J Mater Sci: Mater Med (2008) 19:1479–1484
DOI 10.1007/s10856-007-3299-8

Biofunctionalized poly(ethylene glycol)-*block*-poly(ε-caprolactone) nanofibers for tissue engineering

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Received: 29 June 2007 / Accepted: 2 October 2007 / Published online: 8 November 2007

Abstract Electrospun fibers with contrasting cell adhesion properties provided non-woven substrates with enhanced in vitro acceptance and controllable cell interactions. Poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL) block copolymers with varying segment lengths were synthesized in two steps and characterized by NMR and GPC. A cell adhesive peptide sequence, GRGDS, was covalently coupled to the PEG segment of the copolymer in an additional step. The suitability of polymers with molecular weights ranging from 10 to 30 kDa for electrospinning and the influences of molecular weight, solvent, and concentration on the resulting morphologies were investigated. Generally, electrospun fibers were obtained by electrospinning polymers with molecular weight larger than 25 kDa and concentrations of 10 wt%. Methanol/chloroform (25/75, v/v) mixtures proved to be good solvent systems for electrospinning the PEG-*b*-PCL and resulted in hydrophilic, non-woven fiber meshes (contact angle 30°). The mesh without cell adhesive GRGDS ligands showed no attachment of human dermal fibroblasts after 24 h cell culture demonstrating that the particular combination of the material and electrospinnig conditions yielded protein and cell repellent properties. The GRGDS immobilized mesh showed excellent cellular attachment with fibroblasts viable after 24 h with spread morphology. Electrospun nanofibers based on block copolymers have been produced which are capable of specifically targeting cell receptor binding and are a promising material for tissue engineering and controlling cell material interactions.

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1 Introduction

Non-woven fiber meshes of microfibers—and more recently nanofibers—are investigated as tissue engineering scaffolds, with seeding and in vitro cultivation of cells prior to implantation. Such non-woven meshes are an important type of scaffold developed, aimed to provide a tissue analog for a wide range of tissue types, including bone, cartilage, vascular grafts and subcutaneous implants. [1]. A significant bioresorbable material used in biomedical applications are the polyesters of the poly(α-hydroxy acids) family [2]. Such polyester may have a range of degradation characteristics. One of the polyesters is poly(ε-caprolactone) (PCL) and is often used in tissue engineering where slow degradation is favorable. As PCL will readily adsorb proteins, the reduction of non-specific protein adsorption helps control the biomaterial tissue interaction.

Such non-fouling properties can be supplemented with the enhanced adsorption of specific proteins, or immobilization of cell recognition motives to control the interaction between cells and synthetic substrates [3]. A range of materials can be rendered resistant to non-specific protein adsorption by the surface modification or grafting of PEG to the substrate. While non-specific protein adsorption is an important property of biomaterials, functionalization of an intrinsically protein repellent material to control the cell scaffold interaction has been demonstrated by attaching

natural proteins and small peptides such as RGD. Consequently, the development of functionalized polymers that can elicit specific biological responses is increasing in tissue engineering.

Electrospun nanofibers have attracted interest in biomedical applications. As the literature on electrospinning is expansive and diverse, detailed information on the electrospinning process can be found elsewhere [4–11]. Electrospun fibers offer a means to construct nanometer scale dimensions there has been a surge in the use of electrospinning techniques to create nanofibre scaffolds as substrates from a wide range of different polymers. Electrospun fibers mimic the size scale of fibrous proteins (fibrils) found in extracellular matrices and the three-dimensional nature of the matrix allows for cells to infiltrate the matrix and proliferate [12, 13]. Where a cell may adhere to only one microfiber in a typical biomedical textile, one cell can adhere to multiple electrospun fibers. Hence, there has been a surge in the use of electrospinning techniques to create nanofiber scaffolds as substrates, from a wide range of polymers, for tissue engineering [14]. Besides the small fiber diameter, nanofiber scaffolds offer high surface to volume ratio and the potential for high porosity and interconnected pores. Functionalization with biologically active ligands as well as minimization of unspecific protein adsorption remains challenging tasks for the preparation of electrospun fiber constructs.

Here we report on electrospinning of poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL) copolymers, which enable the immobilization of biologically active sequences (e.g., peptides, proteins and oligosaccharides) in order to produce scaffolds with improved specific cellular interactions. Deprotection of a synthesized *a*-acetal-PEG-*b*-PCL provides the respective functionality for immobilization of a bioactive molecule. Figure 1 shows a schematic illustration of the interaction between functionalized and non-functionalized electrospun fibers and a cell. The covalently attached molecule should be available on the fiber surface while protein resistance is maintained. Combined non-fouling properties and immobilization of bioactive motifs should lead to controlled interactions between the nanofiber surface and cells. We attempt to make electrospun fibers with properties that exhibit diverse cellular interactions—from non- to strong cellular adhesion—with the introduction of a single peptide.

2 Materials and methods

2.1 Materials

Ethylene oxide, 3,3-diethoxypropanol, ε-caprolactone, toluene, methanol, chloroform and diethyl ether were all purchased from Sigma–Aldrich (Germany) while GRGDS was supplied by Bachem (Switzerland). Ethylene oxide, 3,3-diethoxypropanol, ε-caprolactone, and toluene were dried by distillation under nitrogen atmosphere.

2.2 Chemical and morphological characterization

The ^{1}H-NMR and ^{13}C-NMR spectra of polymer samples were measured at 25 °C in $CDCl_3$, containing TMS as internal standard, with an Inova 300 spectrometer (300 MHz, Varian Associates Nuclear Magnetic Resonance Instruments, Palo Alto, USA). Gel permeation chromatography (GPC) measurements were performed at 35 °C using a high-pressure liquid chromatography pump (ERC HPLC 64200) and a refractive index detector (ERC-7215a). The eluting solvent was THF (HPLC grade) with 250 mg/mL 2,6-di-tert-butyl-4-methylphenol and a flow rate of 1 mL/min and five columns with MZ gel were used. The length of the first column was 50 mm, 300 mm for the other four columns. The diameter of each column was 8 mm, the diameter of the gel particles 5 mm, and the nominal pore widths were 50, 100, 1,000 and 10,000 Å, respectively. Calibration was achieved using poly(methyl methacrylate) (PMMA) standards. Infrared spectra were recorded using a Thermo Nicolet Nexus 670 FT-IR spectrometer in transmission mode with a spectral resolution of 4 cm^{-1}. The samples were run as KBr pellets. Contact angle was determined by sessile drop measurements with a goniometer microscope G40 (Krüss, Hamburg, Germany), using electrospun meshes as the substrate and is an average

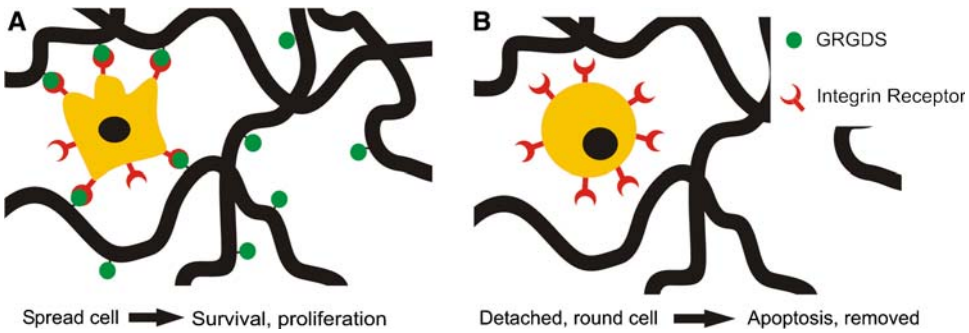

Fig. 1 Schematic illustration of the interactions between nanofibers and cells. Immobilized active molecules lead to cell adhesion to the nanofiber and cell survival (**a**), while cells remain detached on non-functionalized fibers, without immobilized adhesion promoting peptide (**b**)

of 20 measurements. Electrospun fibers were imaged with SEM (Cambridge S360, Leica) using an accelerating voltage of 15 kV, while microscope images were taken with a Zeiss Stemi 2000-C (Zeiss, Oberkochen, Germany).

2.3 Polymer synthesis and chemical functionalization

PEG-*b*-PCL copolymers were synthesized in a two-step synthesis (see Fig. 2 for polymer structure) and the peptide was linked to the PEG-terminus in an additional step. Briefly, first the α-acetal-poly(ethylene glycol) was synthesized starting from 3,3-diethoxypropanol, potassium napthalide and ethylene oxide inside a UniLab Glovebox (Braun, Germany). For a detailed description of the macroinitiator synthesis see Jule et al. [15]. The PCL block was synthesized using the α-acetal-PEG-macroinitiator, pre-dried in an azeotropic distillation, and purified ε-caprolactone with catalytic amounts of tin 2-octoate at 145 °C. The PEG-*b*-PCL was dissolved in DCM and then precipitated into a 50-fold excess of ice-cooled diethyl ether. Successful polymerization was confirmed by ^{1}H-, ^{13}C-NMR and GPC.

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.20 (*t*, *J* = 7.00 Hz, 6H, 3x H-1, H-1′), 1.39 (*m*, H-11), 1.58–1.72 (*kB*, H-10, H-12), 2.31 (*t*, *J* = 7.42, H-9), 3.64 (*s*, H-6), 4.06 (*t*, *J* = 6.68 Hz, H-13), 4.15 (*t*, 1H, H-7) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 24.57 (C-10), 25.47 (C-11), 28.34 (C-12), 34.10 (C-9), 64.13 (C-13), 70.57 (C-6), 173.53 (C-8) ppm.

The α-acetal-group was deprotected by dissolving 500 mg polymer in 10 mL acetonitrile, 1 mL distilled water were added and pH 2 was adjusted by adding 400 µL glacial acid. The solution was stirred for 3 h at room temperature and neutralized to pH 5 by dropwise addition of 0.1 M NaOH. The deprotection of the acetal-group was confirmed by ^{1}H-NMR. A signal at δ = 9.81 ppm indicates the free aldehyde. One mole equivalent GRGDS was added and stirred overnight at room temperature. Sodium cyanoborohydride ($NaBH_3CN$) was added in order to reduce the Schiff base into stable secondary amine. The solution was freeze-dried, the polymer taken up in dichloromethane and reprecipitated in an excess diethyl ether. The product was vacuum-dried and used for electrospinning. The product was characterized by ^{1}H-NMR- and infrared spectroscopy. The disappearing of the aldehyde proton in the ^{1}H-NMR as well as the observation of the characteristic signals of amide bands at 1,656 and 1,565 cm^{-1} in the infrared spectra indicate the immobilized GRGDS.

2.4 Electrospinning

For electrospinning experiments 2 and 10 wt% solutions of the polymers were prepared in a mixture of chloroform and methanol (75/25, v/v). The polymer solution was pumped to the 18-gauge, flat-tipped, stainless steel spinneret at a rate of 0.15 mL/h connected to a voltage source of 25 kV. The fibers were collected on a grounded aluminum cylinder (diameter 80 mm, length 25 mm), rotating at 200 rpm, at a 200 mm distance from the tip of the spinneret [16, 17].

2.5 Cell culture

In vitro experiments were performed with human dermal fibroblasts (hdF) (patient: female, 38 years, passage 8). After isolation, the cells were cultured in a basal medium consisting of Dulbecco's modified eagle serum (DMEM) supplemented with 1% Fetal bovine serum (FBS, Bio Whittaker, Verviers, Belgium) at 37 °C, 5% CO_2 and 95% humidity. Samples were fixed to glass slides (diameter 10 mm) with a biocompatible adhesive, brought into tissue culture vials and pre-incubated overnight in DMEM. The medium was carefully removed and the samples were incubated with 500 µL cell suspension (60,000 cells/mL, hdF) for 24 h at 37 °C. The cell morphology was visualized by haemalum staining whereas cell viability was measured with Live/Dead® staining kits (Invitrogen, Germany).

3 Results and discussion

This study aims to produce non-woven electrospun scaffolds which can be tailored to specifically interact with cells. The first stage towards electrospinning functional nanofibers is the successful synthesis of a block copolymer, consisting of a hydrophilic (PEG) and a bioresorbable hydrophobic segment (PCL). Relatively large hydrophilic segments (10–13 kDa PEG) were combined with the bioresorbable PCL segments.

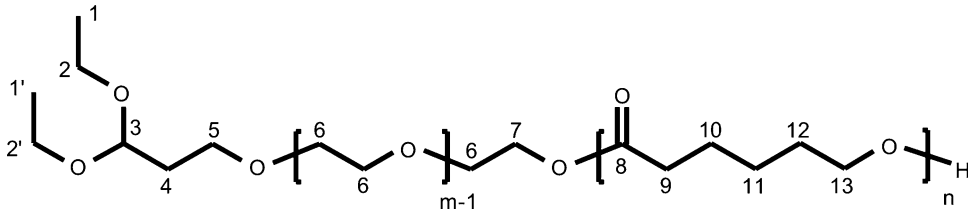

Fig. 2 Structure of α-acetal-PEG-*b*-PCL

Table 1 PEG-*b*-PCL copolymers characterized by NMR and GPC

[a] Molecular weight M_n was calculated from the ^{1}H-NMR

[b] M_n was determined by GPC with THF as eluent and a PMMA standard

Polymer	M_n (PEG)/kDa[a]	M_n (block copolymer)/kDa[a]	M_n (block copolymer)/kDa[b]	M_w/M_n[b]	Yield/%
1	10	13	10.4	1.37	88
2	10	26	19.8	1.66	86
3	13	22	13.3	1.31	89
4	13	24	15.9	1.32	88
5	13	27.5	24.5	1.72	91

First, an α-acetal protected diblock copolymer, based on PCL and PEG was produced in a two-step synthesis. Using 3,3-diethoxypropanol as an initiator, two different α-acetal-PEG macroinitiators, with molecular weights of 10 and 13 kDa, respectively, were prepared by anionic ring-opening polymerization. In order to produce uniform length polymers, the α-acetal-PEG macroinitiators had polydispersity indices (PMI) below 1.1. Five different α-acetal-PEG-*b*-PCL copolymers were synthesized with good yields (≥86%) using tin 2-octoate as catalyst (Table 1). Molecular weights were analyzed by GPC with THF as eluent and narrow molecular weight samples of PMMA as standard. In addition molecular weights were calculated from the end-group analysis by NMR. The resulting PEG-*b*-PCL had molecular weights ranging from 13 to 28 kDa and with relatively narrow molecular weight distributions (M_w/M_n below 1.72). The free aldehyde of the PEG-*b*-PCL was reacted with the amino-group of GRGDS in order to obtain α-functionalized PEG-*b*-PCL copolymers. The Schiff base was reduced to a stable secondary amine and after purification a colorless powder was obtained that could be used for electrospinning of functionalized nanofibers. The successful deprotection of the α-acetal at acidic conditions and the immobilization of the peptide sequence GRGDS was confirmed by ^{1}H-NMR and infrared spectroscopy.

All the five copolymers of PEG-*b*-PCL shown in Table 1 were electrospun or electrosprayed and the molecular weight investigated for fiber/particle formation. Figure 3 shows fibers of a 10 wt% solution of PEG-*b*-PCL with a molecular weight of (a) 14 kDa and (b) 26 kDa. Chain entanglement in a good solvent depends on the molecular weight and its concentration. Above a critical concentration C^*, the chain entanglement acts as force overcoming the Raleigh instabilities associated with the break up of a fluid jet in droplets [18]. Only polymer droplets were observed for dilute solutions regime due to insufficient chain overlap. All of the 2 wt% solutions electrosprayed (Fig. 3a). Molecular weights below 25 kDa lead predominantly to spraying even at higher concentrations. The polymer chains are therefore too short for sufficient entanglement and cannot prevent the break up of the jet in droplets. With increased concentration, and consequently chain entanglement, those polymers with molecular weights above 25 kDa droplets and beaded fibers were formed and further increases in solution concentrations resulted in fiber formation only. In this instance, homogenous electrospun fibers were obtained with molecular weights higher than 25 kDa at concentrations of 10 wt% (Fig. 3b).

To characterize the surface properties of nanofiber meshes in contact with water, sessile drop contact angle measurements were performed. The contact angle of electrospun PEG_{10K}-*b*-PCL_{16K} mesh ($\theta = 30.9° \pm 1.85$) is relatively low indicating the hydrophilic surface of the material. The use of long PEG segments with molecular weights of 10 and 13 kDa as macroinitiators is likely responsible for the hydrophilicity of these polymers. The electrospun PEG-*b*-PCL is readily wetted with aqueous media, which is a useful property when working with frequent media changes in vitro. In comparison, the contact angle of pure PCL electrospun fibers is considerably higher

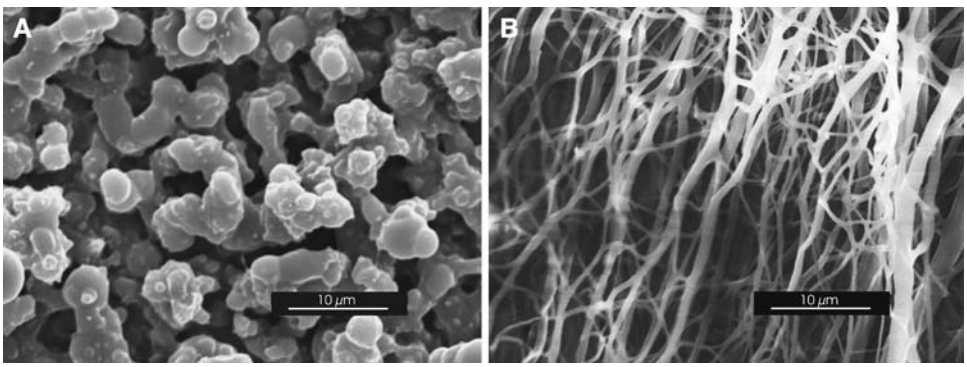

Fig. 3 SEM images showing electrosprayed/electrospun morphologies from 10 wt% solutions of different molecular weights resulting in (**a**) PEG_{10}-*b*-PCL_4 particles and aggregates, and (**b**) PEG_{10}-*b*-PCL_{16} fibers. Original magnification (**a** and **b**) 2500×

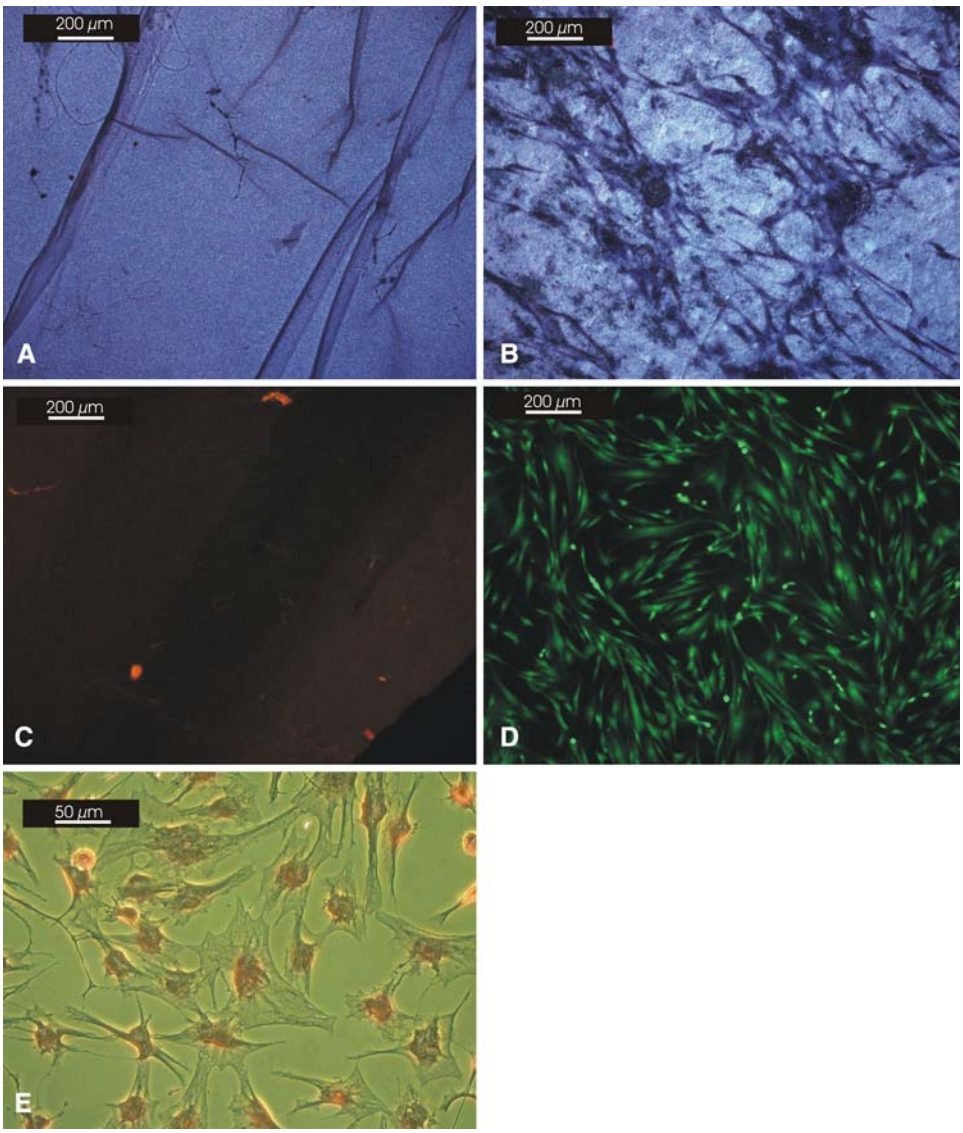

Fig. 4 Optical and fluorescence images of fibers with and without GRGDS. Fibroblasts after 24 h culture and haemalum staining on (**a**) PEG_{10}-*b*-PCL_{16} and (**b**) GRGDS-PEG_{10}-*b*-PCL_{16}. Fluorescence image of fibroblasts after 24 h culture and live/dead staining seeded upon (**c**) PEG_{10}-*b*-PCL_{16}, (**d**) GRGDS-PEG_{10}-*b*-PCL_{16} and (**e**) fibroblasts on glass slide adjacent to electrospun PEG-*b*-PCL. Original magnification (**a**–**d**) 5×, (**e**) 20×

with 119.5°± 0.7, indicating the hydrophobicity of such a mesh (preparation of a PCL mesh is described elsewhere [16]).

In vitro tests indicate the suitability of these electrospun fiber meshes for tissue engineering purposes. Both non-functionalized and GRGDS functionalized electrospun PEG_{10}-*b*-PCL_{16} meshes were seeded with human-derived fibroblasts and kept in cell culture for 24 h and the cell vitality and morphology investigated. Figure 4 shows characteristic optical micrographs including fluorescence images for Live/Dead® staining after 24 h in vitro. The non-functionalized PEG_{10}-*b*-PCL_{16} meshes suppressed cell attachment—they did not adhere on the unfunctionalized mesh substrates (Fig. 4a, c). Since similar electrospun substrates allow some attachment with pure PCL fibers, it is possible that the cells cannot adhere to the fiber surface due to it's hydrophilicity. This is also regarded as an indication that the fibers were repellent against protein adsorption—either FCS from the media or proteins produced by the cells. Adsorption of these proteins would alter the surface properties of the fibers and set up pre-conditions which lead to cell adhesion. Vital, spread fibroblasts were observed attached to all adjacent glass substrates (Fig. 4e), demonstrating that cells cannot adhere, rather than a cytotoxicity issue with the polymeric fibers.

Conversely, the fibroblasts on the electrospun GRGDS immobilized PEG_{10}-*b*-PCL_{16} mesh showed spread morphologies and high viability (see Fig. 4d). These cells display the characteristic spread, flat morphology of fibroblasts on tissue culture plastic, indicating good adherence to the electrospun fibers. Over a 24 h period the cell adherence of fibroblasts onto the fiber was excellent and confirms that the GRGDS and the PEG block is present at the fiber surface. These results and the low contact angle

indicate the presence of the PEG segment and availability of biologically active peptide sequences at the fiber surface.

In summary, we have demonstrated bioresorbable electrospun nanofibres, that are repellent to non-specific protein adsorption and cell adherence. The functionalization of the hydrophilic terminus of the block copolymer with the integrin binding GRGDS peptide in turn provides adhesion sites for cell attachment.

4 Conclusion

Electrospun fibers were produced from block copolymers that could be either functionalized, or prevent cell adhesion. This shows the production of electrospun fibers as scaffolds which provide biologically active sequences on the fiber surface. Block copolymers with low polydispersities and molecular weights from 10 to 30 kDa were synthesized and functionalized with GRGDS. The hydrophilic nature of the resulting fibers is associated with the relatively large hydrophilic component of the copolymers. Electrospinning 10 wt% solutions of these block copolymers with molecular weights higher than 26 kDa resulted in homogeneous electrospun fibers (both functionalized and non-functionalized). Contact angle measurements (30°) confirmed the hydrophilicity of the fibers and suggest that the hydrophilic block is oriented to the fiber surface. The strong adhesion of fibroblasts to the biofunctionalized PEG-*b*-PCL mesh in vitro shows the presence and availability of GRGDS, immobilized to the PEG segment, at the fiber's surface. The electrospun fiber meshes displayed contrasting differences in fibroblast attachment to non-functionalized and GRGDS immobilized mesh. While non-functionalized block copolymer meshes prevent cell attachment, the functionalized non-woven showed enhanced cell attachment, and high cell viability is preserved. The synthesis procedure allows the incorporation of additional bioactive compounds at the last stage, and may be suitable for triggering a host of cell responses and potential applications as artificial scaffolds.

Acknowledgments This work was supported by DFG Graduiertenkolleg 1035 "Biointerface" and BMBF 13N8888. The authors wish to thank DECHEMA, Gesellschaft für Chemische Technik und Biotechnologie e. V., for financial support of the research project "Nanofaservliese für die Therapie von Oberflächenwunden" (AiF-No. 14263) provided from funds of Bundesministerium für Wirtschaft und Technologie (BMWi) via a grant of Arbeitsgemeinschaft industrieller Forschungsvereinigungen "Otto von Guericke" e.V. (AiF).

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