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Effect of Biotin and Galactose Functionalized Gelatin Nanofiber Membrane on HEp-2 Cell Attachment and Cytotoxicity

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Abstract In the present study, we prepared a gelatin nanofiber matrix using an electrospinning technique and cross-linked the nanofibers with 10 % glutaraldehyde vapors. The insoluble nanofibers were functionalized with bioactive molecules like biotin (1 %) and galactose (1 %) by adsorption and coelectrospinning. Surface morphology and fiber dimension were analyzed using atomic force microscopy. The amounts of biotin and galactose bound to the nanofibers before and after adsorption were quantified using high-performance liquid chromatography. Human larynx carcinoma (HEp-2) cell attachment, morphology and cytotoxic characteristics were studied using crystal violet staining and the MTT assay. Cell attachment and viability were highest in biotin- and galactose-embedded nanofibers compared to native nanofibers. Cytotoxicity was less with biotin- and galactose-embedded and adsorbed nanofibers compared to control nanofibers. Hence, we suggest that these biocompatible, nontoxic, biodegradable, functionalized nanofibers could be a potential candidate for application in tissue engineering and scaffold preparation.

Keywords Gelatin nanofiber matrix · Biotin and galactose functionalization · HEp-2 cell · Cell attachment · Cytotoxicity

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Introduction

Tissue engineering represents an emerging interdisciplinary field that applies the principles of biological, chemical and engineering sciences toward the goal of tissue regeneration (Shieh Terada and Vacanti 2004). Tissue-engineering approaches make use of biomaterials, cells and factors either alone or in combination to restore, maintain or improve tissue function. The tissue-engineering strategy generally involves isolation of healthy cells from a patient, followed by their expansion in vitro. These expanded cells are then seeded onto a three-dimensional (3D) biodegradable scaffold that provides structural support, which can also act as a reservoir for bioactive molecules such as growth factors and attachment factors. The scaffold gradually degrades with time to be replaced by newly grown tissue from the seeded cells (Langer and Vacanti 1993). To achieve gradual degradation and efficient growth of cells inside the scaffold, selection of biomaterials for preparation of scaffold and its 3D synthetic frameworks plays a crucial role. These 3D matrix/scaffolds enhance cellular attachment, proliferation and growth, ultimately leading to new tissue formation. A number of novel approaches including emulsion, freeze-drying, gas foaming, phase separation, leaching and rapid prototyping, have been developed for the fabrication of these biomaterial-based 3D scaffolds (Whang et al. 2000; Yoon and Park 2001; Atala and Lanza 2002; Levenberg et al. 2003; Liu and Ma 2009).

Recently, electrospinning has gained an important place in tissue-engineering applications due to its versatility in producing fibrous scaffolds at the nano level with a high surface area to volume ratio, which in turn favors cell adhesion, proliferation, migration and differentiation (Bhattarai et al. 2004; Ma et al. 2005; Shi et al. 2010). Fibrous polymer matrices [such as poly(lactic acid) (PLA)

and poly(lactic-co-glycolic acid) (PLGA)] produced by electrospinning have been widely used for cell culture studies. However, such fibrous architectures alone are not sufficient for mimicking the complex natural extracellular matrix (ECM). Surfaces bearing favorable functionalities (hydrophilic, biomolecule-coated, etc.) are generally needed for cell adhesion and proliferation. Cellular adhesion ligands in ECM play a critical role in cell adhesion and attachment, which affect cell proliferation and differentiation and the maintenance of regular metabolic activities. Differences in cellular response have been reported with changes in distribution and structural presentation of the signals on these functionalized artificial cell scaffolds.

The common cell adhesion biomolecules are selectins, integrins, cadherins, fetal bovine serum, intercellular adhesion molecule 1 (ICAM, also called as CD54), vascular cell adhesion molecule (VCAM, also called as CD106), cadherins, etc. (Pu et al. 2002; Amran et al. 2011). Cell–cell adhesions are mediated by cadherins, whereas cell–matrix adhesions are usually mediated by integrins (Martinez Rico et al. 2009). However, cell attachment can also be achieved using sugar-derivatized polymer surfaces, which are easy to handle and economic compared to the other ligands mentioned above (Liang and Akaike 1998; Yoon et al. 2002). It was reported that the concentration of surface exposed to galactose on the polymer substrate controlled the adhesion of hepatocytes on the surface (Blackburn and Schnaar 1983). In line with this, Yoon et al. (2002) developed a galactose-immobilized PLGA surface for attachment and growth of hepatocytes and found efficient attachment of hepatocytes when compared with control PLGA. Biotin is a coenzyme and has an active role in fatty acid oxidation, leucine metabolism, gluconeogenesis and citric acid cycle (Sriram and Yogeeswari 2010). Biotin is hypothesized to exert a positive effect on cell proliferation, cell signaling, DNA repair and catabolism of leucine (Gropper et al. 2009). Hence, in the present study we used these bioactive molecules to functionalize gelatin nanofibers for tissue-engineering application by adsorption and coelectrospinning processes.

Experimental Procedures

Electrospinning of Gelatin Nanofibers

Briefly, the gelatin/glacial acetic acid solution (10 % w/v solution) held in a 5-ml syringe was delivered into a blunted medical needle spinneret (OD 0.9 mm, ID 0.69 mm) through a Teflon tubing by a syringe pump (Kelly Med, Beijing, China). A polarity-reversible, high-voltage power supply (Glassman High Voltage, Yokohama, Japan) was used to charge the spinning dope of gelatin/glacial acetic acid solution by directly clamping one electrode to the metal needle spinneret (purchased in local market, Coimbatore, India) and another to an aluminum plate (5 × 5 cm) wrapped on a lab rack. The separating distance between the needle tip and the aluminum plate was set to 14 cm. Other operating parameters in a chamber for producing the gelatin nanofibers were as follows: voltage 25 kV, flow rate 0.4 ml/h, ambient temperature 25 °C and humidity 70 %. The obtained nanofibrous membranes were stored in fume hood for 24 h to remove residual solvent and then transferred into a dry cabinet for storage at room temperature.

Glutaraldehyde Vapor Cross-Linking

The cross-linking process was carried out by placing the air-dried gelatin nanofibrous membrane together with a supporting aluminum foil in a sealed desiccator containing 10 ml of aqueous 10 % v/v glutaraldehyde (GTA) solution in a Petri dish for 12 h (Kato et al. 1989). After 12 h, the nanofibers were removed and checked for water insolubility by dipping in water at various time intervals. The residual GTA vapors on the nanofiber mat were removed by keeping them in the fume hood for 2 h, followed by posttreatment at 100 °C for 1 h to remove residual GTA and to partially enhance the cross-linking (Ruijgrok et al. 1994; Zhang et al. 2006). The dried insoluble gelatin nanofibers were used for further experiments.

Dissolvability Test

The dissolvability test was carried out according to Zhang et al. (2006). The cross-linked gelatin nanofibrous membranes were cut to a size of 1 × 1 cm and immersed in warm distilled water (37 °C) for 1 h to test dissolvability. This experimental condition simulates the real situation of gelatin nanofibers in physiological application, including tissue engineering.

Functionalization of Gelatin Nanofibers with Biotin and Galactose

One percent of biotin was dissolved in warm water, and a few drops of 1 N NaOH were added to it until the biotin completely dissolved in the solution. Two different methods were adopted to functionalize gelatin nanofibers with biotin and galactose: adsorption onto cross-linked gelatin nanofibers and coelectrospinning into the 10 % (w/v) gelatin–acetic acid solution before electrospinning, followed by cross-linking. In adsorption-based studies, the following combinations were tried: (1) 1 % (w/v) biotin, (2) 1 % (w/v) galactose and (3) 1 % (w/v) biotin and galactose. Similar combinations were tried with coelectrospinning of gelatin solution. Adsorption of biotin and galactose onto

cross-linked gelatin nanofibers was carried out for a constant contact time of 8 h at room temperature. After 8 h, the gelatin nanofibers were removed from the solution and washed with sterile distilled water until the solution pH turned neutral.

Estimation of Biotin and Galactose by HPLC

The residual biotin and galactose in the solution after adsorption was estimated using high-performance liquid chromatography (HPLC) with the respective columns, mobile phase and detectors.

Estimation of Biotin

The percentage of biotin adsorption onto the nanofibers was estimated using HPLC (Agilent 1100; Agilent, Santa Clara, CA) by calculating the residual biotin level in the solution after adsorption. The estimation was carried out according to the Agilent user manual (http://www.chem.agilent.com/library/applications/59682971.pdf). A reverse-phase gradient column (4.6 × 75 mm Zorbax SB-C18, 3.5 μm) was used with two types of mobile phases, 0.05 M KH_2PO_4 (pH 2.5) and acetonitrile, at a constant flow rate of 1 ml/min. The temperature in the column compartment was maintained at 15 °C, and a variable wavelength UV detector was used for the estimation.

Estimation of Galactose

The percentage of galactose adsorption onto the nanofibers was estimated using HPLC (Agilent 1100) by calculating the residual galactose level in the solution after adsorption. The estimation was carried out according to the Agilent user manual. The Bio-Rad HP XP column (0.3 × 7.8 mm; Bio-Rad, Hercules, Ca) was used with ultrapure water as the mobile phase at a constant flow rate of 0.7 ml/min. The temperature in the column compartment was maintained at 80 °C, and a refractive index detector was used for the estimation.

Characterization of Gelatin Nanofibers Using Atomic Force Microscopy

Gelatin nanofibers (native and coelectrospun) were characterized using atomic force microscopy (AFM). A dried section of nanofibers was mounted onto a safire platform using a double-sided Scotch tape and inserted into the sample platform. Images were recorded by a multimode scanning probe microscope (Ntegra Aura; NT-MDT, Moscow, Russia) at ambient conditions (25 ± 2 °C) using single crystal silicon N-type probes (NSG 03-A) with a radius of curvature of 10 nm. The cantilever with long tips (aspect ratio 3:1), with force constants of 0.35–6.06 N/m and resonance frequencies of 47–150 kHz, was used to image the surface morphology. The wings were scanned using noncontact (tapping) mode AFM in different sizes starting from 50 × 50 μm and then gradually reducing the scan area. The surface morphology, fiber diameter, average roughness and surface skewness as well as grain analysis were measured using NT-MDT image analysis software.

In Vitro Cell Culture Studies

Human larynx carcinoma (HEp-2) cells (National Centre for Cell Science, Pune, India) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10 % fetal calf serum (FCS) at 37 °C in a humidified incubator with 5 % CO_2. Gelatin nanofibers (native, adsorbed and coelectrospun nanofibers) were exposed to UV radiation for 30 min and then prewetted with 70 % ethanol for a period of 30 min. The sterile, dry nanofibers were placed in a 24-well culture plate and seeded with HEp-2 cells (5×10^4/100 μl) with uniform cell density and incubated further to investigate the time period required for HEp-2 cell attachment onto nanofibers. HEp-2 cell attachment was measured using crystal violet staining according to the BD Bioscience (San Jose, CA) cell adhesion protocol. Cytotoxicity was estimated using the standard 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay (Jeong et al. 2010).

Statistical Analysis

All of the quantitative results were obtained from triplicate samples. Data are expressed as mean ± SD. Statistical analysis was carried out using Microsoft (Redmond, WA) Excel and NT-MDT image analysis software.

Results and Discussion

Natural polymers offer many advantages over synthetic polymers, being nontoxic and similar or often identical to macromolecular substances present in the human body. Some of the natural polymers used as biomaterials are collagen, hyaluronic acid, gelatin, chitosan, elastin, silk and wheat protein (Yannas 2004). Among these natural polymers, gelatin is a protein biopolymer derived from partial hydrolysis of native collagens, which are the most abundant structural proteins found in the skin, tendon, cartilage and bone of animal bodies (Ward and Courts 1977). Gelatin polymers are mostly preferred for their bioavailability, nonimmunogenicity, biodegradability, biocompatibility and commercial availability at relatively low cost (Guidoin et al. 1987; Jonas et al. 1988; Marois et al.

1995). The conversion of this naturally available gelatin to high-surface area nanofibers and nanofibrous composites by coelectrospinning with polymers and apatites for tissue-engineering scaffold preparation has been widely studied. These nanofibers and nanocomposites have been reported to enhance cell adhesion and mimic the properties of ECM (Zhang et al. 2006; Liu et al. 2009). However, such fibrous architectures alone are not sufficient for mimicking the complex natural ECM. Surfaces bearing favorable functionalities (hydrophilic, biomolecule-coated, etc.) are generally needed for cell culture studies. Functionalization of nanofibers with various biomolecules like recombinant human bone morphogenetic protein (rhBMP-2), transforming growth factor beta 1 (TGF-β1) and cell adhesion factors have been studied for cell attachment (Kim 2003; Lee 2004; Tamai et al. 2005). However, very few studies have depicted the importance of selection of the mode of functionalization of nanofibers in tissue engineering. In this study, we analyzed the impact of adsorption and coelectrospinning of biofunctional molecules like biotin and galactose on cell attachment and cytotoxicity.

Preparation and Characterization of Native and Cross-Linked Gelatin Nanofibers

Gelatin nanofibers were produced by electrospinning using 10 % (w/v) gelatin biopolymer in glacial acetic acid and characterized using AFM in noncontact mode. The nanofibers formed were bead-free and appeared to be randomly arrayed uniform fibrous nonwovens with average fiber diameters of 220 nm (Fig. 1a). However, when the gelatin nanofibers were cross-linked with GTA, the fiber diameter was reduced to 146 nm. After GTA vapor cross-linking, the membranes became visibly yellowish and slightly shrunken. The color change was due to the establishment of aldimine linkages (CH=N) between the free amine groups of protein and GTA (Harland and Peppas 1989; Pezron et al. 1991). Cross-linking of collagenous materials with GTA involves the reaction of free amino groups of lysine or hydroxylysine amino acid residues of the polypeptide chains with the aldehyde groups of GTA (Olde Damink et al. 1995). The reduction in fiber diameter may be due to this shrinking of fibers as a result of vapor cross-linking. The non-cross-linked gelatin nanofibers dissolved within a few minutes. However, when the nanofibers were cross-linked with GTA for 12 h, all the fibers remained insoluble. The morphology of nanofibers totally differed with coelectrospinning of gelatin with biotin and galactose (Fig. 1b–d). The blending of biotin and coelectrospinning with gelatin resulted in the formation of beads covered by shredded nanofibers (Fig. 1b). The fiber diameter could not be measured since the nanofibers were not uniform in shape and size and only beads covered by tiny shredded nanofibers could be observed. But when the gelatin was coelectrospun with galactose, the nanofibers appeared like bamboo with a slight decrease in diameter at regular intervals (Fig. 1c). The average diameter of the galactose-coelectrospun nanofibers was 256 nm, which was measurable and distinct compared to biotin-coelectrospun nanofibers. When gelatin was coelectrospun with both biotin and galactose, the fiber morphology differed again. The fiber diameter varied from 95 to 290 nm (Fig. 1d). The fiber morphology showed similarities between biotin-coelectrospun (encircled area 1) and galactose-coelectrospun (encircled area 2) nanofibers (Fig. 1d). The average roughness also varied from coelectrospinning. The average roughness is the arithmetic mean of the absolute values of the surface departures from the mean plane. The roughness of the surface was calculated using NT-MDT image analysis software. The overall roughness was found to decrease with coelectrospinning; however, combined coelectrospinning with biotin and galactose increased the roughness considerably when compared to individual coelectrospinning. The average roughness of native gelatin nanofibers was 163.63 nm, whereas for biotin, galactose and combined coelectrospinning it was 81.63, 93.90 and 101.30 nm, respectively.

Adsorption of Biotin and Galactose onto Cross-Linked Gelatin Nanofibers

Adsorption of biotin and galactose onto cross-linked gelatin nanofibers (1 × 1 cm) was carried out at a constant contact time of 8 h at room temperature. The percentage of adsorption was calculated from the residual biotin and galactose solution and estimated using a suitable HPLC method. The biotin was estimated using a reverse-phase C18 column at 204 nm in UV, and galactose was estimated using the Bio-Rad HP column in RI mode with the Agilent HPLC system. The HPLC results clearly indicated a decrease in biotin and galactose concentration after immersing the nanofiber matrix for 8 h. The standard biotin showed two peaks, at a retention time of 3.088 min with a peak area of 407,587,394 mAu and a retention time of 5.098 min with an area of 429,403,070 mAu. After adsorption, the 5.098-min peak was not found. However, at the retention time of 3.083 min, there was a decrease in peak area. One percent of biotin was used as standard, and the residual biotin level in the solution was calculated to be 0.14 %. This indicates that the biotin adsorption was very high; i.e., 85.94 % of the biotin was adsorbed onto the gelatin nanofibers. Similarly, standard galactose showed a maximum peak at a retention time of 9.159 min with a peak area of 27,917,926 mAu. After adsorption, a decrease in peak area was observed at a retention time of 9.152 min. One percent of galactose was used as standard,

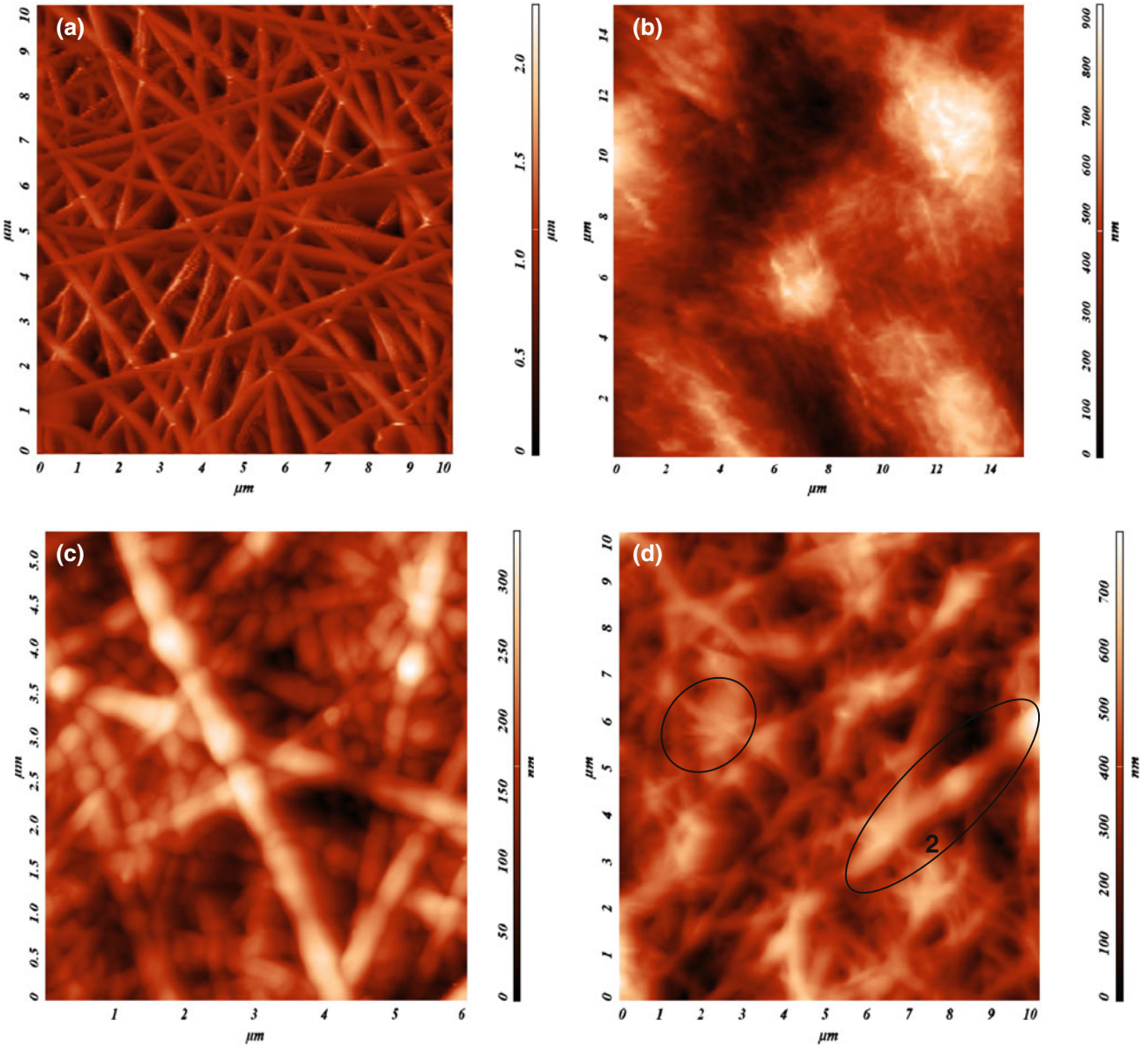

Fig. 1 AFM image of native and coelectrospun gelatin nanofibers. **a** Native gelatin nanofibers. **b** Coelectrospun gelatin nanofibers with 1 % biotin. **c** Coelectrospun gelatin nanofibers with 1 % galactose. **d** Coelectrospun gelatin nanofibers with 1 % biotin and 1 % galactose

and the residual galactose level in the solution was calculated to be 0.099 %. We found 90.04 % of galactose to be adsorbed onto the gelatin nanofibers. The adsorption may be due to the GTA cross-linking of gelatin nanofibers. GTA acts as a bifunctional linker that can functionalize the amine group on the surface, which subsequently binds with biotin (Cooper and Lorenz Meyer 2011). Galactose adsorption may also be due to the GTA cross-linking of gelatin nanofibers. GTA acts as a bifunctional cross-linker through covalent bonding (Altankov et al. 1991), which can influence the binding of galactose. Similar modification of gelatin sponge using galactose has been carried out by Hong et al. (2003).

In Vitro Cell Culture Studies with Functionalized Gelatin Nanofibers

Nanofibrous scaffolds have many advantages that make them well suited for tissue-engineering applications. Besides mimicking the architecture of natural ECM, a higher surface area to volume ratio of nanofibers leads to more cellular attachment in comparison to larger fibers (Ghasemi-Mobarakeh et al. 2008). However, these nanofibrous scaffolds alone are not sufficient to promote cell attachment and proliferation. The favorable functionalities displayed at the surface of nanofibers always play a critical role in cell differentiation, attachment and growth

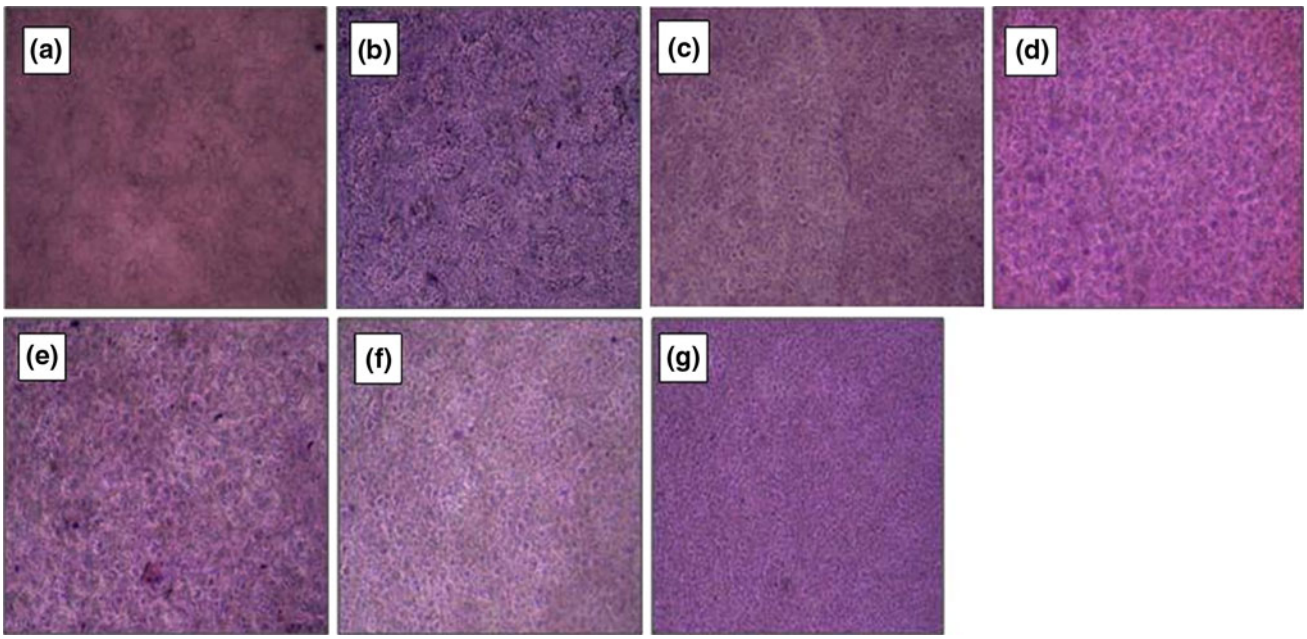

Fig. 2 Cell attachment onto functionalized gelatin nanofibers. **a** Control. **b** One percent biotin coelectrospun gelatin nanofiber. **c** One percent galactose coelectrospun nanofiber. **d** One percent biotin and galactose coelectrospun gelatin nanofiber. **e** Biotin adsorbed gelatin nanofiber. **f** Galactose adsorbed gelatin nanofiber. **g** Biotin and galactose adsorbed gelatin nanofibers. Magnification ×400

along with the surface morphology of the nanofiber. In the present study, HEp-2 cells were used to study the attachment of cells to the biotin and galactose functionalized nanofibers and compared with nonfunctionalized nanofibers. The cell proliferation/attachment and cytotoxicity tests were performed using crystal violet staining and the MTT assay, respectively. HEp-2 cells were cultured in DMEM supplemented with 10 % FCS and incubated along with nanofibers to elucidate the HEp-2 cell attachment property of gelatin nanofibers. In continuous observations, HEp-2 cells were found attached at 88 h. Hence, further studies were conducted by incubating the cells for 88 h in gelatin nanofibers. The control gelatin nanofibers showed poor cell density compared to the other functionalized nanofibers (Fig. 2a). The cell attachment was very prominent in both adsorbed and coelectrospun gelatin nanofibers with higher cell density (Fig. 2b–g). In almost all treatments, cells were dispersed throughout the scaffold except with 1 % biotin-coelectrospun gelatin nanofiber. These cells (Fig. 2b) were grouped as a cluster, mimicking the fiber morphology in the 1 % biotin-coelectrospun nanofiber (Fig. 1b). These results clearly indicate that not only surface functionalization but also coelectrospinning creates a favorable environment for cell attachment, differentiation and proliferation. Crystal violet staining clearly indicated uptake of crystal violet by the cells adhered to the matrix after washing. Fluorescence microscopy clearly indicated the presence of cells attached to the nanofibers, similar to the light microscopic

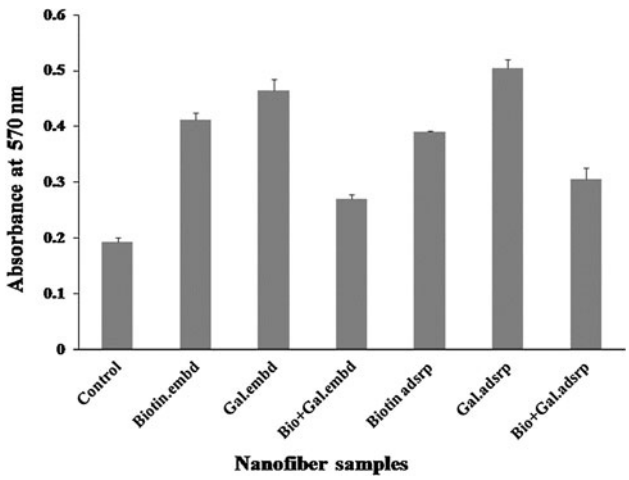

Fig. 3 Cell attachment studies using crystal violet staining

images. The fluorescence microscopic images also indicated uniform distribution of biotin and galactose (figure not shown). Maximal attachment was observed in galactose-adsorbed nanofibers, which was followed by biotin-embedded, galactose-embedded and biotin-adsorbed nanofibers (Fig. 3).

Our results correlate with earlier reports (Hong et al. 2003; Park 2002). Biotin is necessary for cell growth, production of fatty acids and metabolism of fats and amino acids. It plays a role in the citric acid cycle, which is the process by which biochemical energy is generated during aerobic respiration. Biotin not only assists in various metabolic reactions but also helps to transfer carbon

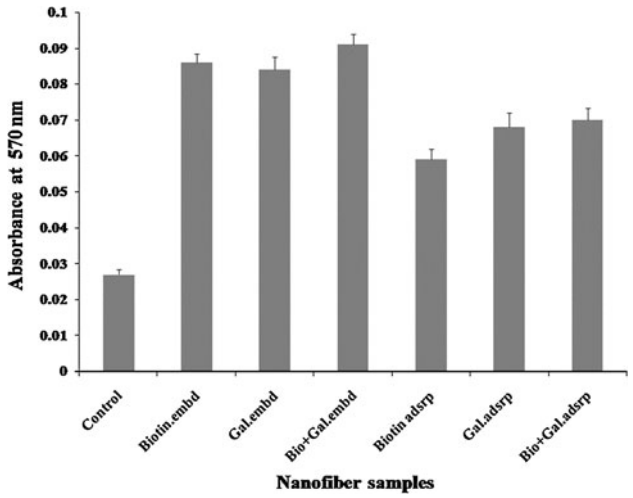

Fig. 4 Cell viability test using MTT assay

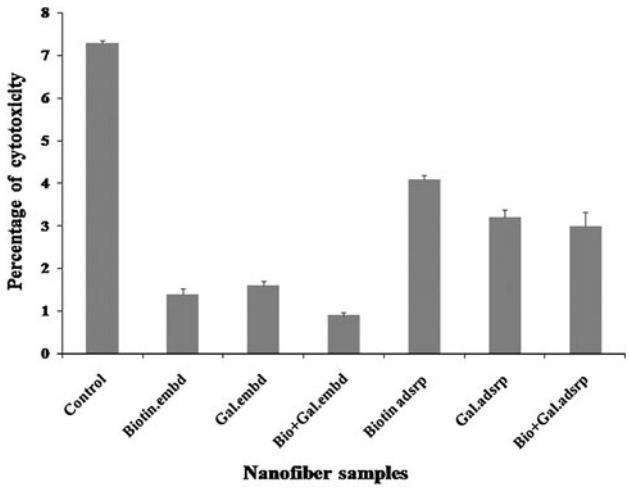

Fig. 5 Cytotoxicity of control and functionalized nanofibers

dioxide. Galactose is recognized by mammalian hepatocytes through the asialoglycoprotein receptor, leading to regulation of a degradative pathway in glycoprotein homeostasis (Chung and Park 2007). Galactose is also necessary in the upkeep of proper cell-to-cell communication. Porous scaffolds immobilized with galactose showed very good improvement in hepatocyte attachment, viability and metabolic functions. Gelatin sponges modified with galactose were shown to support hepatocyte adhesion and function such as release of lactate dehydrogenase, albumin secretion and urea synthesis. Perfusion culture of hepatocytes with galactose-derivatized PLGA scaffolds further improved the viability and functional activity of cells (Hong et al. 2003). Hong et al. (2003) modified the gelatin sponge using galactose residues, which was reported to significantly increase the attachment of hepatocytes on the substrate. Hence, our study clearly indicates that the coelectrospinning or adsorption of biotin and galactose onto gelatin nanofibers enhances cell attachment and proliferation throughout the scaffold.

The MTT assay was used to compare the number of cells in the nanofiber scaffold and tissue culture plate. The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells (Ghasemi-Mobarakeh et al. 2008). Figure 4 shows the viability graph of HEp-2 cells in various gelatin nanofibers prepared in this study. The viability of the cells was highest in biotin- and galactose-embedded nanofiber compared to other treatments. The embedding technique proved to be efficient compared to the adsorption technique in the case of cell viability. The control nanofiber was found to support lower cell adhesion compared to both embedding and adsorption techniques. The cytotoxicity of the nanofibers toward HEp-2 cells was also assayed using the MTT assay with suitable controls. The percentage of cytotoxicity was less with biotin- and galactose-embedded and adsorbed nanofibers compared to control nanofibers. Cytotoxicity was even less with embedded samples than the adsorbed sample (Fig. 5). This cytotoxicity of control nanofibers may be attributed to the presence of residual level of GTA on the gelatin, as mentioned by Ulubayram et al. (2012). They reported that the cross-linking agents are responsible for such cytotoxicity and vary from one cross-linking agent to the other. Kim and Kwon (2007) evaluated the cytotoxicity of cross-linked gelatin membranes and reported that such cross-linked matrix supported the proliferation of human dermal fibroblasts.

Conclusion

From this study we conclude that cross-linked gelatin nanofibers could be an ideal candidate for functionalization. The coelectrospinning and adsorption of biotin and galactose separately as well as jointly improves cell attachment and proliferation. The cytotoxicity, viability and attachment studies clearly indicate that biotin-/galactose-adsorbed/coelectrospun gelatin nanofibers could be ideal candidates for scaffold development and tissue-engineering applications.

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