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Coating of Polyelectrolyte Multilayer Thin Films on Nanofibrous Scaffolds to Improve Cell Adhesion

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ABSTRACT: The adhesion of L929 cells to poly(ε-caprolactone) (PCL) nanofibers was successfully improved via coating with polyelectrolyte multilayer thin films (PEMs), which enhanced the potential of this material as a scaffold in tissue engineering applications. With the electrostatic self-assembly technique, poly(diallyldimethylammonium chloride) (PDADMAC) and poly(sodium 4-styrene sulfonate) (PSS) were formed as four-bilayer PEMs on electrospun PCL nanofiber mats. Because PDADMAC and PSS are strong polyelectrolytes, they provided stable films with good adhesion on the fibers within a wide pH range suitable for the subsequent processes and conditions. PDADMAC and gelatin were also constructed as four-bilayer PEMs on top of the PDADMAC- and PSS-coated nanofibers with the expectation that the gelatin would improve the cell adhesion. L929 cells from mouse fibroblasts were then seeded on both uncoated and coated scaffolds to study the cytocompatibility and *in vitro* cell behavior. It was revealed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that both the uncoated and coated nanofiber mats were nontoxic as the cell viability was comparable to that of those cultured in the serum-free medium that was used as a control. The MTT assay also demonstrated that cells proliferated more efficiently on the coated nanofibers than those on the uncoated ones during the 48-h culture period. As observed by scanning electron microscopy, the cells spread well on the coated nanofibers, especially when gelatin was incorporated. The surface modification of PCL nanofiber mats described in this research is therefore an effective technique for improving cell adhesion. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 1574–1579, 2009

Key words: biocompatibility; fibers; polyelectrolytes; self-assembly; thin films

INTRODUCTION

Tissue engineering, an emerging field in the area of human heath care, has attracted growing interest in the last few decades. One of the challenges in the development of tissue engineering applications is the need to preserve a cell's ability to grow on synthetic scaffolds and maintain tissue-specific function; both depend critically on factors such as cell/scaffold and cell/cell interactions.[1] The key factors involved during the *in vivo* growth of tissue formation and maturation are the viability, proliferation, and spreading of cells. To improve each of these parameters, increasing efforts have been made to develop new coatings to improve the biocompatibility of a given surface.[2] Surface modification using polyelectrolyte multilayer thin films (PEMs) to develop biocompatible materials has attracted attention lately because of several advantages.[3] This technique is very simple because it relies only on a dipping process, and it can be applied to surfaces with a very complex morphology and variable chemistry. The assembly occurs most of the time in aqueous media and does not require the use of organic solvents, which can be a problem in further cytocompatibility studies. Finally, biopolymers, oligomers, and other proteins, which are key components of biocompatible surfaces, can be readily used because of their electrostatic nature. The stability of the coating is important especially when one tries to achieve surface modification before cell adhesion. A loss of electrostatic interaction due to the neutralization of polyelectrolytes in PEMs can lead to decomposition or excessive swelling of the coating, as previously

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reported.[4] In this article, we suggest that PEMs assembled from a less pH-sensitive polyelectrolyte such as poly(diallyldimethylammonium chloride) (PDADMAC) could lead to more stable films. The non-pH dependence of PDADMAC provides fully charged polyelectrolytes in a pH range of 1–14.[5] Although enhanced stability is of interest, the overall cell interaction with the film should be as good in the case of PDADMAC and gelatin.[6,7]

Meanwhile, several researches have indicated that electrospun nanofibers are very promising as cell scaffolding materials because nanofibrous scaffolds provide a high level of surface area to which cells can attach on account of their three-dimensional feature and high surface area to volume ratio.[8] Among the various types of nanofibrous scaffolds, poly(ε-caprolactone) (PCL) is one of the most interesting materials because of its biocompatibility, low cost, easy processability, and slow hydrolytic degradation rate. It has been reported that PCL is a good substrate for promoting cell attachment and proliferation.[9,10] Its application as a scaffold in cell transplantation is of great interest.

To explore the possibility of using PCL nanofibers as nanofibrous scaffolds for tissue engineering, this work was aimed at improving cell adhesion on PCL nanofibers by coating them with PEMs from PDADMAC, poly(sodium 4-styrene sulfonate) (PSS), and gelatin. L929 cells were used to study the cytocompatibility of the nanofibers and the PEMs. The effects of PEMs on L929 cell behavior in terms of cell attachment and proliferation were also investigated.

EXPERIMENTAL

Electrospinning of PCL nanofibers

It is commonly known that the concentration of a polymer solution or the corresponding viscosity is one of the most important variables in controlling fiber morphology[11] as well as other electrospinning parameters, including the applied voltage, the distance between the needle tip and collector, the solution flow rate, the syringe and needle configuration, and the rotational speed. In this study, the process parameters were selected from preliminary work done in the laboratory and in a previous study[10] that identified a 10.5% (w/v) PCL solution (Sigma, St. Louis, MO) in 1 : 3 (v/v) methanol/chloroform (Labscan, Bangkok, Thailand), a distance of 20 cm from the needle tip, and an applied voltage of 13 kV as the acceptable spinning parameters to obtain continuous and bead-free PCL fibers. The morphology of the obtained nanofiber mats was observed with a Phillips (Eindhoven, The Netherlands) XL-30CP scanning electron microscope.

PEM formation by the electrostatic self-assembly technique

PDADMAC, PSS, and gelatin (type B from bovine skin) were purchased from Sigma–Aldrich (St. Louis, MO). The uncoated and coated nanofiber mats were manually immersed alternately at pH 7 in a 10 m*M* PDADMAC solution as a positively charged polyelectrolyte for 2 min and in a 10 m*M* PSS solution as a negatively charged polyelectrolyte for 2 min. After each was dipped in the polyelectrolyte, the nanofiber mats were rinsed for 20 s three times in deionized water to remove excess polyelectrolyte. The dipping was repeated until four bilayers of PEMs (a total of eight layers of polyelectrolytes) had been obtained, and these were labeled PDADMAC/PSS. Our preliminary study indicated that PDADMAC/PSS was stable within a wide range of pHs because both are strong polyelectrolytes. With the same procedure, four-bilayer PEMs were constructed from 10 m*M* PDADMAC and gelatin at pH 9 on top of the previously coated nanofibers, and these were labeled PDADMAC/PSS+PDADMAC/gelatin. It was expected that when gelatin was incorporated, the cell adhesion would be enhanced. Our preliminary study also pointed out that, although PDADMAC/gelatin was not as stable as PDADMAC/PSS because gelatin is a weaker electrolyte, the primer coating with PDADMAC/PSS provided film stability suitable for subsequent processes and conditions.

Cell culture

L929 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT) supplemented by 10% fetal bovine serum (FBS; Gibco, Invitrogen, Grand Island, NY), 1% L-glutamine (Invitrogen), and a 1% antibiotic and antimycotic formulation containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen), and they were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until the cells reached 80% confluence.

Cytocompatibility test

Extraction media were prepared by the immersion of samples cut from the uncoated and coated fiber mats into a serum-free medium (SFM) containing the same composition as DMEM but without FBS for 24 h. L929 cells were cultured in DMEM for 16 h and then in SFM for 24 h. The cells were then reincubated for 24 h in the extraction media. The viability of the cells cultured with fresh SFM (as a control) and with extraction media was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay to evaluate the cytocompatibility.

Cell viability by the MTT assay

For the MTT assay, the culture medium was aspirated and replaced with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide obtained from Sigma–Aldrich. After that, the plate was incubated for 30 h at 37°C. The yellow tetrazolium salt was reduced to purple formazan crystals by the dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The solution was then aspirated, and dimethyl sulfoxide (DMSO; 900 μL/well) containing a glycine buffer (125 μL/well) was added to dissolve the formazan crystals. After 10 min of rotary agitation, the absorbance of the DMSO solution at 540 nm was measured with a Thermospectronic (Rochester, NY) Genesis 10 ultraviolet–visible spectrophotometer to determine the amount of purple formazan crystals formed, which was proportional to the number of viable cells.

Cell adhesion and proliferation assessments

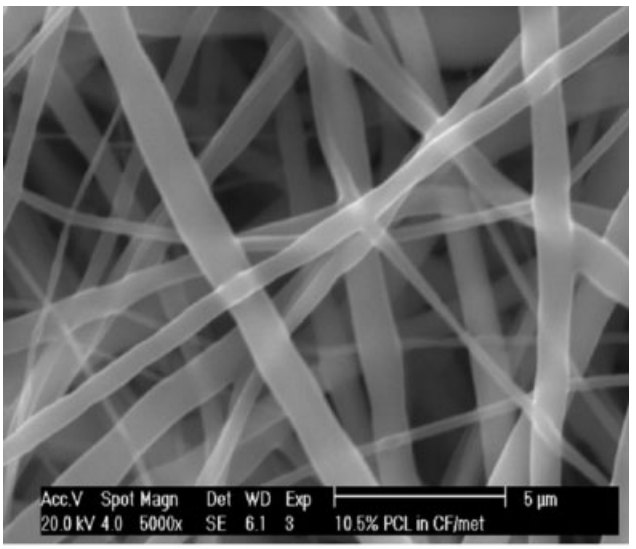
The uncoated and coated nanofiber mats were cut into circular specimens 15 mm in diameter. The specimens were placed in wells of a 24-well tissue-culture polystyrene plate (TCPS; Nunc, Rockford, IL), which were later sterilized in 70% ethanol for 10 min. The specimens were then rinsed with autoclaved deionized water and subsequently air-dried. L929 cells were seeded in the wells containing the uncoated and coated nanofiber specimens as well as the empty wells of TCPS, which were used as controls, at an initial density of 5×10^4 cells per well. After seeding for 4, 8, 24, and 48 h, each specimen was rinsed with phosphate-buffered saline (PBS; Sigma–Aldrich) to remove unattached cells before the MTT assay. Cell attachment was evaluated at 4–8 h, whereas cell proliferation was evaluated at 24 and 48 h.

Morphological analysis by scanning electron microscopy (SEM)

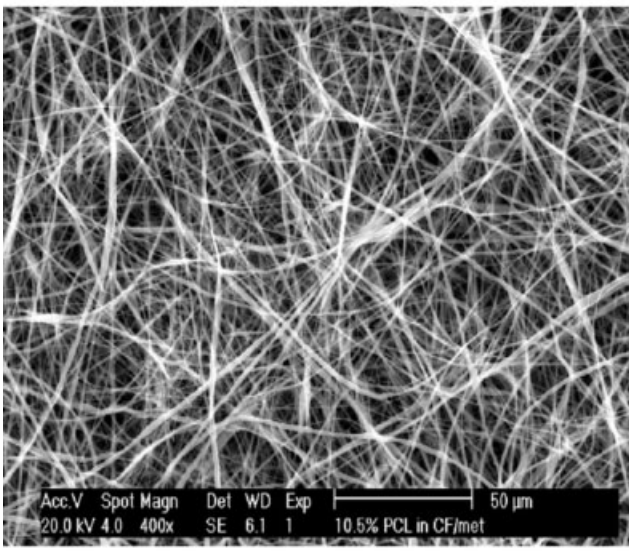
The morphological appearance of the cells during the attachment and proliferation periods was observed with a JEOL (Tokyo, Japan) JSM-6400 scanning electron microscope. The cell-seeded scaffolds were rinsed twice with PBS and fixed with 3% glutaraldehyde in 1% calcium chloride for 30 min at 5°C. After being washed in PBS, the scaffolds were dehydrated consecutively in 30, 50, 70, 90, and 100% ethanol for 2 min for each concentration. Furthermore, substitution with hexamethyl disiloxane (Sigma–Aldrich) was done. Samples were then critically point-dried and covered with a thin layer of gold–palladium through sputtering under an argon atmosphere before SEM observation.

RESULTS AND DISCUSSION

SEM micrographs of bead-free PCL nanofibers are shown in Figure 1. The three-dimensional fibrous mesh consisted of fibers with diameters ranging from 400 nm to 2 μm. Most of the fiber diameters were less than 1 μm, and the average diameter was 507 ± 251 nm. In addition to the broad fiber diameter distribution, the fibers often had a nonuniform



(a)



(b)

Figure 1 SEM micrographs of PCL nanofibrous scaffolds: (a) 5000× (scale bar = 5 μm) and (b) 400× (scale bar = 50 μm).

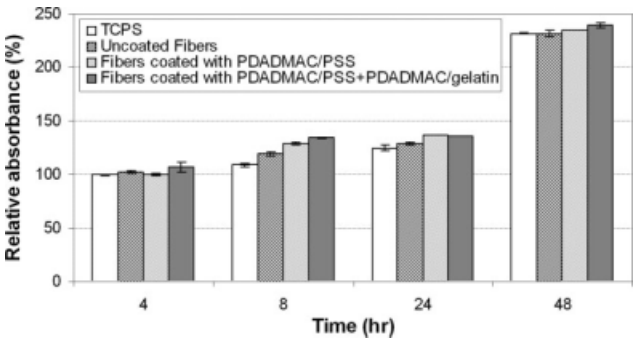


Figure 2 Viability of the L929 cell culture on fibrous scaffolds at 4, 8, 24, and 48 h as indirectly quantified by relative absorbance.

diameter; that is, the diameter varied along an individual fiber. The fibers were randomly oriented, and interconnected voids were presented.

The cytocompatibility evaluation, based on the viability of L929 cells as determined indirectly by the MTT assay, indicated that, when the relative absorbance in the case of L929 cells cultured with fresh SFM for 24 h was normalized at 100%, the relative absorbance in the case of L929 cells cultured for the same period of time in the extraction media from the uncoated nanofibers, the nanofibers coated with PDADMAC/PSS, and the nanofibers coated with PDADMAC/PSS+PDADMAC/gelatin was comparable at 103.5, 110.2, and 108.8%, respectively. The absorbance values implied the cytocompatibility of the uncoated and coated nanofiber mats with L929 cells.

The attachment and proliferation of L929 cells on the uncoated and coated nanofiber mats were signified by the viability of the attached L929 cells at 4, 8, 24, and 48 h after seeding on the uncoated and coated scaffolds and on TCPS as the control. The number of viable cells for each sample was quantified by its relative absorbance, as shown in Figure 2. During the first 24 h, the attachment of L929 cells on nanofiber mats was comparable to that on TCPS. The proliferation of L929 cells could be assessed from the absorbance values after the cells were cultured for 24 and 48 h. At 24 h after seeding, the viability of attached L929 cells on the nanofiber mats

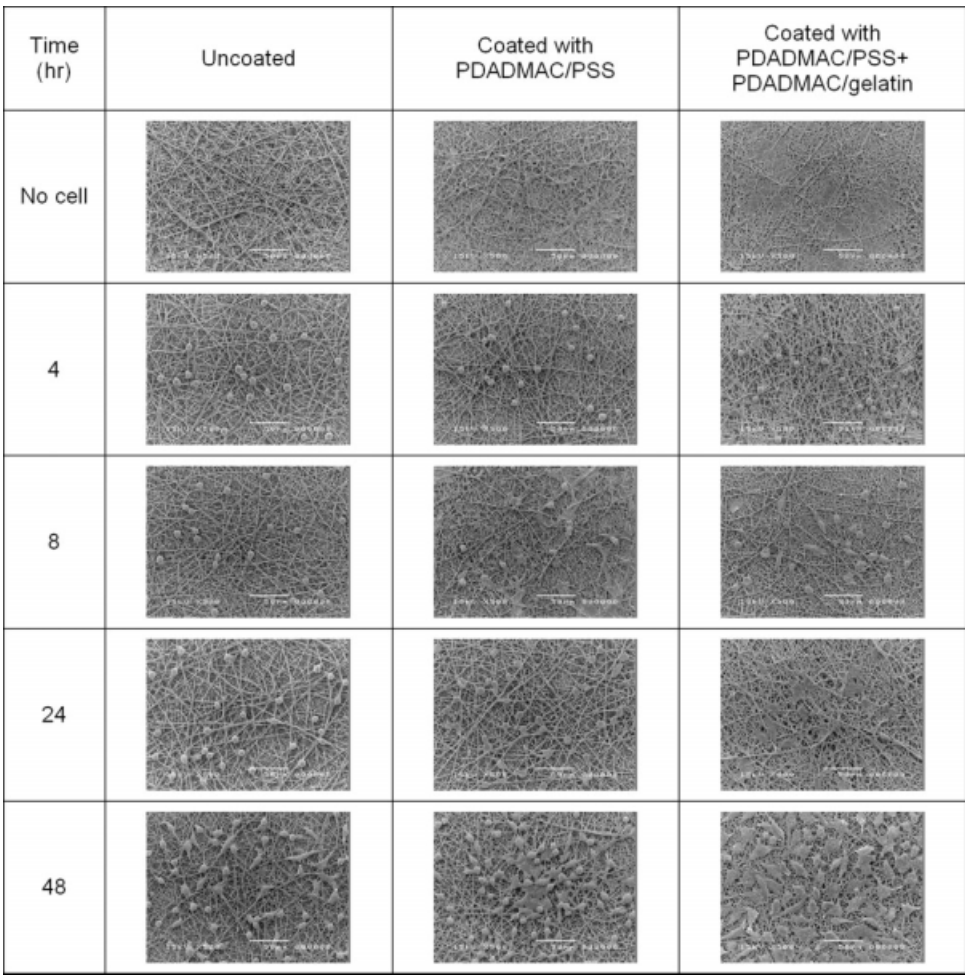


Figure 3 SEM micrographs of the L929 cells on nanofibrous scaffolds after culture periods of 4, 8, 24, and 48 h at 500× (scale bar = 50 μm).

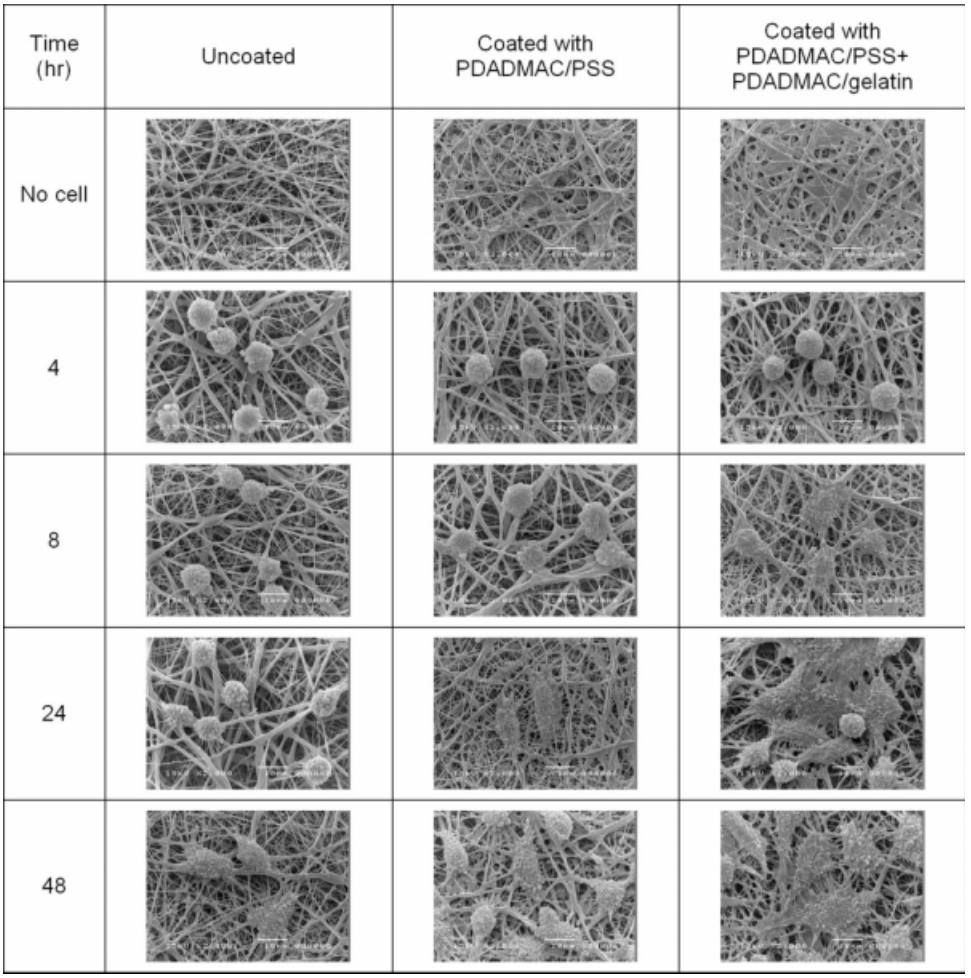


Figure 4 SEM micrographs of the L929 cells on nanofibrous scaffolds after culture periods of 4, 8, 24, and 48 h at 2000× (scale bar = 10 μm).

was comparable to that on TCPS. At 48 h, the number of viable attached cells on TCPS and all the nanofiber mats increased significantly. It was obvious that the viability of the attached cells on the coated nanofiber mats was greater than that on TCPS and the uncoated one, especially for the nanofibers coated with PDADMAC/PSS+PDADMAC/gelatin. This noticeable increase in cell proliferation when gelatin was incorporated into the PEMs may be explained by the presence of basic, negatively charged lysine and arginine residues in the denatured collagen molecule, which promoted electrostatic interactions with the cell surface, and the presence of specific cell adhesion sites such as RGD sequences. Moreover, fibroblast binding to gelatin-modified PCL fibers was anticipated directly via the prominent $\alpha_1\beta_1$ and $\alpha_2\beta_2$ integrin receptors.[12]

It can be observed from the SEM micrographs shown in Figures 3 that the area on the nanofiber mats that the cells occupied increased with increasing culture time. In Figure 3, the SEM micrographs at a low magnification of 500× show the morphologies of cells that were cultured on the uncoated nanofibers, the nanofibers coated with PDADMAC/PSS, and the nanofibers coated with PDADMAC/PSS+PDADMAC/gelatin for different culture periods. After the first 8 h, some of the cells were spread and flattened, but many were still round, and this indicated an absence of strong cell–fiber interaction at this time point. After 48 h, the adherence of cells to the nanofibers coated with PDADMAC/PSS+PDADMAC/gelatin was densely distributed in comparison with that of the uncoated nanofibers.

SEM micrographs at a higher magnification of 2000× are shown in Figure 4. Clearly, during the first 4 h of cell culture, the morphology of the cells was mostly round with a slight trace of filopodia. At a longer time, the cells on coated nanofiber mats, especially those coated with PDADMAC/PSS+PDADMAC/gelatin, expanded more, with evidence of the anchoring ligands reaching out to help support them on the fiber surface.

The high porosity of nanofibrous scaffolds provides more structural space for cell accommodation

and makes the exchange of nutrient and metabolic waste between the scaffold and environment more efficient. The reason that cells enter the matrix through small pores may be that the pores in an electrospun structure are formed by differently oriented fibers lying loosely on one another.[11] When cells perform an amoeboid movement to migrate through the pores, they can push the surrounding fibers aside to expand the holes as the small fibers offer little resistance to cell movement. These characteristics are fundamental criteria for successful tissue engineering scaffolds. According to the results, cells seeded on the nanofibrous scaffolds had an appropriate interaction with their environment on the basis of the following observations. First, the cells maintained a normal phenotypic shape, and this suggested that the cells functioned biologically within this structure. Second, the cells favored this structure, so they attached to the fibers and proliferated in the nanofibrous network. Third, these cells crosslinked the nanofibers and integrated with the surrounding fibers to form a three-dimensional cellular network.

From this evidence, it can be concluded that cell adhesion on PCL nanofibrous scaffolds can be improved via coating with PDADMAC/PSS as the primer to provide film stability and via further coating with PDADMAC/gelatin as a top coating to promote cell attachment, cell proliferation, and proper cell spreading.

CONCLUSIONS

Bead-free PCL nanofibers were successfully prepared by electrospinning. With the electrostatic self-assembly technique, the as-spun nanofiber mats were coated with four bilayers of PEMs constructed from PDADMAC and PSS (denoted PDADMAC/PSS) and with four bilayers of PEMs constructed from PDADMAC/gelatin on top of four bilayers of PEMs constructed from PDADMAC and PSS (denoted PDADMAC/PSS+PDADMAC/gelatin). These uncoated and coated nanofiber mats exhibited cytocompatibility with L929 cells. Various cell behaviors revealed by the MTT assay and SEM—cell attachment, proliferation, spreading, and morphology—demonstrated an outstanding improvement of cell adhesion on the coated nanofiber mats over the uncoated ones, especially when the nanofibers were coated with PDADMAC/PSS+PDADMAC/gelatin. These results suggest that surface modification with PEMs is an effective technique for increasing the potential for using electrospun fiber mats as nanofibrous scaffolds for tissue engineering.

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