and surface-modified nanofibrous mats were used to confirm successful surface modifications of mats.

2.4. Evaluation of the biological activities

2.4.1. Culture of mesenchymal stem cells (MSCs)

The MSCs of bone marrow were obtained from Stem Cell Technology Research Center. The cells were cultured in a Dulbecco's modified eagles medium (DMEM, Bioidea, Iran) containing 10 wt.% of fetal bovine serum (FBS, Gibco, Germany) in an incubator with a CO_2 injection capability of 5% and a humidity of 95% at 37 °C.

2.4.2. Cell seeding and culture on nanofibrous scaffolds

After sterilizing the scaffolds by UV-rays for 20 min, they were incubated in culture medium overnight before cell seeding in order to make sure for removal of surface contaminants and facilitate protein adsorption and cell attachment onto the scaffold surface. The MSCs were seeded on the scaffolds at the density of $1Í10^4$ cells cm^{-2} in culture medium supplemented with 10% FBS up to 72 h. Moreover, the cells tendency to the scaffold was measured by reverse microscope. The cellular culture was maintained in an incubator at 37 °C with 5% CO_2.

2.4.3. Cell morphology on scaffolds

Furthermore, the morphology of seeded MSCs on the surface-modified PCL (SM-PCL) nanofibrous mat was examined using SEM. After washing the cells that were seeded ($1Í10^4$ cells/well) on scaffolds with PBS, the attached cells were fixed by paraformaldehyde solution (2.5% v/v). To dehydrate the cells, scaffolds were placed in a series of EtOH with concentrations from 60% to 100%. Then, SEM images were obtained at accelerating voltage of 2 kV after gold sputter coating.

2.4.4. *In vitro* cytocompatibility evaluation of nanofibrous scaffolds

To evaluate the MSCs viability and proliferation on nanofibrous scaffolds, the MTT assay was used. Briefly, MSCs were seeded at the density of $1Í10^4$ cells cm^{-2} scaffolds. At three time points of 24, 48 and 72 h, the samples were transferred into new wells, and the MTT solution was added to each well, after

which the plates were incubated in the dark at 37 °C for 3 h. The absorbance of the solution was measured at 490 nm. The color absorbance was measured with an ELISA Reader at a wavelength of 570 nm (BioTek EL Í800). The experiments were run in triplicate.

2.4.5. Chondrogenic differentiation of MSCs on the surface of scaffolds

The MSCs at a density of 1Í10^4 cells cm^{-2} were cultured in 6-well plates from each of two groups with chondrogenic differentiation media (+S) and without chondrogenic differentiation media (-S) (three replicates) up to 21 days. After 24 h of cultivation, the differentiation medium was added to the wells (dexamethasone (1×10^{-7} μM), ascorbic acid (0.1 M) and Insulin Transferrin Selenium (ITS 1%), all prepared from Sigma Aldrich). After that, on determined time points, the peripheral medium of each well was removed, and the 10% MTT solution was added and incubated for 3 h. Then, the sediments were dissolved in DMSO. Ultimately, the absorbance of the solution was calculated with ELISA reader instrument at a wavelength of 570 nm.

2.4.6. Alcian blue staining of differentiated MSCs

To confirm *in vitro* differentiation of MSCs towards chondrocytes on SM-PCL nanofibrous scaffolds, the alcian blue staining was performed. For this aim, the MSCs at a density of 1Í10^4 cells cm^{-2} were cultured on scaffolds in the presence of a chondrogenic differentiation medium. After predetermined time points of adding the differentiation medium, i.e., 24 h, 7, 14 and 21 days, the scaffolds were fixed by paraformaldehyde, at room temperature for 20 min, then washed with PBS and located in the vicinity of alcian blue (10-15 μL) for 30 min. After that, scaffolds were washed with aqueous HCl (0.1 mol), and observed on the lamella by an inverted microscope.

2.4.7. Reverse transcription polymerase chain reaction (RT-PCR)

In order to extract RNA from differentiated cells on the scaffold, the MSCs at a density of 1Í10^5 cells cm^{-2} were cultured in each well of 6-well plates for 21 days in two groups comprising with and without differentiation medium. The total RNA was extracted using the Aria Tous Extraction Kit according to the manufacturer's protocol (Arya Tous, Iran). The first step of RT-PCR relies on primer extension conversion of RNA to complementary DNA (cDNA) by RT enzyme. Then, the polymerase chain reaction (PCR) is performed on samples. Synthesis kit of Arya Tous was used to synthesize cDNA. All primers including type 2 collagen, AGGTCACAGGTTATCCAG R, AGGTCACAGGTTATCCAG *β2M* F, TGCTGTCTCCATGTTTGATGTATCT R, and TCTCTGCTCCCCACCTCTAAGT were ordered to Takapu-Zist after being designed. All stages were performed to determine the expression of *β*2M gene and collagen

type II with their primers. Finally, they were placed in thermocycler, and the temperature protocol of the kit was conducted for 2 min at 95 °C, 30 s at 94 ° C, 30 s at the specified temperature for the device based on the melting temperature (T_m) of desired genes, and further 30 s at 72 °C to perform the RT-PCR test. The PCR products were taken on a 1.5 wt.% agarose gel, and the gel was stained with SYBR green and photographed with a photo document device.

2.4.8. Immunocytochemistry of differentiated cells

Immunocytochemistry test was performed to confirm the differentiation of MSCs towards cartilage cells. The MSCs at a density of $1Í10^4$ cells cm^{-2} were cultured for 21 days on the scaffolds in two groups comprising with and without differentiation medium in a 6-well plate. At the designated time, samples were examined in terms of type II collagen (Col II) protein expression. At first, the samples were washed with PBS and placed in paraformaldehyde in a cool place for 20 min. Then, they have rewashed with PBS and incubated first with type II collagen antibody (Santa Cruz Biotechnology, USA) at 4 °C. In order to block the secondary antibody (conjugated with phycoerythrin, Chemicon Temecula, USA) response, the goat serum (10 wt.%) was added for 30 min to the background as an additional color. After constant washing, DAPI compound was added to the samples and immediately removed. After that, they were charged onto the PBS solution and kept at 4 °C. Finally, the samples were observed with an Olympus fluorescent microscopy (Nikon, Japan) to confirm desired markers.

2.4.9. Statistical analysis

The data were statistically analyzed using One-Way ANOVA test to evaluate the statistical significance. The results were analyzed by SPSS 18 software, and the variance was in the significant level of $p \leq 0.05$.

3. Results And Discussion

3.1. Surface modification and characterization of PCL Scaffolds

Since sulfated alginate can promote chondrogenic differentiation of MSCs and accelerate cartilage tissue repair, we used it as an organic coating on PCL nanofibers. Therefore, we used the nitrite/bisulfite-mediated sulfation reaction for synthesis of sulfated alginate due to its accordance with *green chemistry* regulations, ease of reaction and good reaction controllability[36]. Contrast to the common sulfating agents which degrade sodium alginate polymer chain during a reaction and lead to pollution problems[32]; this method is non-toxic, little pollution and low cost. Based on the literature, the first step of sulfation reaction refers to the formation of sulfation agent ($N(SO_3Na)_3$). After that, the hydroxyl functional groups of uronic acid residues react with the sulfating agent in aqueous medium through a substitution reaction to obtain sodium sulfated alginate (SSA) (Fig. 1a). The degree of sulfation (DS) for three different

batches of SAA was 0.65 approximately equaled to two sulfate group per three uronic acid residues. Number-average molecular weights of SA and SSA determined by GPC were 32 kDa and 42.5 kDa, respectively (Fig. 1b). The sulfation reaction of alginate was confirmed through an appearance of deterministic bands of associated asymmetrical S = O stretching vibration and symmetrical C–O–S vibration associated to a C–O–SO_3 group at 1256 cm^{-1} and 833 cm^{-1}, respectively (Fig. 1c) [37]. To further characterize the chemical structure of SSA, the ^1H NMR spectra of neat SA and SSA were studied (Fig. S1, S2). The assignment of new peaks at 4.01 ppm and 4.18 ppm in ^1H NMR showed a substitution preference for sulfation of the hydroxyl groups on C2 on both M and G monosaccharides [38].

After synthesis and physicochemical characterization of SSA as a surface modifying agent, the PCL was electrospun, surface activated by HCAP and surface-modified with different concentrations of sulfate alginate including 3, 5 and 7 mg mL^{-1}. The SEM was used to investigate the scaffold morphology. The SEM images showed that the electrospun PCL scaffold has randomly oriented nanofibrous morphology where the diameter of fibers were ranged from 110 to 250 nm (Fig. 2a). According to the SEM images, HCAP-treated PCL nanofibers also illustrated a randomly oriented and smooth morphology with a significant increase in nanofiber diameter, approximately 128 nm (Fig. 2b). On the other hand, sulfated alginate surface-modified nanofibers showed a heterogeneous and sticky morphology with an average diameter of 409 nm (Fig. 2c). A summary of the morphology of neat and modified PCL nanofibers are listed in Table 1. The FTIR-ATR of neat and surface-modified PCL (SM-PCL) electrospun nanofibers was performed to confirm the successful surface coating of SSA on the PCL surface.

A shoulder at 1638 cm^{-1} appeared in surface-modified samples was assigned to the asymmetric stretching vibration of the carboxylate group. Furthermore, the finger print of mannuronic acid residue was observed at 812 cm^{-1} in modified samples[39]. However, the characteristic band associated with the sulfate group at 1250–1260 cm^{-1} was not observed in spectra of the modified sample due to its overlapping with bands of PCL functional group in this region. Based on characterization results, the concentration of 5 mg mL^{-1} of SSA was selected as the optimal concentration for surface modification of PCL nanofibers and all of cell and molecular analyses were applied according to this sample (Fig. 3).

Table 1
Average diameter for neat and surface-modified PCL nanofibers

Sample	Neat PCL	Plasma treated PCL	SM-PCL
Morphology	Smooth	Smooth	Sticky
Fiber diameter	182 ± 69	310 ± 68	409 ± 157

3.2. Cytocompatibility of surface-modified scaffolds

To explore the effects of sulfated alginate coating on cell growth, we cultured the fresh MSCs on surface of the scaffolds and performed the MTT assay at 24, 48 and 72 h time points (Fig. 4). The higher optical density (OD), and consequently higher initially adhered cells for SM-PCL scaffold at 24 h were related to

the presence of bioactive sulfate functional groups. Expectedly, this trend was observed for 48 and 72 h time points. Öztürk et al., has previously shown that the sulfated alginate induce high cell viability and spread morphology of chondrocytes mediated by integrin and cell synthesize collagen[40]. The results showed that MSCs proliferation on PCL nanofibrous scaffold coated by sulfated alginate (SM-PCL) is considerably higher than that on the non-surface-modified scaffold on the third day ($p < 0.000$). The inverted microscopy images of cultured MSCs on the scaffolds after 72 h showed that the cells grew and proliferated alongside the scaffold. They were also proliferated on the scaffold surface and grew towards the scaffolds (Fig. S3). Our results showed that the surface-modified scaffolds are non-toxic.

3.3. Chondrogenic differentiation

In the next step, we evaluated the potential of sulfated alginate as a coating on PCL nanofibers to differentiate the MSCs into the chondrocytes. The MTT assay was used to evaluate the growth and reproduction and survival of differentiated MSCs to semi-cartilage cells for 21 days. The results showed that the MSCs are viable on SM-PCL scaffold over time and the sulfated alginate coating is capable of supporting cells growth, proliferation and differentiation during the studied intervals. The results showed a significant difference in the cell growth 24 h, 7, 14 and 21 days after cell culture on surface-modified PCL scaffold compared to the control group and untreated scaffold ($p \leq 0.005$).

The presence of differentiation medium and surface modification of scaffolds afforded an improving in the switch function between cellular proliferation and differentiation (Fig. 5). The similar results of modified alginate role as an artificial ECM was previously reported for chondrogenic differentiation of MSCs[41]. One of the most important notes in chondrogenic differentiation process is proliferation limit of the cells in lung-term culturing. Results of MTT assay showed that the number of the cells on all samples increases during 21 days, however; the viability of cells cultured on SM-PCL scaffolds with sulfated alginate demonstrated lower proliferation and metabolic activity. This decrease in metabolic activity was assigned to the chondrogenic differentiation of MSCs to semi-cartilage cells. As shown in Fig. 5, the optical density (OD) of cultured MSCs on Un-PCL scaffold is significantly higher than that on SM-PCL sample because of higher un-differentiated MSCs which are proliferating, while the lower OD of cultured MSCs on SM-PCL scaffold reveals their differentiation to the semi-cartilage cells. These results confirmed that sulfated alginate when used as a coating on polymeric nanofibers can alter the fate of MSCs to the chondrocytes.

To probe the morphology and cell adhesion on the SM-PCL scaffold after 7 days, SEM was used. The SEM images showed that the cell adhesion, cell-cell bindings and morphological transformation is well on the SM-PCL scaffold (Fig. S4a). Further characterization of cell adhesion on SM-PCL scaffold was performed by DAPI staining (Fig. S4b).

In order to evaluate the secretion of ECM components such as GAGs from MSCs cultured on surface-modified scaffolds in the presence of a differentiation medium, alcian blue staining was employed. In this staining method, more color absorption in the sample indicates a more considerable amount of GAG secretion by the cultured cells on that sample. The higher color intensity of the SM-PCL scaffold in the

third week compared to the 24-h time-point indicated the differentiation tendency of the MSCs towards the chondrocytes (Fig. 6a-d). No color absorption was observed for unmodified and plasma-treated PCL scaffolds (Fig. 6e,f). The increase of color intensity in nanofibrous scaffold surface-modified by sulfated alginate coating, and also the absence of color absorption in non-surface-modified nanofibrous scaffold showed that the sulfated alginate where used as coating can play a similar role to the sulfated alginate bulk hydrogels.

3.4. Chondrogenic marker of semi-cartilage cells

The chondrogenesis effects of polysaccharides such as alginate, sulfated alginate and gellan gum were previously reported[35,40,42]. Chondrogenic differentiation was assessed by evaluating the expression of *type II collagen* (*Col. II*) gene in the cells through RT-PCR. The expression of β-2-microglobulin (*β2M*) gene was recorded as a control gene. The *Col. II* gene is one of the important factors in chondrogenic differentiation process. Sulfated alginate can assist chondrocytes to synthesize own ECM and maintain them in cartilage phenotype. Proper ECM could enhance cell attachment, proliferation and chondrogenic differentiation. RT-PCR data showed the expression of *Col. II* gene (band at 85 bp) for SM-PCL scaffold after 7, 14 and 21 days. The results revealed that the SM-PCL scaffold effects on the chondrogenic differentiation of seeded MSCs and reserve *Col. II* expression in mRNA level up to 21 days without any growth factor and chondrogenic medium (Fig. 7A). Recently, Re'em et al. reported that the sustained release of transforming growth factor beta 1 (TGF-β1) from sulfated alginate scaffolds improve chondrogenesis of entrapped MSCs compared with those lacking sulfated alginate[43]. However, growth factor-free chondrogenic differentiation of MSCs to chondrocytes is an interesting demand in cartilage tissue engineering due to the high cost, intrinsically low stability and low active half-life of most growth factors.

Immunocytochemistry (ICC) assay was also performed to determine the expression of the main protein of cartilage ECM, i.e., *Col. II*. The results demonstrated the expression of chondrogenic differentiation of seeded MSCs on the SM-PCL scaffold after 21 days (Fig. 7B). The expression of this cartilage marker was also observed, which confirmed the positive effects of sulfated alginate coating in the differentiation process. Furthermore, sulfated alginate, due to its heparin-mimicking biochemical structure, leads to MSCs differentiation towards chondrocytes even without the presence of differentiation medium.

4. Conclusion

In this study, the fabrication of sulfated alginate surface-modified PCL nanofibrous scaffolds was reported. For this aim, electrospun PCL nanofibers were first surface-activated by cold atmospheric plasma and further surface-modified with sulfated alginate. Sodium sulfated alginate was synthesized through the green nitrite/bisulfite-mediated sulfation reaction. The sulfation of sodium alginate was confirmed by appearance of asymmetrical S = O stretching vibration band of sulfate groups and chemical shifts of uronic acid residues in FTIR and ^{1}H NMR analyses, respectively. The concentration of 5 mg mL^{-1} of SSA in aqueous medium was selected as the optimal concentration for surface modification of PCL

nanofibers. After surface modification of PCL nanofibrous mat with SSA, its in vitro cytocompatibility and capability for chondrogenic differentiation of MSCs were studied. The results of MTT assay, DAPI staining of MSCs and SEM images of cells cultured on the scaffolds showed that the surface-modified scaffolds can support the growth and proliferation of MSCs. Furthermore, the SM-PCL mats led to the chondrogenic differentiation of MSC into cartilage cells due to the presence bioactive coating of sulfated alginate. Finally, the RT-PCR and immunocytochemistry results proved the successful differentiation of MSCs and reaching to the semi-cartilage cells by confirming the expression of type 2 collagen gene on cell-cultured SM-PCL nanofibrous mats. These results confirmed that sulfated alginate when is used as coating can still maintain its ability to chondrogenic differentiate of MSCs to the chondrocytes.

Declarations

Competing interests:

The authors declare no competing interests.

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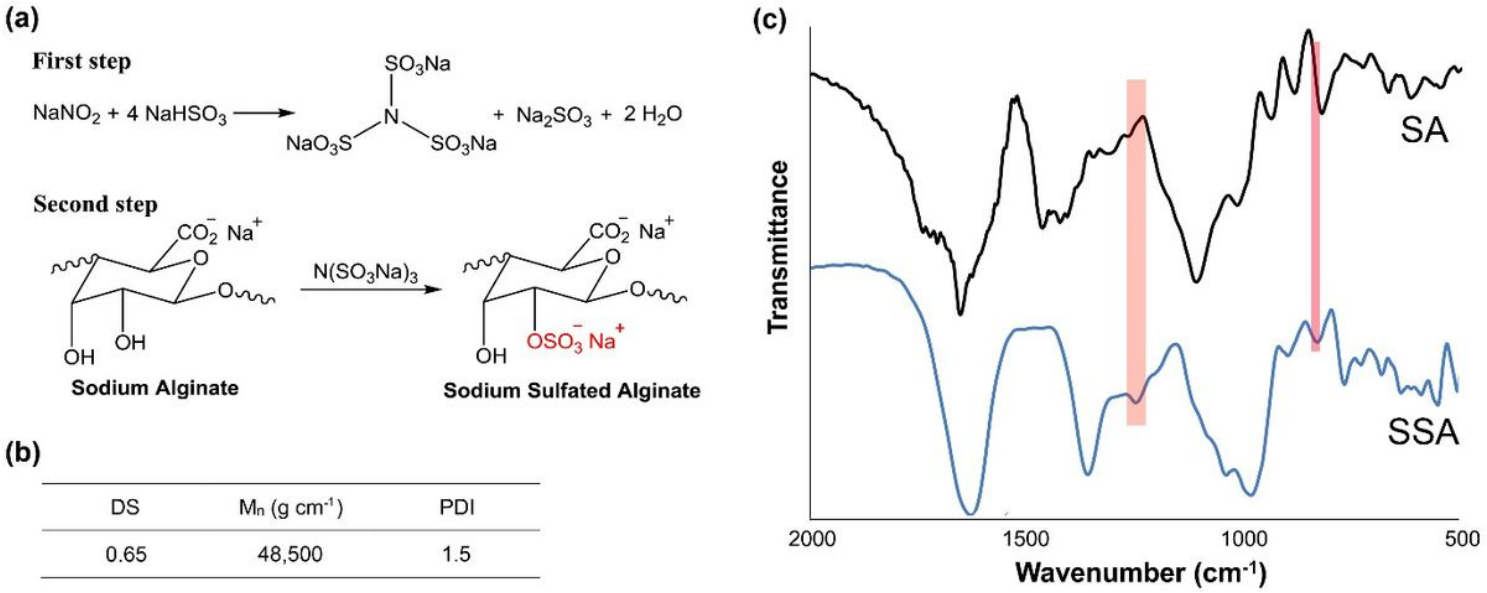
Figures



(b)

DS	M_n (g cm^{-1})	PDI
0.65	48,500	1.5

Figure 1

Chemical characterization of sodium sulfated alginate (SSA) salt: (a) Chemical procedure used for synthesis of SSA. (b) Physicochemical characterization of SSA. (c) FTIR-ATR spectra of neat sodium alginate (SA) and SSA.

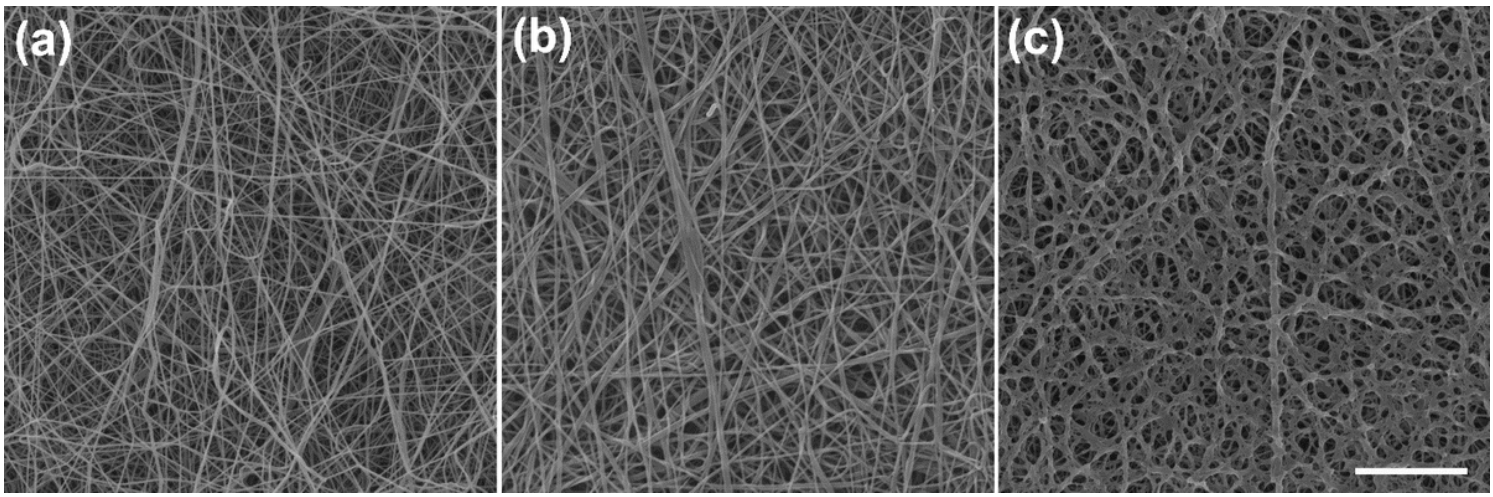


Figure 2

The morphology of PCL nanofibrous scaffold and its surface-modified samples: (a) PCL scaffold, (b) PCL scaffold surface-activated by cold atmospheric plasma, (c) Sulfated alginate surface-modified PCL scaffold.

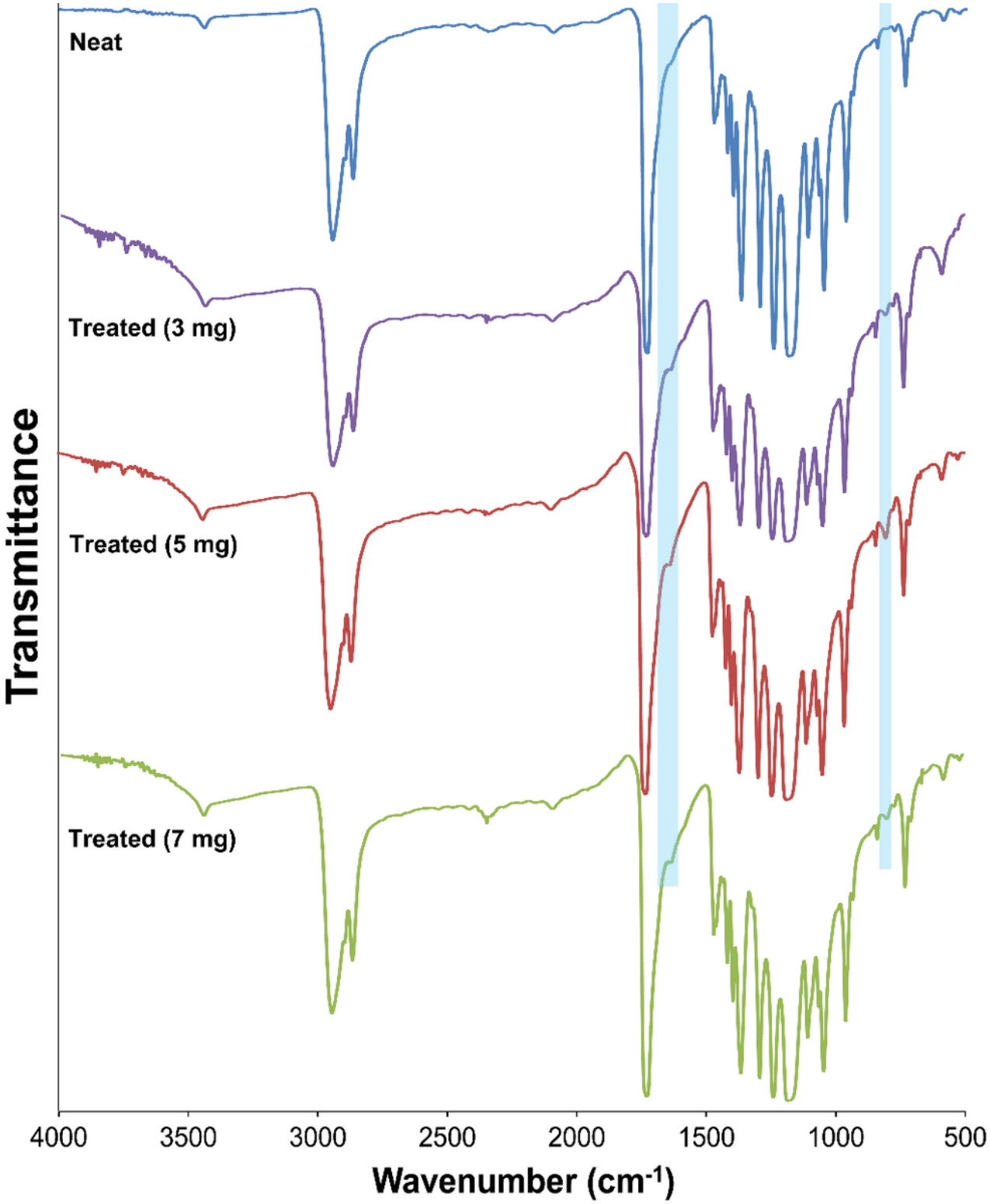


Figure 3

FTIR analysis of SM-PCL scaffolds with different concentrations of SSA (3, 5 and 7 mg mL-1) as the surface modifying agent. The concentration of 5 mg mL-1 was considered as the optimal concentration of SSA.

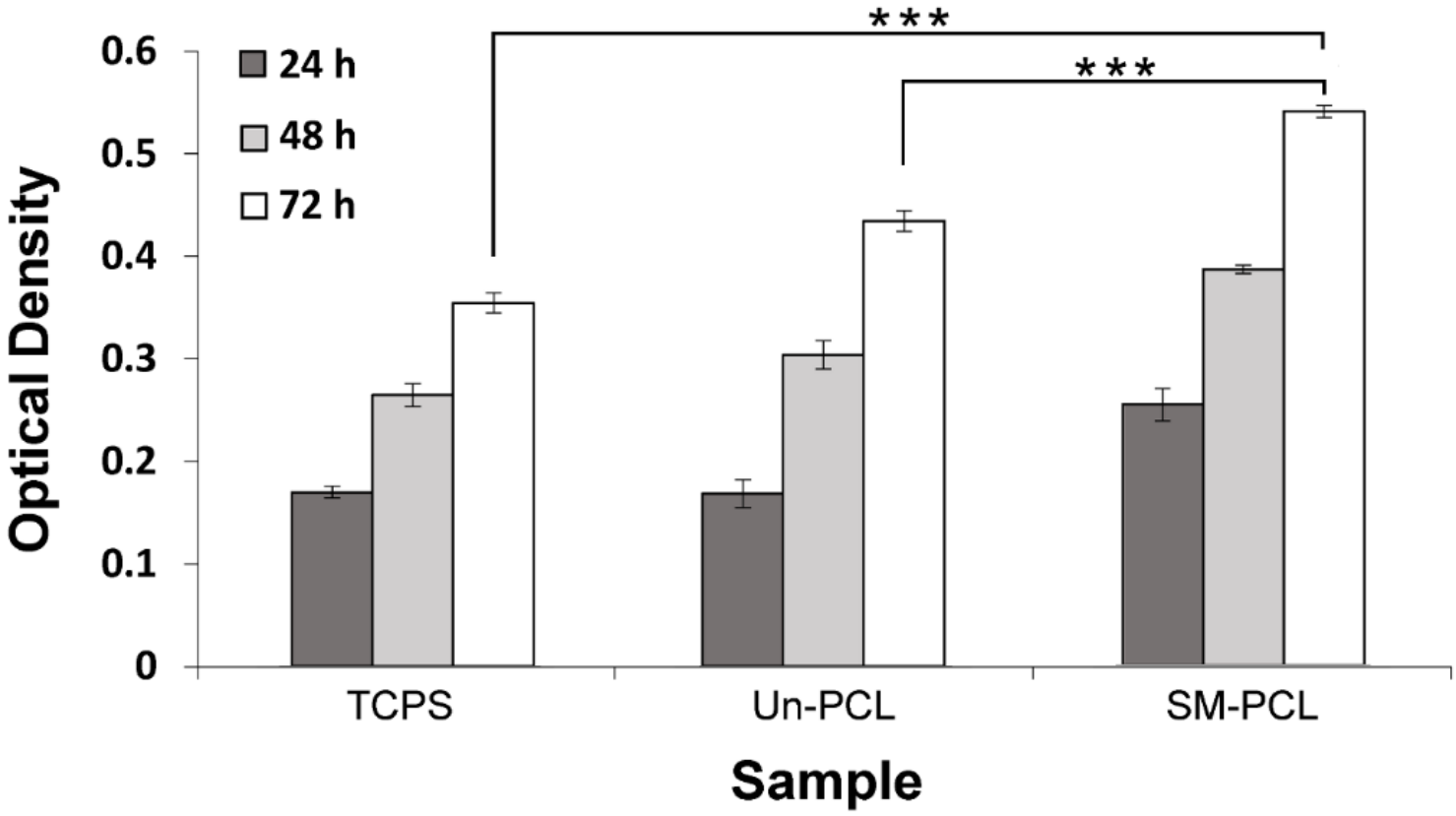


Figure 4

The MSCs metabolic activity and viability were performed on tissue cultured polystyrene plate (TCPS), untreated PCL scaffold (Un-PCL) and surface-modified PCL (SM-PCL) scaffolds after 24, 48 and 72 h by MTT assay. Results showed the statistically significant higher cell viability of SM-PCL scaffold compared to unmodified and control samples (n=3) (*** $p < 0.001$).

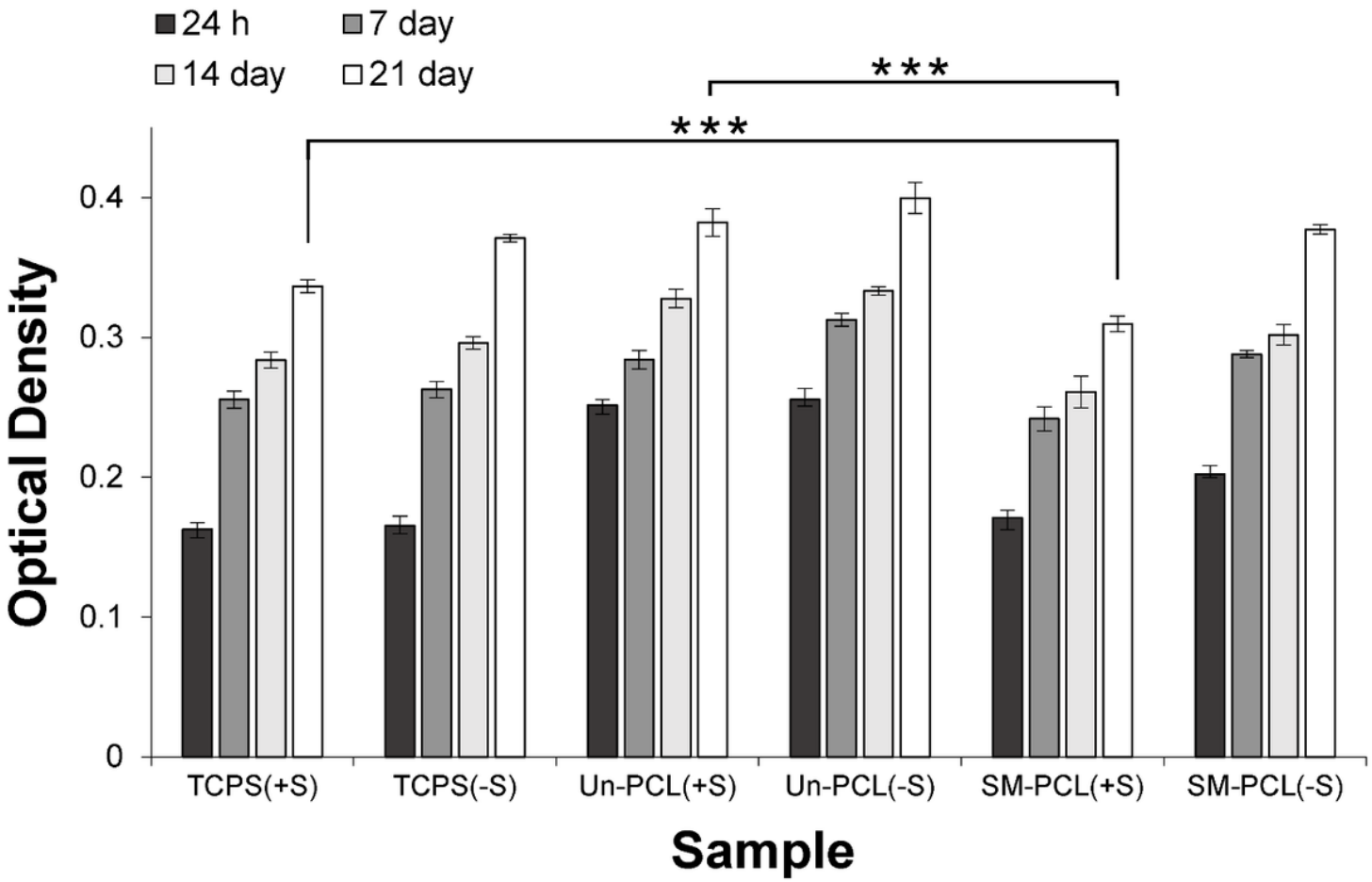


Figure 5

The MSCs metabolic activity and viability were performed on TCPS, Un-PCL and SM-PCL scaffolds after 24 h, 7, 14 and 21 days by MTT assay to illustrate the chondrogenic differentiation capability of substrates in the absence or presence of differentiation medium (n=3, S stands for differentiation supplement) (*** $p < 0.001$).

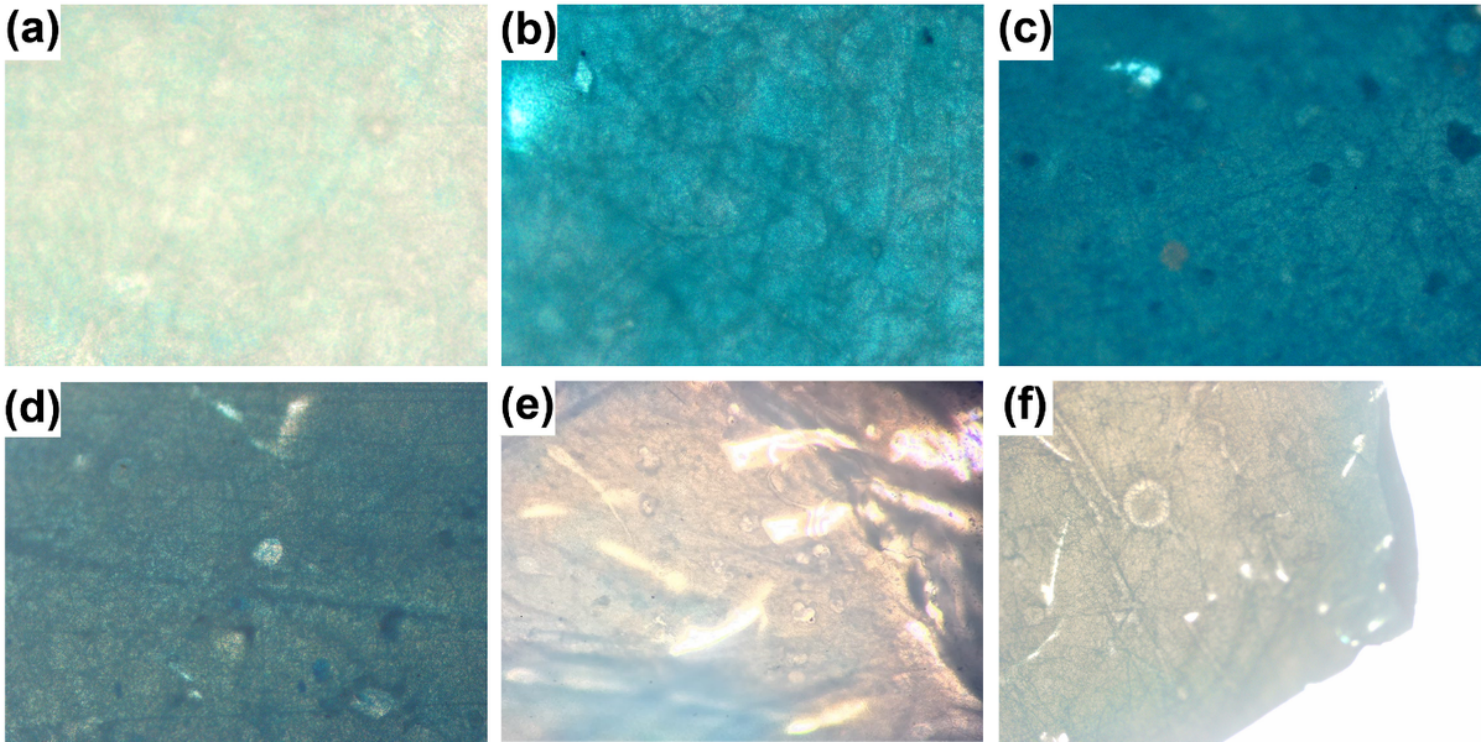


Figure 6

Alcian blue staining of MSCs cultured on SM-PCL scaffold after 24 h, 7, 14 and 21 days (a-d), unmodified PCL (Un-PCL) sample and (e) plasma-treated PCL scaffold after 21 days.

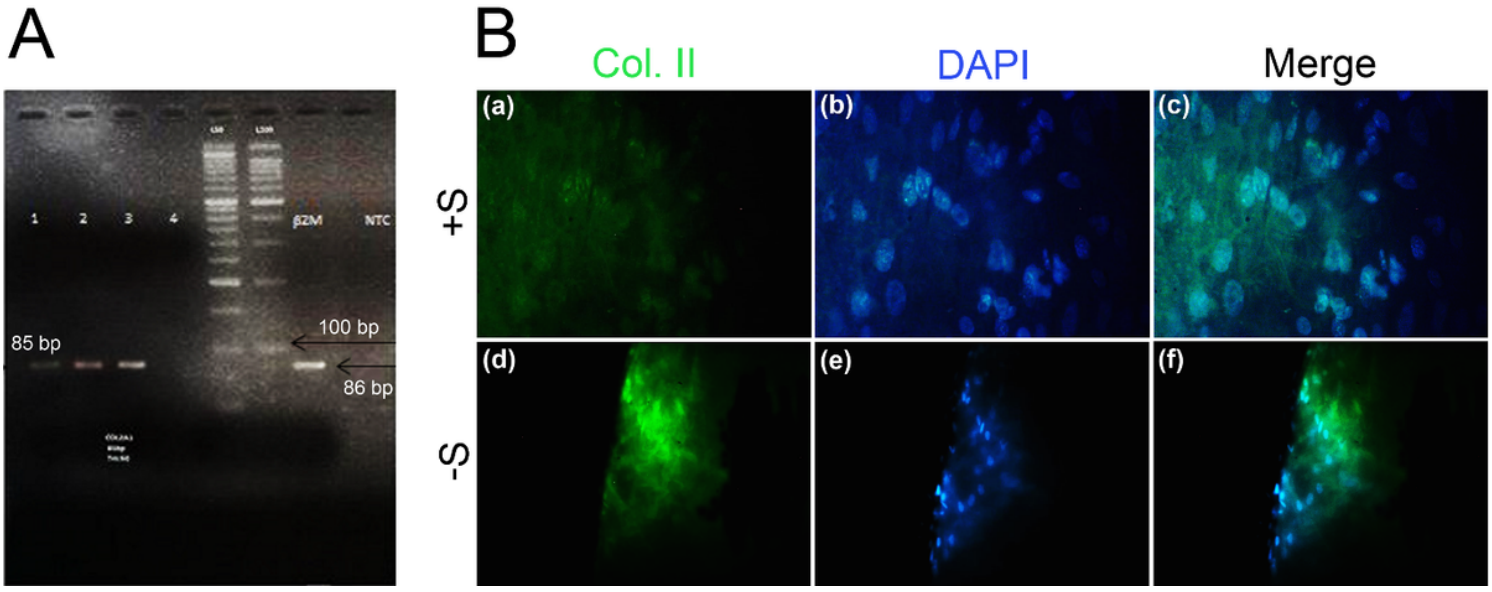


Figure 7

Molecular and cellular characterization during chondrogenic differentiation of MSCs into the chondrocytes. A) Gel electrophoresis analysis of polymerase chain reaction (PCR) products: SM-PCL scaffold in the presence of a differentiation medium, the type 2 collagen gene is expressed in this group. Samples 1, 2 and 3 refer to the days of 7, 14 and 21, respectively (Ladder = 50; 100). B) Expression of

type 2 collagen in differentiated MSCs to the semi-cartilage cells on SM-PCL scaffold in the presence (a-c) and absence (d-f) of differentiation medium (S stands for differentiation supplement).

Supplementary Files

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