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Fabrication of multifunctional microfibrous and nanofibrous cellulose carriers and comparison of cell adhesion and spreading potential on them

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ABSTRACT

Fibrous biomaterials have received much attention in tissue engineering and regenerative medicine due to their morphology, resembling extracellular matrix. In comparison to synthetic fibers, cellulose based fibers have interesting properties for cellular applications such as biodegradability, biocompatibility, simple preparation and their potential for chemical modification. Among cellulose derivatives, carboxymethyl cellulose and quaternized cellulose are the most important and valuable cellulose ethers which have anionic and cationic surface charge. In this research, we report the fabrication of multifunctional cellulose microfibrous and nanofibrous scaffolds and the comparison of adhesion and spreading potential of human fibroblast cell on them. The fabricated fibrous scaffolds were characterized by several instrumental techniques. The results showed that multifunctional cellulose nanofibers and microfiber had 8.6 and 8.2 mV surface potential, 7.1 and 6.8 MPa tensile strength, 560 and 510 MPa Young modules, 610 and 595% water uptake and 41o and 44o contact angle, respectively. The MTT assay showed that proliferation of fibroblast cells was enhanced in nanofibrous, compared to microfibrous mat. The SEM analysis of fixed cells on scaffolds showed that cells spreading on nanofibrous samples became more noticeable than microfibrous ones.

Keywords: *Anionic cellulose; Cationic cellulose; Microfiber; Nanofiber; Fibrous carriers; Surface charge.*

1. INTRODUCTION

Fibers are continuous structure materials that have an extremely high ratio of length to width [1]. Over the past few decades, the use of fibrous biomaterials has met with success in tissue engineering and regenerative medicine since fibrous structures exibit morphology similar to extracellular matrix [2]. This type of biomaterial supports cell-biomaterial interactions and allows cell growth by gases, nutrients and regulatory factors transport [3]. Regardless of fibrous scaffold compositions, they can be categorized into two major groups, microfibrous and nanofibrous scaffolds, according to the size of the fibers.

Cellulose is a naturally occurring linear polymer from polysaccharide which is the most abundant renewable source available on the earth [4]. In comparison to synthetic materials, cellulose based fibers have interesting properties such as biodegradability, biocompatibility, network structure, appropriate water absorption capacity and good mechanical strength [5, 6]. The use of this material in biomedical applications has gained attention due to the multifuncionality of cellulose, for example, the possibility of chemical and physical alternations [7, 8].

Etherification is a commonly used chemical treatment for cellulose surface modification. Carboxymethyl cellulose and quaternized cellulose, as negatively and positively surface charged biomaterials, are fabricated by etherification of alkali cellulose. In this treatment, monochloroacetic acid and quaternary epoxides act as anionization and cationization reagents that react with hydroxyl groups of cellulose to produce carboxymethyl and quaternary ammonium based cellulose ethers, respectively [9, 10]. Carboxymethyl cellulose is the most important and valuable cellulose ether which has attracted considerable attention in advanced fields such as tissue engineering, drug delivery, dye adsorbents and lithium ion batteries, besides its conventional various applications in textile, foods, ceramic and oil drilling [11, 12]. Quaternized cellulose has also emerged as a potential material in tissue engineering and regenerative medicine, as a promising protein and nonviral gene carrier in drug delivery systems, cellular carrier in biopharmaceutical industries and wound-dressing applications [13-17].

Among different natural micro scale cellulose fibers, cotton has historically been the most used material in medical products such as sutures, absorbent pads, dressings and bandages [18]. With the development of nanotechnology, cellulose nanofibers have been produced and great attention has been paid to them. Various techniques can be used to produce cellulose nanofibers but electrospinning approach may be considered as an ideally simple, highly versatile and cost-effective method [19-22].

In this work, we report the fabrication of multifunctional microfibrous and nanofibrous cellulose based scaffolds and comparison of adhesion and spreading potential of human fibroblast cell on them. The fabricated fibrous scaffolds were also characterized by several instrumental techniques.

2. MATERIALS AND METHODS

2.1. Materials.

Cellulose acetate was provided by Aldrich Co. The average molecular weight was 30 kDa, the acetyl content was 39.8 wt% and the degree of acetyl substitution was 2.4. The anionization and cationization agents, monochloroacetic acid (MCA) and 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHPTAC, aqueous solution of 69 wt%), respectively, were purchased from Sigma and microfibrous cotton were supplied by Fluka. All Chemicals were provided from Aldrich Co. Solvents such as acetone, ethanol, isopropanol, dimethyl sulfoxide (DMSO) and N-dimethylacetamide (DMAc) were received from Merck. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylphenyltetrazolium bromide) reagent (98%), Dulbecco's modified eagle's medium (DMEM)-F12, fetal bovine serum (FBS), penicillin-streptomycin (pen-strip) and phosphate-buffered saline (PBS, pH 7.4) were purchased from GIBCO Invitrogen (Carlsbad, CA, USA). For cell culture study, human fibroblast cells were obtained from Tofigh Daru Research & Engineering Company, Tehran, Iran.

2.2. Production and chemical treatment of cellulose nanofibers and microfibers.

2.2.1. Electrospinning of cellulose acetate (CA).

CA was dissolved in 2:1 (w/w) acetone/DMAc mixture for preparing 15 wt.% CA electrospinning solution with contineous stirring at room temperature until the solution became clear. The obtained CA solution was filled in a plastic syringe connecting a stainless steel needle as the nozzle. The injection speed was set at 1 mL/h by the syringe pump. The applied voltage and tip to collector distance were 14 kV and 15 cm, respectively [23].

2.2.2. Deacetylation of electrospun CA nanofibers.

The fabricated nanofibers were soaked in 0.05 M NaOH ethanolic solution at room temperature for 24 h to deacetylate CA electrospun and regenerate cellulose nanofibers.

2.2.3. Simoultanous anionization and cationization of cellulose nanofibers and microfibers.

The multifunctional cellulose nanofibers and microfibers (cotton) were fabricated by dissolving 0.8 g monochloroacetic acid in alkaline solutions containing mercerized cellulose fibers at 60 °C for 2 h, then 5 g CHPTAC solution was added to the mixture and stirred at 80 ℃ for 2 h.

2.3. Characterization of multifunctional nanofibers and microfibers.

Field Emission Scanning Electron Microscopy (FESEM, Tescan – mira 3, Czech Republic) was used to investigate the surface morphology of prepared nanofibers and microfibers before and after cell culturing. The FESEM accelerating voltage was set at 15 kV. Chemical structures of the cellulose samples were characterized by Fourier transform infrared (FT–IR) spectrometer (Nicolette 6700, Thermo Fisher Scientific Co. Ltd., MA, USA). Contact angle measurements were performed at room temperature (G10 goniometer from Krüss, Germany). To measure swelling behavior, dry weight (Wd) of each sample was recorded, after immersion in water, the sample weight was measured again to record its wet weight (Ww). The water uptake ability of the samples was obtained by Equation (1) [24]:

$$\text{Water uptake } \% = \frac{W_w - W_d}{W_w} \times 100 \qquad (1)$$

An electro kinetic analyzer (EKA, Anton Paar KG, Graz, Austria) was used to measure surface zeta potential at 25 ℃. The mechanical properties of multifunctional cellulose nanofibers and microfibers (Young's modulus and tensile strength) were obtained by BOSE mechanical testing machine (Model ELF 3200).

2.4. Cell adhesion and MTT assay.

UV light was applied for 1 h on each side of multifunctional cellulose nanofibers and microfibers to sterilize them. The samples were circular shaped and placed into 24-well plates. The standard cell culture treated 24-well plates was used as a control. The cell culture density of 16,000 cells/cm3 was used as seeding content on each sample. 1000 µL of (DMEM)-F12 medium supplemented with 10% FBS and 1% pen-strip was poured to each well. Cells were maintained at 37 ℃ in a humidified atmosphere containing 5% CO2 and the culture medium was replaced every 2 days. MTT assay was carried out after 1, 3, and 5 days of cell culturing on each sample. At test days, 50 µL of MTT solution with 0.5 mg/mL concentration in PBS (pH 7.4) was replaced with 500 µL of medium in each plate. The MTT containing medium was incubated at each plate for 4 h and then gently removed. After this period of time, purple formazan crystals formed in live cells and for dissolving them 150 µL DMSO was added to the solution at room temperature and kept for 15 min. The absorbance of each solution was measured at 570 nm using a microplate reader (Synergy HTX, Biotek).

Cell adhesion assay was performed to investigate the attachment of human normal fibroblast cells on surface of multifunctional cellulose nanofibers and microfibers. After cellular incubation on the scaffolds for 24 h, 2.5% crosslinking agent (glutaraldehyde) was introduced to each sample and left at 4 oC for 2 h. After fixation of the cells, an increasing gradient of ethanol was used to dehydrated cells, then samples were air dried at 4 °C. The morphology of fixed cells on the scaffolds was observed by FESEM.

3. RESULTS

Cellulose acetate nanofibers were fabricated by electrospinning process and then deacetylated to convert into electrospun cellulose nanofibers as illustrated in Figure1a. Electrospun cellulose acetate nanofibers were completely regenerated to cellulose nanofibers by soaking in 0.05 M NaOH/ethanol alkaline solution at ambient temperature for hydrolyzing acetate functional groups. The hydrophobic acetyl groups on the surface of cellulose acetate were substituted by hydrophilic hydroxyl groups and resulted in the increase of the hydrophilic property of cellulose. The properties of cellulose scaffolds were improved for biomedical applications by functionalizing them with carboxymethyl and trimethylammonium groups. The mechanism for introducing carboxyl and trimethylammonium groups on the cellulose nanofiber and microfiber surface was based on the reaction of cellulose with monochloroacetic acid and CHPTAC under the catalytic action of sodium hydroxide as shown in Figure 1b [25].

The surface morphologies of the fibers were investigated by FESEM. As shown in Figure 2, FESEM images represent the smooth, continuous and irregular surface of multifunctional cellulose nanofibers and microfibers.

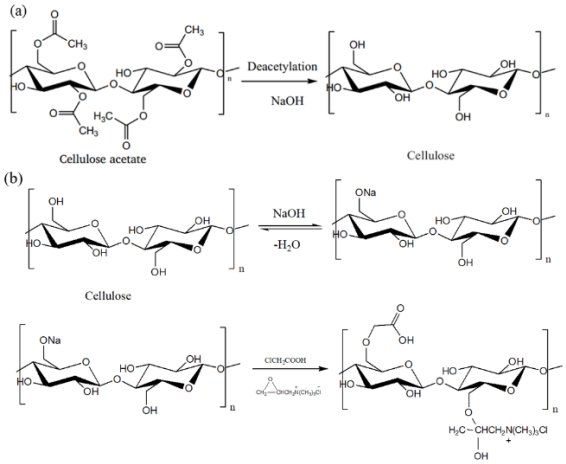

Figure 1. (a) Mechanism of fabrication of regenerated cellulose nanofibers. (b) Mechanism of simultaneous anionization and cationization of cellulose nanofibers and microfibers.

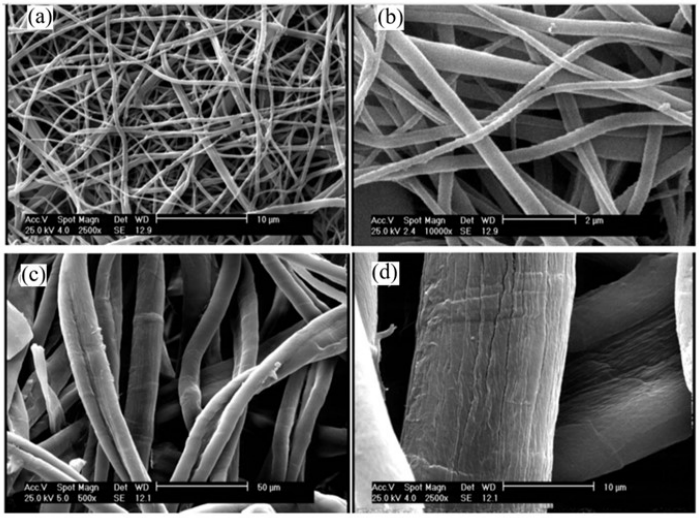

Figure 2. FESEM images of (a) and (b) cellulose nanofibers, (c) and (d) cellulose microfibers.

FTIR spectroscopy was applied to approve the regeneration of cellulose nanofibers from deacetylation of CA nanofibers and to identify the presence of anionized and cationized functional groups on the surface of chemically treated cellulose nanofibers and microfibers. As presented in Figure 3, the acetate group vibrations vanished from the CA spectrum and a broad absorption band at 3320 cm^{-1} was observed which represents the O–H group stretching vibration. This evidence indicate the presence of more hydroxyl groups in the regenerated cellulose. The FTIR spectrum of multifunctional cellulose nanofibers and microfibers exhibit further absorption bands at 1594, 1414 and 1059 cm^{-1} which is related to carboxylate group (–COO) asymmetrical and symmetric stretching vibration sand ether bonds (C–O–C) asymmetric stretching vibration, respectively. These results indicate that carboxymethyl group was successfully introduced on cellulose surface. The appearance of new bands at 1045 and 2920 cm^{-1}, which respectively represent the C–N stretching vibration of the quaternary ammonium groups and methyl groups stretching vibration on the cellulose surface, indicates the successful cationization of multifunctional cellulose scaffold [25].

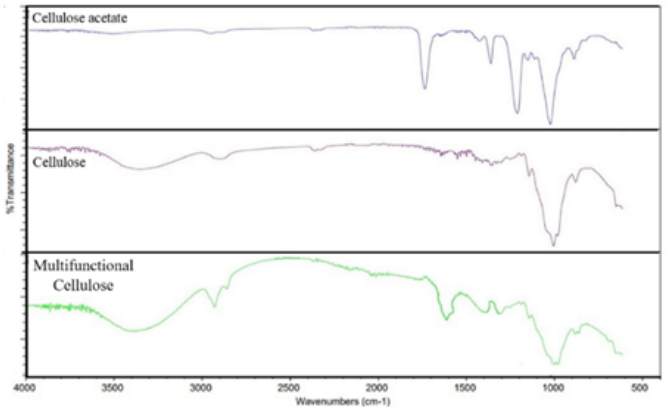

Figure 3. FTIR spectra of fabricated fibers

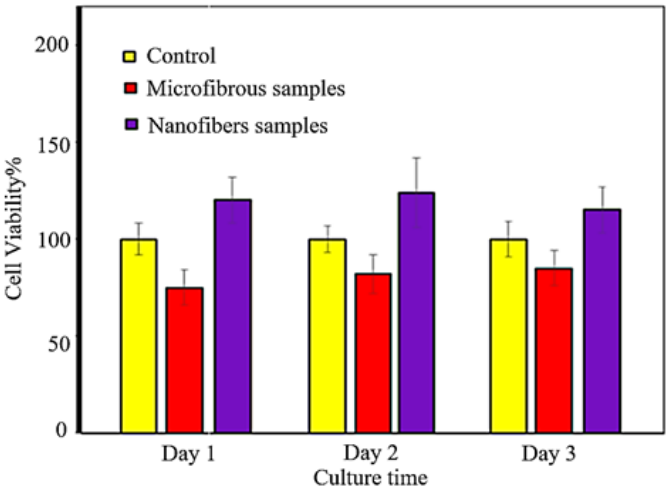

Figure 4. Normal fibroblast cell viability and proliferation on multifunctional cellulose nanofibers and microfibers.

As presented in Table1, the zeta potential measurements of multifunctional cellulose nanofibers and microfibers showed that a noticeable number of negatively charged groups was introduced on fibrous cellulose structure before cationization (-12.5 mV for nanofibers and -12.1 mV for microfibers at pH 7) due to the presence of carboxymethyl groups on the surface. After introducing quaternary ammonium group on fibers, zeta potential increased to +8.6 mV for nanofibers and +8.2 mV for microfibers at pH 7 which indicates the cationization of surface.

The multifunctional cellulose samples were additionally characterized by analyzing their water uptake ability in distilled water. The results revealed that the water uptake of multifunctional cellulose nanofibers and microfibers was 610% and 595%, respectively. It was observed that the contact angle of multifunctional cellulose nanofibers and microfibers was 41^{o} and 44^{o}. The tensile strength of nanofibers and microfibers was 7.1 and 6.8 MPa and their Young's modulus was 560 and 510 MPa, respectively. When comparing the mechanical properties of the nanofibers and microfibers, it is obvious that nanofibers had higher tensile strength and Young's modulus due to their high surface area to volume ratio.

Table 1. Some physico-chemical properties of multifunctional cellulose nanofibers and microfibers

Property	Nanofibers	Microfibers
Zeta potential (before cationization), (mV)	-12.5±0.3	-12.1±0.2
Zeta potential (after cationization), (mV)	+8.6±0.3	+8.2±0.3

Contact angle, (°)	41±2	44±2
Water uptake, (%)	610±20	595±15
Tensile Strength, (MPa)	7.1 ±0.2	6.8 ±0.1
Young's modulus, (MPa)	560 ±15	510 ±20

The results of MTT assay (Figure 4) indicated the high viability of cells on nanofibrous samples compared to microfibrous samples. This is propably due to the fact that nanofibrous structures support higher anchorage for cell attachment and proliferation. The MTT assay tests showed that multifunctional cellulose nanofibers and microfibers did not have any toxic effect on the human fibroblast cell, so they can be used as a carrier to support cell proliferation for biomedical applications.

The SEM analysis of fixed fibroblast cells on scaffolds (Figure 5) showed the average length of cells on nanofiber surface is considerably higher than that of microfibers, indicating that nanofibrous scaffolds may enhance the cell outgrowth.

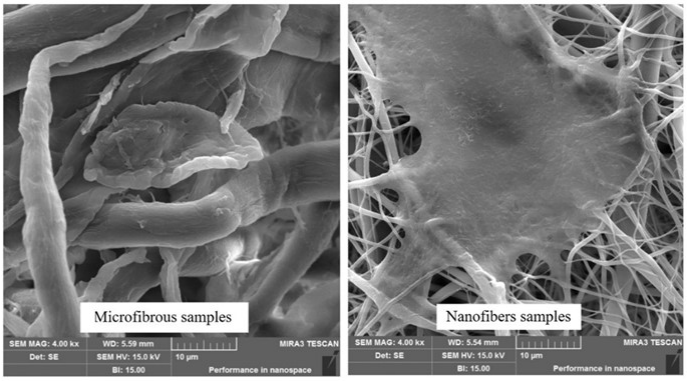

Figure 5. SEM images of fixed fibroblast cells on the surface of multifunctional cellulose nanofibers and microfibers.

4. CONCLUSIONS

In this work, we focus on the fabrication of multifunctional microfibrous and nanofibrous cellulose scaffolds and comparison of their properties which could be useful for scientists who intend to use fibrous scaffold in tissue engineering and regerative medicine. The results of this research showed that nanofibrous multifunctional cellulose had higher water uptake, wettability and mechanical strength than microfibrous one. The MTT assay showed that proliferation of fibroblast cells increased on nanofibrous multifunctional cellulose, compared to microfibrous mat. The SEM image of fixed fibroblast on scaffolds showed that spreading of cells on nanofibrous samples was more noticeable than microfibrous one.

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6. ACKNOWLEDGEMENTS

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