J Mater Sci: Mater Med (2010) 21:2637–2651
DOI 10.1007/s10856-010-4112-7

Functionalization of electrospun fibers of poly(ε-caprolactone) with star shaped NCO-poly(ethylene glycol)-*stat*-poly(propylene glycol) for neuronal cell guidance

Kristina Klinkhammer · Julia Bockelmann · Chariklia Simitzis · Gary A. Brook · Dirk Grafahrend · Jürgen Groll · Martin Möller · Jörg Mey · Doris Klee

Received: 27 February 2010 / Accepted: 29 May 2010 / Published online: 22 June 2010

Abstract Microfibers produced with electrospinning have recently been used in tissue engineering. In the development of artificial implants for nerve regeneration they are of particular interest as guidance structures for cell migration and axonal growth. Using electrospinning we produced parallel-orientated biocompatible fibers in the submicron range consisting of poly(ε-caprolactone) (PCL) and star shaped NCO-poly(ethylene glycol)-*stat*-poly(propylene glycol) (sPEG). Addition of the bioactive peptide sequence glycine-arginine-glycine-aspartate-serine (GRGDS) or the extracellular matrix protein fibronectin to the electrospinning solution resulted in functionalized fibers. Surface characteristics and biological properties of functionalized and non-functionalised fibers were investigated. Polymer solutions and electrospinning process parameters were varied to obtain high quality orientated fibers. A polymer mixture containing high molecular weight PCL, PCL-diol, and sPEG permitted a chemical reaction between hydroxyl groups of the diol and isocyanante groups of the sPEG. Surface analysis demonstrated that sPEG at the fiber surface minimized protein adhesion. In vitro experiments using dorsal root ganglia explants showed that the cell repellent property of pure PCL/sPEG fibers was overcome by functionalization either with GRGDS peptide or fibronectin. In this way cell migration and axonal outgrowth along fibers were significantly increased. Thus, functionalized electrospun PCL/sPEG fibers, while preventing non-specific protein adsorption, are a suitable substrate for biological and medical applications.

K. Klinkhammer · C. Simitzis · D. Grafahrend · J. Groll · M. Möller · D. Klee (✉)
Institute of Technical and Macromolecular Chemistry and DWI e.V., RWTH Aachen University, Pauwelsstr. 8, 52056 Aachen, Germany
e-mail: klee@dwi.rwth-aachen.de

K. Klinkhammer
e-mail: klinkhammer@dwi.rwth-aachen.de

C. Simitzis
e-mail: xara_si@yahoo.gr

D. Grafahrend
e-mail: dirk.grafahrend@freudenberg.de

J. Groll
e-mail: groll@dwi.rwth-aachen.de

M. Möller
e-mail: moeller@dwi.rwth-aachen.de

J. Bockelmann · J. Mey
Institute of Biology II, RWTH Aachen University, Mies-van-der-Rohe-Str. 15, 52074 Aachen, Germany
e-mail: Julia@Bockelmann.biz

J. Mey
e-mail: mey@bio2.rwth-aachen.de

J. Bockelmann · J. Mey
EURON Graduate School of Neuroscience, Maastricht, Netherlands

G. A. Brook
Institute of Neuropathology, Medical Faculty, RWTH Aachen University, Pauwelsstr. 30, 52074 Aachen, Germany
e-mail: gbrook@ukaachen.de

G. A. Brook
IZKF-BIOMAT, Aachen University Hospital, RWTH Aachen University, Pauwelsstr. 30, 52074 Aachen, Germany

Abbreviations

BSA	Bovine serum albumin
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DCM	Dichloromethane
DIV	Days in vitro
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
ECM	Extracellular matrix
FCS	Fetal calf serum
GRGDS	One letter code of the peptide sequence GlyArgGlyAspSer
Mw	Molecular weight
NF200	Neurofilament 200 kDa
NGS	Normal goat serum
NMP	*N*-Methylpyrrilidone
PBS	Phosphate buffer saline
PCL	Poly(ε-caprolactone)
PCL-ol	PCL diol
PEG	Poly(ethylene glycol)
PNS	Peripheral nervous system
S100	Protein antibody, marker for Schwann cells
SC	Schwann cells
SEM	Scanning electron microscopy
sPEG	Star shaped NCO-poly(ethylene glycol)-*stat*-poly(propylene glycol)
THF	Tetrahydrofuran
TBS-T	Tris-buffered saline with 1% Triton-X 100
wt%	Weight percent
XPS	X-ray photoelectron spectroscopy

1 Introduction

Tissue engineering strategies are directed to restore physiological functions using artificial implants. Synthetic scaffolds are intended to repair the damage caused by injuries. This has a special importance in the case of the peripheral nervous system (PNS). In vivo cells are surrounded by a three-dimensional network of filaments, the extracellular matrix (ECM), which provides a growth permissive substrate for axonal regeneration. Biomaterials for nerve repair therefore have to mimic the properties of the ECM including its fibrillar structure and the presentation of biochemical signals to the cells. Specifically, the ECM of lesioned peripheral nerves confers information to induce Schwann cell (SC) proliferation, migration, and differentiation and to guide nerve fiber regeneration. After peripheral nerve injury, if proximal and distal nerve stumps remain in contact with each other, Schwann cells reorganize and align to form the axon growth promoting bands of Büngner [1]. Artificial substrates consisting of single fibers or bundles of fibers can serve as attractive guidance structures for oriented SC migration and neurite outgrowth [1–3].

The method of electrospinning offers the possibility to produce microfiber scaffolds with diameters similar to that of ECM fibrils (0.5–5 μm) to act as cell adhesive substrates. With this technique, a high voltage is applied to a solution of (synthetic or natural) polymers generating a liquid jet. During its way to the grounded target the jet is continuously stretched due to electrostatic repulsion between surface charges whereas evaporation of the solvent leads to a solid fiber being collected on the target [4].

For the application as guidance structures in a regenerating peripheral nerve the prepared fibers were desired to be aligned in parallel, of homogenous thickness and free of defects. Solution properties, including polymer composition, molecular weight of the polymer, solution concentration, surface tension, and polarity of the solvent, influence the quality of the resulting fibers. However, governing parameters such as applied voltage, flow rate, and distance between spinneret and target are also important [5]. Using different collector set-ups result in different fiber arrangements. Two parallel bars (referred to as the gap-method) allow the collection of orientated fibers which are suspended in air. This offers the possibility to deposit the fibers on various substrates such as chemically reactive hydrogel layers [6].

In the development of an artificial scaffold for nerve regeneration, one must consider not only shape and mechanical strength of the implant but also biocompatibility of the material and its surface and bulk properties. Following implantation into the body, endogenous proteins immediately interact with the biomaterial according to their particular affinities to the material, and host cells respond to these adsorbed protein layers [7]. Nevertheless, unspecific protein adsorption is often undesirable because it can cause problems such as acute or chronic inflammation [8] as well as fibrous encapsulation [9]. Protein resistant surfaces, mainly in the form of hydrogels and hydrogel coatings, should be hydrophilic, uncharged, and flexible. Polyethylene glycol (PEG) fulfils these demands and is capable of masking the foreign nature of implants within the host organism. PEG is non-toxic and non-immunogenic and has demonstrated protein repellence in several studies [10, 11]. Electrospun fibers consisting of poly(ethylene glycol)-*block*-poly(D,L-lactide) block copolymers have already shown their biocompatibility and protein resistance properties [12]. Star shaped NCO-poly(ethylene glycol)-*stat*-poly(propylene glycol), is named "sPEG" in the following text instead of the chemical more correct description of "sP(EO-*stat*-PO)" to improve the readability of this paper, allows the preparation of a dense polymer network by cross-

linking of the arms via functional groups. This prevents unspecific protein binding and has been proven to be superior to linear PEG-coatings [13].

In contrast to undesired non-specific protein adsorption, specific protein-, and cell-substrate interactions are required for successful integration and tissue repair. These special interactions can be triggered by surface functionalization with special biological signals (e.g., ECM proteins, peptides, or polysaccharides). Such biological signals showed the highest activity when immobilized on the scaffold, while soluble proteins including collagen and laminin, are known to support cell adhesion via activation of integrin receptors [14].

In the present study we have developed a method for the production of aligned biodegradable electrospun fibers based on a blend of poly(ε-caprolactone) (PCL) with functionalized sPEG. The hydrophilic properties of sPEG prevented unspecific protein adsorption while covalent binding with the RGD peptide or with the fibronectin protein within the electrospinning solution resulted in favorable biological properties, as revealed by in vitro assays using dorsal root ganglia (DRG) explants.

2 Materials and methods

2.1 Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Munich, Germany). Toluene and tetrahydrofuran (THF) were dried by distillation over lithium aluminium hydride and stored in a nitrogen atmosphere. Acetone was dried statically over molecular sieve (3 Å) for 3 days and then distilled in a nitrogen atmosphere. It was stored over molecular sieve and nitrogen. Star shaped NCO-poly(ethylene glycol)-*stat*-poly(propylene glycol) (with a backbone of 80% ethylene glycol and 20% propylene glycol and isocyanate (NCO) end groups, Mw = 12000 g/mol; sPEG) was synthesized in our laboratory from hydroxyl-terminated star polymer (DOW-chemicals, Terneuzen, Netherlands) by reaction with isophorone diisocyanate according to details published elsewhere [15]. All other chemicals were used as received.

2.2 Solution preparation

Two different systems were chosen to determine electrospinning ability and cellular response on PCL/sPEG fibers. The solutions were prepared with a variety of solvents whose properties are summarized in table 1. Based on preliminary experiments these were the solvent mixtures best suited for the electrospinning of high quality fibers in parallel orientation. In the first part of experiments, fibers consisting of PCL (Mw = 65000 g/mol) with sPEG were produced. PCL was dissolved (17 wt%) in a mixture of dry dichloromethane (DCM) (p.a., crown capped) and dry acetone (1/1 v/v). Then a specific amount of sPEG (15 or 27 wt% of the solid fraction) dissolved in dimethyl sulfoxide (DMSO) or *N*-methylpyrrolidone (NMP) was added. The volume of DMSO or NMP containing sPEG was 5% of the total solution. The mixture was stirred for 1 min. These mixtures were called "P-PEG" according to the combination of PCL and sPEG and the solution compositions have been summarized in Table 2.

Table 1 Properties of the solvents used in the electrospinning experiments

	Boiling point[a] (°C)	Vapor pressure[a] (20°C, hPa)	Surface tension[b] (dyne/cm)
Acetone	56	233	25
DCM	40	475	27
DMSO	202	0.32	41
NMP	189	0.56	43[c]
THF	66	173	26

[a] www.merck.de

[b] www.surface-tension.de

[c] www.bulkmsm.com

In the second approach, different solutions were prepared first from three polymers: (1) a 15 wt% solution of PCL in a mixture of dry DCM and dry acetone (1/1 v/v); (2) poly(ε-caprolactone)-diol (PCL-ol) (Mw = 2000 g/mol) was dissolved with the same solvents, resulting in solutions with 40.2–57.9 wt%; (3) sPEG was dissolved in THF resulting in a 50% (w/v) solution. To prepare the electrospinning solution with a total solution concentration of 17 or 22 wt%, the PCL-ol solution was added to the sPEG, stirred for 1 min, followed by adding the PCL solution and stirring again for 1 min. Finally NMP [5% of total solution (v/w)] was added as a fourth solvent and the mixture was stirred for another 30 s. These blends were called "PPol-PEG" according to the combination of PCL, PCL-diol and sPEG. The solution compositions are summarized in table 2. For biologically functionalized fibers, the peptide sequence GRGDS (Bachem, Bubendorf, Switzerland; PPol-PEG3 + RGD) or the ECM protein fibronectin (Biopur AG, Switzerland; PPol-PEG4 + Fn) was mixed into the NMP prior to the use of the solvent. The amount of GRGDS used was one-fifth of the amount of sPEG [n(GRGDS) = 1/5 n(sPEG)]. Fibronectin was applied at a weight ratio of 1:250 (fibronectin to all polymer solids), which resulted in a ratio of sPEG to fibronectin of 2:1. Additionally, a 9 wt% solution of PCL in methanol/chloroform (1/3 v/v) was prepared, because pure PCL fibers served as reference.

Table 2 Solution concentrations and polymer concentrations of solid fibers for the different PCL/sPEG mixtures

Name	P-PEG1	P-PEG2	P-PEG3	P-PEG4	PPol-PEG1	PPol-PEG2	PPol-PEG3	PPol-PEG4
Solvents								
1. solvent	Acetone	Acetone	Acetone	Acetone	Acetone	Acetone	Acetone	Acetone
2. solvent	DCM	DCM	DCM	DCM	DCM	DCM	DCM	DCM
3. solvent	DMSO	NMP	DMSO	NMP	THF	THF	THF	THF
4. solvent	–	–	–	–	NMP	NMP	NMP	NMP
Solutions which were mixed to prepare electrospinning solution								
c(solution 1, PCL-65_k-solution) (wt%)	17	17	17	17	15	15	15	17
c(solution 2, PCL-diol) (wt%)	–	–	–	–	40.2	53.1	57.9	54.7
c(solution 3, sPEG) (wt%)	57	57	54	54	50	50	50	36
c(total electrospinning solution) (wt%)	23	23	26	26	17	17	17	22
Concentration of polymers in electrospun fibers								
wt% (PCL-65_k in fiber)	85	85	73	73	90	75	60	57.4
wt% (PCL-diol in fiber)	–	–	–	–	3	8	13	13.1
wt% (sPEG in fiber)	15	15	27	27	7	17	27	29.5

Table 3 gives details about the solution properties, calculated for a sPEG amount of 0.05 g each. Fibers were termed "PCL/sPEG" when containing PCL, PCL-diol, and sPEG or PCL and sPEG and were investigated without distinguishing between them (in contrast to pure PCL fibers).

2.3 Electrospinning

For electrospinning, the solution was filled in a 1 ml syringe with a single-use blunt-end 21 gauge needle (Hamilton, Bonaduz, Switzerland) and ejected with a defined flow rate. The needle was connected to a high voltage supply (Bertan, Spellman high voltage electronics corporation, Hauppauge, NY, USA) and a high voltage was applied to the polymer solution. Various voltages (10–25 kV), flow rates (0.5–2 ml/h) and working distances (5–25 cm) between the needle and target were used. A scanning electron microscopy (SEM) stub (diameter = 14 mm) covered with aluminum foil was used for random fiber collection. Two parallel grounded bars with a distance of 2 cm between them were used for oriented fiber collection. Pure PCL fibers served as controls and were produced by electrospinning with a voltage of 20 kV, a flow rate of 0.5 ml/h and a distance between needle and target of 20 cm.

2.4 Substrate preparation

Glass coverslips (diameter = 12 mm) and silicon wafers were surface modified with a protein resistant sPEG-layer as described before [2]. Briefly, after cleaning the coverslips with ethanol and surface activation by UV-ozone-treatment for 12 min, they were transferred into a glove box. Here they were aminosilanisated with a solution of 0.3 ml of *N*-[3-(trimethoxysilyl)propyl] ethylene diamine (97%) in 50 ml dry toluene for 2 h. Afterwards the coverslips were washed with dry toluene and stored in toluene in a nitrogen atmosphere. Prior to spin-coating a solution of 20 mg sPEG in 0.2 ml THF and 1.8 ml of deionized water was prepared and crosslinking allowed for 5 min. Subsequently, the coverslips were placed onto a spin-coater and covered with the filtered solution (0.2 μm, Whatman, Maidstone, Great Britain). The coverslips were accelerated within 5 s to 4000 rpm for 40 s. For protein adsorption assays, silicon wafers were treated in the same way.

Table 3 Solution composition for the different PCL/sPEG mixtures

Name	P-PEG1	P-PEG2	P-PEG3	P-PEG4	PPol-PEG1	PPol-PEG2	PPol-PEG3	PPol-PEG4
m (sPEG) (g)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
V (THF for sPEG) (ml)	–	–	–	–	0.1	0.1	0.1	0.1
m (PCL-65_k) (g)	0.275	0.275	0.133	0.133	0.639	0.219	0.110	0.097
m (PCL-diol) (g)	–	–	–	–	0.025	0.025	0.025	0.022
m (PCL-65_k-solution) (g)	1.617	1.617	0.833	0.833	4.262	1.461	0.735	0.571
m (PCL-diol-solution) (g)	–	–	–	–	0.062	0.047	0.043	0.044
V (3. solvent) (ml) (5%)	0.038	0.038	0.042	0.042	0.224	0.083	0.046	0.038

Oriented fibers were deposited onto the substrates by drawing the substrate through the mat of suspended fibers. Random fibers were directly electrospun onto the reactive substrates.

2.5 Fiber characterization

2.5.1 Scanning electron microscopy

The electrospun fibers were visualized by SEM (S360 Zeiss NTS, Oberkochem, Germany and Hitachi S3000 N, Hitachi High Technology Europe GmbH, Krefeld, Germany), using 15 kV and working distances of 5–15 mm after sputtering with gold for 90 s (S150B, Edwards, Crawley, Great Britain). The SEM images were used to measure fiber diameters. For each preparation at least 30 fibers were measured and data are presented as mean ± standard deviation. The same images were used to estimate the orientation distribution of the fibers.

2.5.2 Protein adsorption

Random PCL/sPEG fibers and fibers of PCL were collected onto sPEG coated silicon wafers. Four samples were prepared for fibers of each composition. Half of the samples were incubated in water for 5 days, washed with fresh distilled water afterwards, and dried in a nitrogen stream. The second half remained untreated. Protein adsorption of the incubated and non-incubated fibers was determined using fluorochrome-labeled bovine serum albumin (BSA, Invitrogen, Karlsruhe, Germany) as described previously [6, 16]. Briefly, the various substrates were incubated for 20 min with rhodamine red-labeled BSA (50 μg/ml) diluted in phosphate buffered saline buffer (PBS) followed by three washing steps (20 min each) in PBS. The samples were then incubated for 60 min in PBS and washed thoroughly with distilled water to remove not adsorbed proteins and dried with nitrogen. The samples were kept in the dark during incubation and washing. Protein adsorption was examined by fluorescence microscopy (Axioplan 2 imaging, Zeiss, Germany), and images were taken with a constant exposure time of 20 s.

2.5.3 X-ray photoelectron spectroscopy

Small fiber mats (2 × 8 mm) were examined by XPS (Ultra Axis^{TM} spectrometer, Kratos Analytical, Manchester, Great Britain) to investigate the atomic composition of the surfaces of the different PCL and PCL/sPEG fibers. Untreated samples and fiber samples which were incubated in water for 5 days and dried in a vacuum afterwards were investigated. The samples were irradiated with monoenergetic Al Kα1,2 radiation (1486.6 eV) and the spectra were taken at a power of 144 W (12 kV × 12 mA). The elemental concentration is given in atom%, but it should be stated that this method can detect all elements except hydrogen and helium and that therefore, the determination of the composition does not include either of these elements.

2.6 In vitro assays

For in vitro cell experiments, orientated electrospun fibers were deposited onto glass coverslips slips which had been surface modified with a thin layer of sPEG hydrogel. To investigate migration of Schwann cells and regeneration of sensory axons on microfibers, explants of DRG were placed onto PPol-PEG3 fibers, GRGDS-functionalized PPol-PEG3 fibers, fibronectin-functionalized PPol-PEG4 fibers, and PCL fibers. All substrates were sterilized with 70% ethanol for 10 min and washed three times (15 min each) with PBS before cell experiment application.

2.6.1 DRG preparation and explants

DRG were prepared from embryonic day 10 chick embryos as published previously [2]. Two to three DRG per coverslip were explanted onto the microfibers. First 100 μl cell culture medium [Dulbecco's modified eagle medium (DMEM), Gibco, Invitrogen, Paisley, United Kingdom], containing antibiotics, antimycotica with 10% fetal calf serum (FCS), were added to allow the explants to adhere to the substrate. After a few hours in the incubator, an additional 400 μl medium was given to ensure sufficient nutrition of the tissue. Explants were incubated for 4 days in vitro (DIV), then fixed 30 min with 4% paraformaldehyde and stained immunocytochemically prior to microscopical analysis.

2.6.2 Neurite outgrowth

Neurite outgrowth was measured using image J software on a montage of neurofilament stained images taken from DRG samples. Axon lengths were measured from the border of the DRG to the tips of the outgrowing neurites. For every explant the axons were separately evaluated on the two sides of the explant in parallel with the underlying microfibers. For each side, the 20 longest axons (if less: all axons) were measured.

2.6.3 Schwann cell migration

The same DRG explants were also used for Schwann cell migration measurements, using S100/DAPI staining. Regions with a width of 50 μm were selected on the two opposite sides of the explants, where microfiber orientation

was perpendicular to the edge of the explant, and cells were able to migrate in parallel to the fiber substrate. On these sides, most cells migrated and the farthest migration distance was measured. The distance of the 10 farthest migrating cells of each side was determined using Image J software.

2.6.4 Immunocytochemistry

Standard immunocytochemical staining procedures were used. The blocking solution used was 2% normal goat serum (G9023, Sigma) in Tris-buffered saline with 0.4% Triton X-100 (Carl Roth, Karlsruhe, Germany). We used primary antibodies against S100 (polyclonal rabbit serum, S2644, Sigma; 1:100 dilution) to visualize Schwann cells, and against neurofilament (NF200; mouse monoclonal antibody, N0142, Sigma; 1:300 dilution) to mark neurons and axons. Samples were incubated with primary antibodies for 1 h at 37°C. As secondary antibodies Alexa Fluor546 goat anti rabbit IgG (Molecular Probes, Invitrogen, Paisley United Kingdom; A-110106) and Alexa Fluor™ 488 goat anti-mouse IgG (Molecular Probes, Invitrogen, Paisley United Kingdom; A-11001) were applied in dilution of 1:500, also for 1 h at 37°C. Cell nuclei were stained using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.5 µg/ml, Sigma), 5 min at room temperature. Results were evaluated using Zeiss Cell Observer Microscope coupled to MosaiX software and Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) connected to an on-line digital camera (Spot Basic software).

3 Results

3.1 Electrospinning

Electrospinning was performed with two solution systems (P-PEG and PPol-PEG) as described earlier. Figure 1 shows a schematic illustration of the electrospinning process, indicating the chemical reactions assumed to take place during fiber preparations. Since high quality electrospinning is necessary for the deposition and collection of the parallel fibers, needed for the regeneration assays, much effort was expended in determining the optimal parameters for this purpose. The high quality fibers were indicated by alignment and suspension of fibers between the two parallel target bars and a low variability of fiber diameter. Variations of solvent mixtures, sPEG content and spinning parameters including voltage, flow rate, and distance between needle and target were tested systematically. It was found that different process parameters had to be selected for the individual chemical compositions. In general, a voltage of 20 kV and a needle-target distance of 20 cm resulted in the best fibers.

In the first approach, fibers were produced by electrospinning from mixtures of high molecular weight PCL with sPEG (P-PEG). Different concentrations of sPEG (15 and 27 wt%) and different solvent compositions were used (see table 2). Blends of PCL with 15 wt% sPEG resulted in high quality fibers collected in an oriented manner using the gap-method, independently of which solvent was used. Figure 2 shows that a concentration of 15 wt% sPEG in the electrospun fibers resulted in highly oriented fibers with

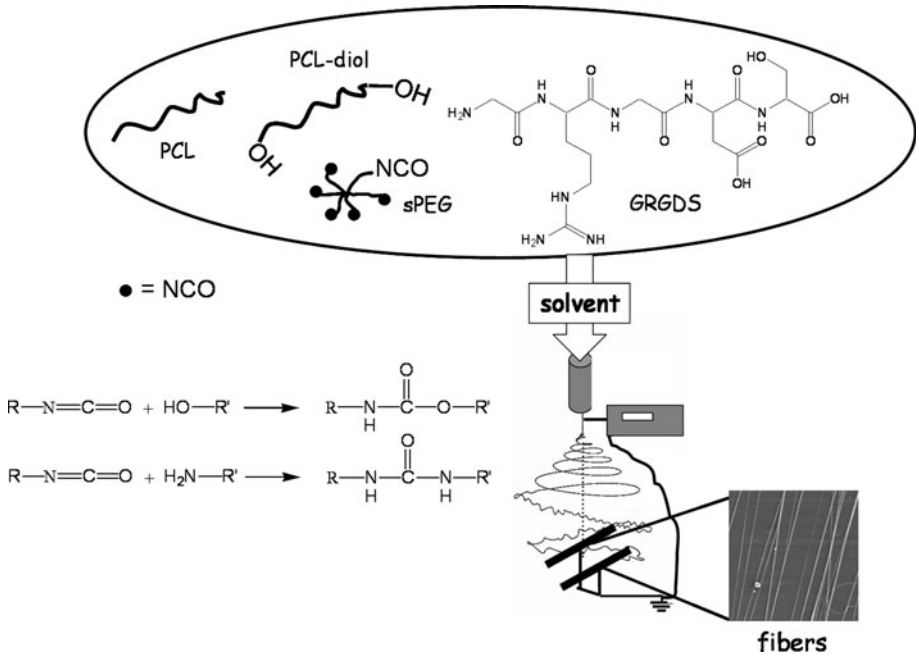

Fig. 1 Preparation of electrospinning solution and chemical reaction between components. For the preparation of the electrospinning solution the different compounds PCL, PCL-diol, sPEG, and GRGDS were mixed according to table 2 and dissolved in a solvent mixture. These solutions were electrospun to oriented fibers. Chemical reactions between isocyanate groups of the sPEG molecules and amine or hydroxyl groups of PCL and GRGDS form urethane and urea groups, respectively and resulted in covalent binding between the molecules. The same reactions were assumed to take place with functional groups of fibronectin

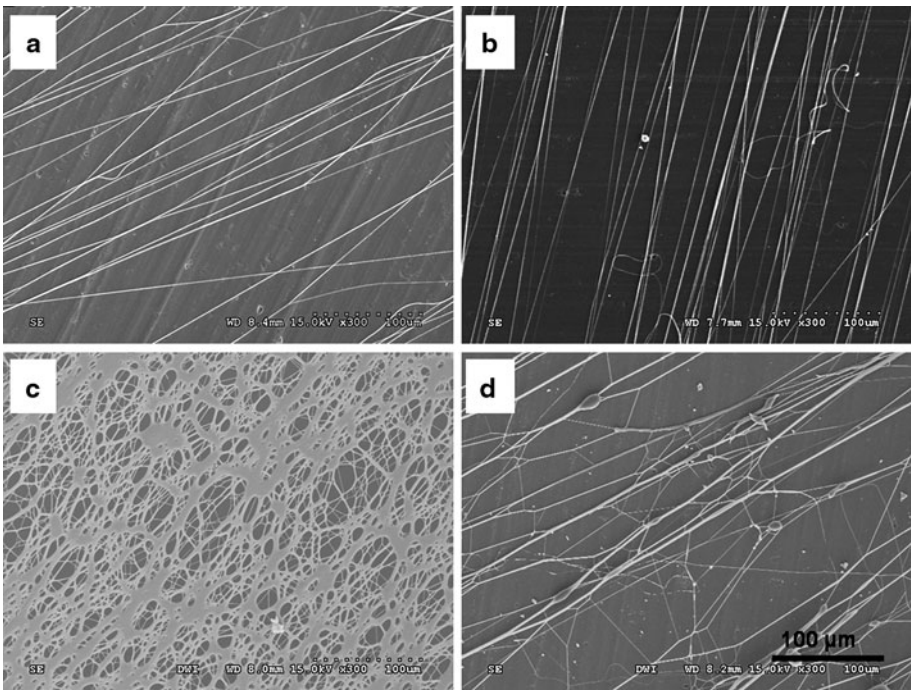

Fig. 2 Electrospun P-PEG fibers produced of blends PCL with 15 or 27 wt% sPEG from different solvents. **a** 15 wt% sPEG, acetone/DCM + DMSO (P-PEG1), **b** 15 wt% sPEG, acetone/DCM + NMP (P-PEG2), **c** 27 wt% sPEG, acetone/DCM + DMSO (P-PEG3), **d** 27 wt% sPEG, acetone/DCM + NMP (P-PEG4), scale bar = 100 µm

both solvent mixtures (DMSO or NMP as third solvent). With both solvents orientated fibers with diameters of 0.92 ± 0.29 µm (DMSO, Fig. 2a, P-PEG1) and 0.64 ± 0.11 µm (NMP, Fig. 2b, P-PEG2) were obtained. Although electrospinning with DMSO tended to produce fibers with larger diameters, this effect was not statistically significant. When the sPEG concentration was increased to 27 wt%, a fiber network of so-called "wet fibers" occurred from electrospinning with solvent system acetone/DCM/DMSO (Fig. 2c, P-PEG3) while oriented fibers were obtained from electrospinning with NMP. Here orientated fibers with diameter of 1.68 ± 0.87 µm (Fig. 2d, P-PEG4) were achieved. P-PEG2 and P-PEG4 fibers were used for further investigations.

In the second system, fibers were electrospun from solutions of PCL, PCL-ol, and different amounts of sPEG. Figure 3 shows SEM images of these fibers. With all sPEG concentrations, good quality fibers with parallel orientation were easily obtained. Low sPEG concentrations of 7 wt% (PPol-PEG1) resulted in the deposition of smaller fibers (0.45 ± 0.14 µm, Fig. 3a) than concentrations of 17 wt% (PPol-PEG2) (0.66 ± 0.21 µm, Fig. 3b), 27 wt% (PPol-PEG3)

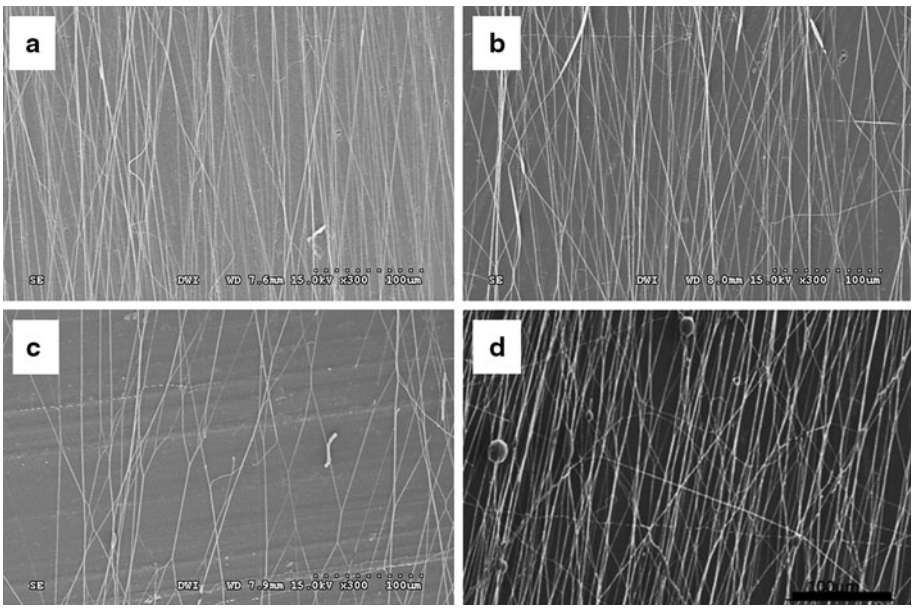

Fig. 3 Electrospun PPol-PEG fibers produced of PCL, PCL-ol, and different amounts of sPEG in acetone/DCM/NMP. **a** 7 wt% sPEG (PPol-PEG1), **b** 17 wt% sPEG (PPol-PEG2), **c** 27 wt% sPEG (PPol-PEG3), **d** 29.5 wt% sPEG (PPol-PEG4). With the PPol-PEG system high quality oriented fibers were obtained independent on the sPEG concentration, scale bar = 100 µm

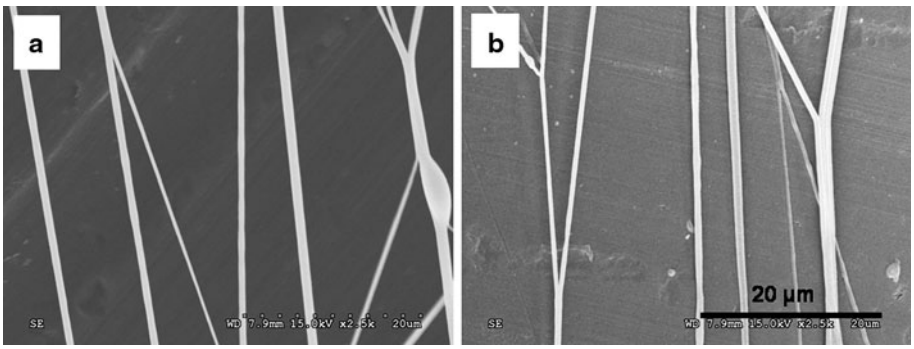

Fig. 4 SEM images of PPol-PEG3 fibers electrospun with (**a**), and without (**b**) the inclusion of GRGDS, scale bar = 20 µm

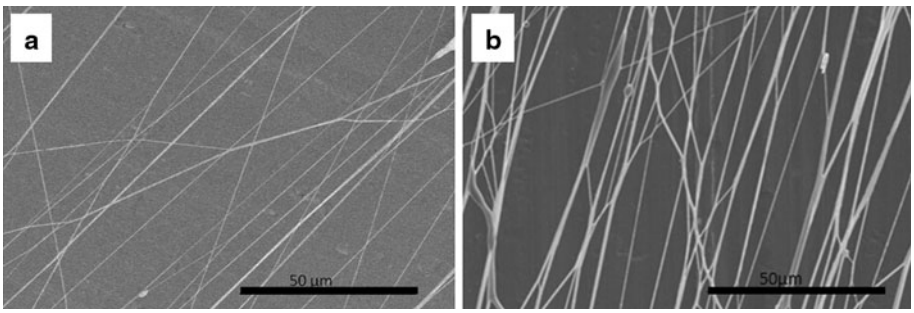

Fig. 5 SEM images of PPol-PEG4 fibers electrospun with (**a**), and without (**b**) the inclusion of fibronectin, scale bar = 50 µm

(0.67 ± 0.17 µm, Fig. 3c) and 29.5 wt% (PPol-PEG4) (0.57 ± 0.28 µm, Fig. 3d). Increasing the sPEG concentration caused a slight decrease of fiber quality, and concentrations above 37 wt% failed to generate any fibers. PPol-PEG3 and PPol-PEG4 fibers were used for further investigations. To overcome the cell repellent properties of the sPEG-containing fibers, GRGDS, a peptide sequence which is known to promote cellular adhesion (PPol-PEG3 + RGD), or the whole ECM protein fibronectin (PPol-PEG4 + Fn) were incorporated into the fibers and tested in in vitro assays. SEM was used to determine changes in fiber quality following the inclusion of bioactive molecules. Figures 4 and 5 show examples of such images. No difference in fiber quality could be observed in the presence (Fig. 4a) or absence of GRGDS (Fig. 4b), however fiber diameter increased slightly (0.85 ± 0.28 µm) with inclusion of GRGDS. Occasional swellings of peptide-containing fibers was observed in in vitro experiments. Similar results were achieved with PPol-PEG4 fibers. The fiber quality did not change visibly when fibronectin was incorporated (diameters 0.67 ± 0.27 µm; Fig. 5), but occasional intersections between the fibers were noticed. In table 4 the properties of the different fiber types are summarized. Table 5 gives information about the orientation distribution of the fibers and the volume porosity. The best parallel orientation of the fibers was achieved with biofunctionalized PPol-PEG + RGD and PPol-PEG + Fn fibers. Here the majority of the fibers (64, respectively 65%) were parallel to each other or deviate maximum 5° from each other. PPol-PEG3 fibers had the

Table 4 Properties of fibers from both fiber systems

Name	P-PEG 1	P-PEG 2	P-PEG 3	P-PEG 4	PPol-PEG1	PPol-PEG2	PPol-PEG3	PPol-PEG4
sPEG content % in solid fraction	15	15	27	27	7	17	27	29.5
Diameter (µm) mean ± SD	0.92 ± 0.29	0.64 ± 0.11	n.a.	1.68 ± 0.87	0.45 ± 0.14	0.66 ± 0.21	0.67 ± 0.17	0.57 ± 0.28
Fiber quality	Good	Good	Very poor	Poor	Good	Good	Good	Fair
Parallel fibers?	Yes	Yes	No	No	Yes	Yes	Yes	Variable
Protein adsorption	n.d.	Fair	n.d.	Low	n.d.	n.d.	Low	Low
Functionalization	PPol-PEG3 + GRGDS				PPol-PEG4 + fibronectin			
Diameter (µm)	Increased to 0.85 ± 0.28 (127%)				Increased to 0.67 ± 0.27 (118%)			
Fiber quality	Fair-swellings in fibers				Good			
Parallel fibers?	Yes, but crossing fibers occur				Variable, crossing fibers occur			

n.a. not applicable

n.d. not determined

Table 5 Orientation distribution and volume porosity of various fibers

Name/orientation distribution (%)	PPol-PEG3	PPol-PEG3 + RGD	PPol-PEG4	PPol-PEG4 + Fn
Degree of deviation (°)				
0–4	44	64	56	63
5–10	19	12	21	13
11–15	16	3	7	13
16–20	12	12	7	3
>21	9	9	9	8
Volume porosity (%)	12.3 ± 4.7	19.9 ± 11.1	6.6 ± 3.5	6.7 ± 3.3

worst orientation distribution with only 44% of the fibers in a deviation range of 5° and with PPol-PEG4 fibers 56% of the fibers were in this range. The volume porosity named the area of a sample which is covered with fibers in comparison to the total sample area. As it can also be seen from the SEM images of the fibers, fibers were mainly clearly separated from each other and the volume porosity was calculated to be between around 6% for the PPol-PEG4 and PPol-PEG4 + Fn fibers and a bit higher for the PPol-PEG3 (12%) and the PPol-PEG3 + RGD (19%) fibers.

3.2 Fiber characterization

Fibers were further characterized by contact angle determination, protein adsorption assays, and XPS measurements. The contact angle of pure PCL fibers was 141.7°. For PPol-PEG3 and PPol-PEG4 fibers the measurement of a contact angle was not possible because the water drop was fully adsorbed immediately after its deposition on the surface, demonstrating the hydrophilicity of these fibers. Protein adsorption was investigated because non-specific protein adsorption to the fibers may enhance adverse physiological reactions in vivo and should therefore be avoided. Figure 6 presents results of the protein adsorption tests with PCL and different PCL/sPEG fibers. All images were taken with the same exposure time of 20 s. Therefore, a qualitative comparison between the samples based on the intensity of fluorescence was possible. PCL fibers showed the strongest protein adsorption (Fig. 6a, b). It did not change after incubation in water. The fibers with 15 wt% sPEG (P-PEG2, Fig. 6c, d) showed weaker protein adsorption than PCL fibers, and this also did not change after incubation in water. The inclusion of 27 wt% sPEG (P-PEG4) in the fibers resulted in an even stronger reduction of protein adsorption (Fig. 6e) compared to pure PCL fibers. After incubation in water, however, the protein adsorption increased significantly (Fig. 6f) and was comparable with that of fibers with 15 wt% sPEG. Fibers prepared with PCL-ol (PPol-PEG3) showed reduced protein adsorption comparable with P-PEG4 fibers. After incubation the protein adsorption did not change, indicating that the sPEG was still present at the fiber surface. Because PPol-PEG4 fibers nearly contained the same polymer concentrations in the solid state (see table 2), protein adsorption assays were not performed with these fibers.

X-ray photoelectron spectroscopy measurements of the fibers were performed to investigate surface composition. Table 6 shows atomic concentration of oxygen, carbon, nitrogen, and silicon of the fiber surfaces. Nitrogen, which may be used as an indicator for the existence of sPEG at the fiber surface, was measured in PPol-PEG3 fibers before and after incubation in water. Interestingly, the nitrogen content increased slightly from 0.5% prior to water incubation to 0.9% after incubation. We conclude that sPEG remained bound and may have been concentrated on the surface of the electrospun fibers. With PPol-PEG4 fibers, similar results were observed. Based on the XPS data and comparative protein absorption, we conclude that the best blend of PCL/sPEG was achieved with the PPol-PEG3 and the PPol-PEG4 preparations. These types of fibers were therefore tested in bioassays.

3.3 Cell experiments

In the context of the intended application of microfibers for peripheral nerve repair, two biological properties are considered to be most important: (i) the ability to support Schwann cell migration, and (ii) the ability to guide axonal regeneration. Both properties were evaluated by placing DRG explants onto fibers of various compositions. DRG explants were analyzed with immunocytochemical staining after 4 DIV. S100 immunoreative Schwann cells were found to have migrated from all explants. Axons from the sensory neurons within the explants had also grown out and were visualized using NF200 immunostaining (Fig. 7). Schwann cell migration from the explants, as well as neurite outgrowth, was more pronounced on GRGDS containing PPol-PEG3 than on the other types of substrate (PCL fibers, PPol-PEG3 fibers without peptide). Axons grew parallel to the GRGDS-functionalized PPol-PEG3 fibers (Fig. 7a) and the PCL fibers (Fig. 7b). In contrast, only few, very short axons were seen on PPol-PEG3 fibers

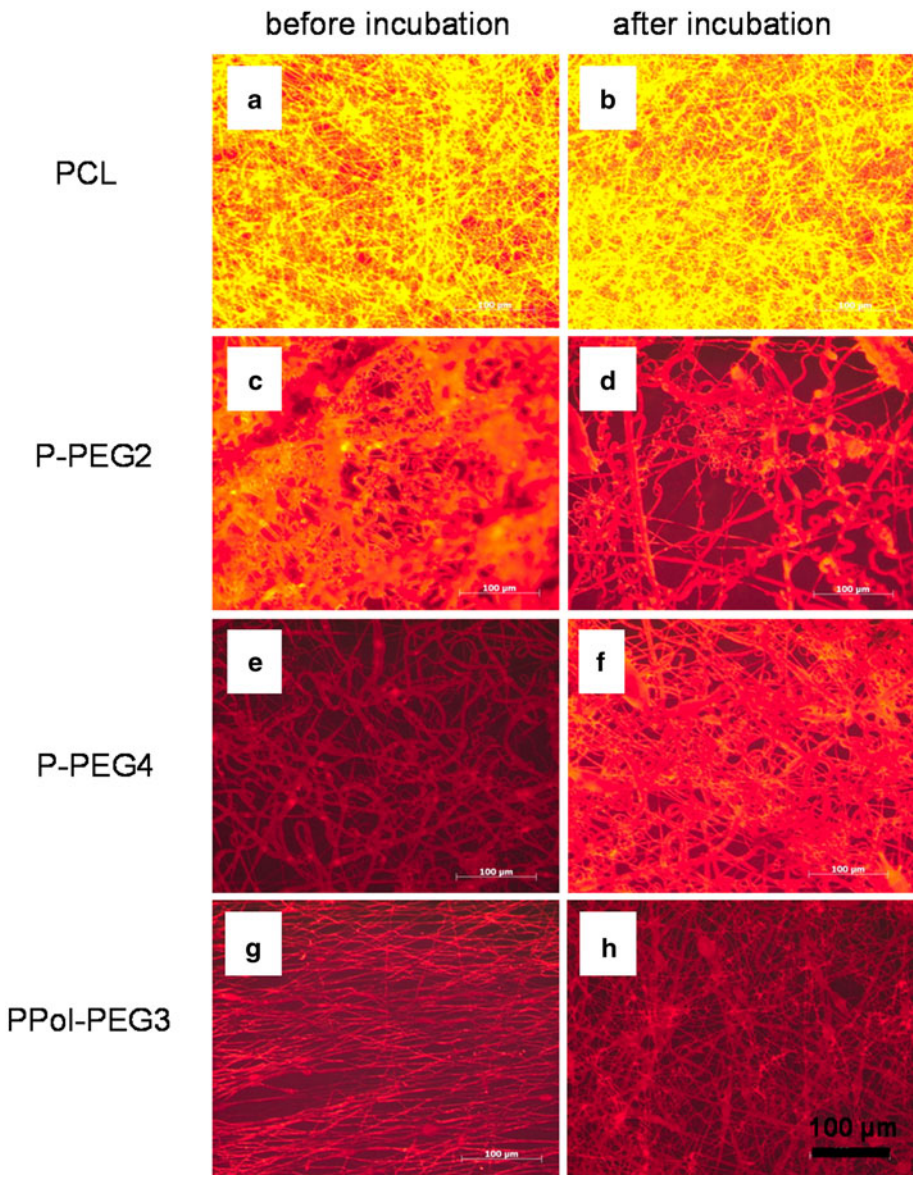

Fig. 6 Protein adsorption on fiber samples before and after incubation in water. **a** PCL fibers before incubation, **b** PCL fibers after incubation, **c** P-PEG2 before incubation, **d** P-PEG2 after incubation, **e** P-PEG4 before incubation, **f** P-PEG4 after incubation, **g** PPol-PEG3 before incubation, **h** PPol-PEG3 after incubation, all images were taken with the same exposure time of 20 s, scale bar = 100 µm

Table 6 XPS data showing atomic concentrations of elements from PCL and PCL/sPEG blends before and after incubation in water

	PCL	P-PEG2		P-PEG4		PPol-PEG3		PPol-PEG4
Incubation	Before	Before	After	Before	After	Before	After	Before
O	19.0	20.1	26.0	21.4	23.4	21.2	25.0	17.8
C	80.2	79.9	71.5	78.1	73.3	77.1	72.6	81.8
N	0.0	0.0	0.0	0.0	0.0	0.5	0.9	0.3
Si	0.8	0.0	2.5	0.6	3.3	1.3	1.4	0.0

without functionalization (Fig. 7c). With PPol-PEG4 + Fn fibers neurites detached from the substrates and therefore an analysis of axonal growth was not possible. However, Schwann cell migration did occur on these fibers (Fig. 8), and this was similar to GRGDS containing fibers. Schwann cells did not grow in a preferred direction but spread equally around the explant. The reason for this might be the fact that the fibers were not very parallel but frequently intersected (Fig. 5b).

Cell migration and neurite outgrowth were quantified (Fig. 9). Axonal growth was best on PPol-PEG3 + RGD fibers, where the longest 10% neurites reached a mean

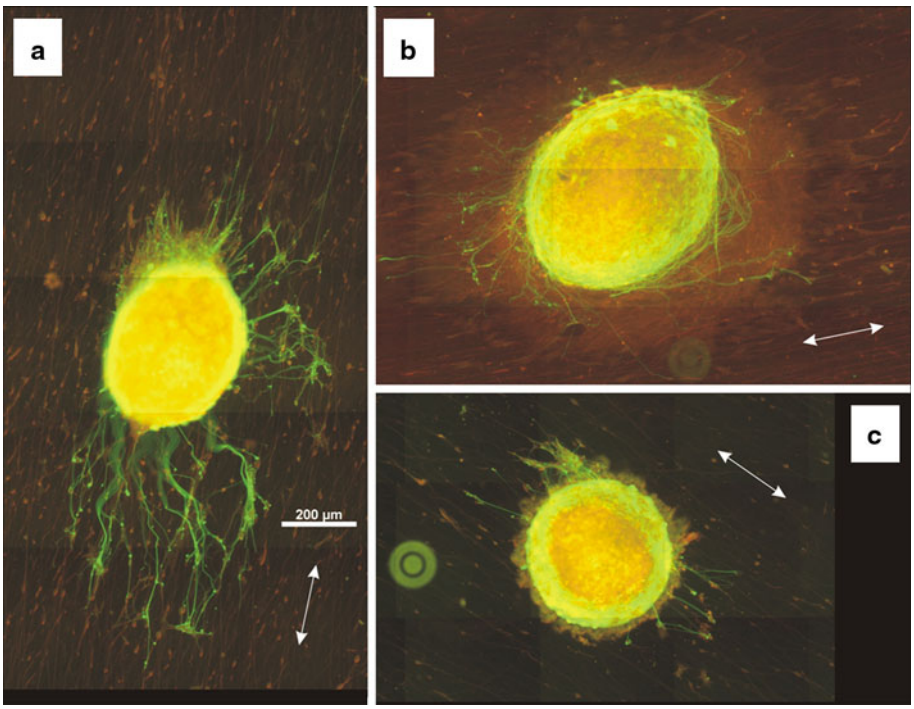

Fig. 7 Fluorescence images of DRG explants on different fibers, dorsal root ganglia were explanted from E10 chick embryos and placed onto **a** PPol-PEG3+RGD, **b** PCL, and **c** PPol-PEG3 fibers and incubated for 4 DIV, outgrowing axons were stained with NF200 antibody and Schwann cells were visualized by immunocytochemical staining with S100 the *arrows* indicate fiber orientation, scale bar = 200 µm

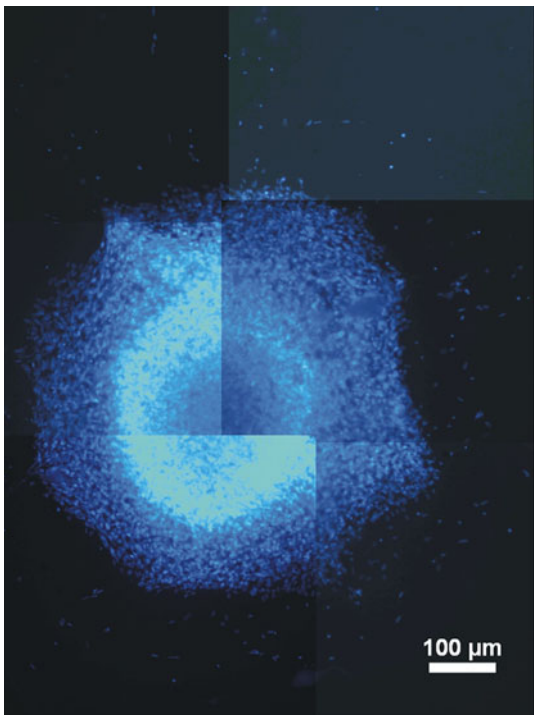

Fig. 8 Fluorescence image of DRG explant on PPol-PEG4 + Fn fibers, nuclei of migrating cells were stained with DAPI, the DRG explant itself, brightly stained in the centre, is surrounded by a dense halo of migrating cells (reminiscent of an egg, sunny side up), scale bar = 100 µm

length of 325 ± 84 µm (mean ± standard error of mean), while the 10% longest neurites on PCL fibers had 230 ± 34 µm and only 140 ± 30 µm on PPol-PEG3

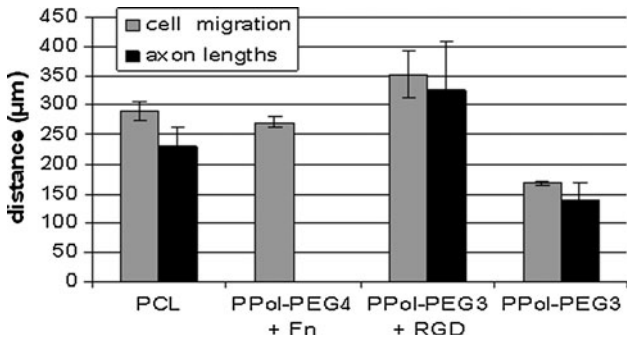

Fig. 9 Length of outgrowing axons and distance of Schwann cell migration from DRG explants on different fiber materials, after 4 DIV the distance of 10% cells that had migrated farthest from the explants and the lengths of the 20 longest axons/explant were measured, *t*-test, $P < 0.05$, *error bars* standard error of the mean

fibers. To assess Schwann cell migration, the distances of the 20 cells that had migrated farthest from every explant was determined. Similar to the effect on axons, migration distances were largest on PPol-PEG3 + RGD fibers (352 ± 84 µm), and slightly shorter on PPol-PEG4 + Fn fibers (272 ± 10 µm) and on PCL fibers (290 ± 16 µm) and reached only half of this value on non-functionalized PPol-PEG3 fibers (168 ± 5 µm). With inspection after 4 DIV, it appears that cell migration occurred within the same time frame as axonal growth. The results also show that functionalization of PPol-PEG3 with the GRGDS peptide substantially improved their biological properties.

4 Discussion

4.1 Electrospinning and fiber characterization

Fibers consisting of PCL and sPEG were produced, analyzed, and tested using in vitro assays in this study. It is already known that polyethylene glycol containing fibers [12, 16] as well as surfaces covered with sPEG [17] prevent unspecific protein adsorption and cell adhesion. Here these properties of sPEG were exploited to produce electrospun microfibers that can guide cell movement but are resistant to non-specific adsorption. Compared to the previously described approach, this process leads to functionalizable fibers in a simple manner, providing similar advantages of the minimized unspecific protein adsorption without the time consuming block copolymer synthesis. A more detailed comparison of these approaches is presented elsewhere [18].

Since electrospun fibers cannot be generated using pure sPEG (due to its low molecular weight) PCL was added to the electrospinning solution. It is likely that a chemical reaction occurred between hydroxyl groups of PCL and the isocyanate groups of the sPEG polymer, resulting in the formation of urethane groups and the building of a block copolymer. Although sPEG is, to a certain extent, soluble in aqueous solutions, the above reaction should have prevented its diffusion into the cell culture medium. In the hypothesized reaction, schematically shown in Fig. 1, both peptide- and protein functionalization may occur by the formation of urea groups resulting from the reaction of amine groups of the peptide/protein with the isocyanate groups of sPEG.

Electrospinning of two different PCL/sPEG fiber types was performed. The first type consisted of high molecular weight PCL, mixed with sPEG (P-PEG). The second type contained in addition low molecular weight PCL-diol (PPol-PEG). The higher amount of hydroxyl groups in the latter fiber type offered more binding sites for reactions with the isocyanate groups.

For electrospinning of the P-PEG fibers two different solvent compositions were assessed, consisting of acetone/DCM mixed with either DMSO or NMP. At the lower sPEG concentration of 15 wt%, small oriented fibers could be produced from both solvent mixtures (Fig. 2a, b). Since protein adsorption (Fig. 6) was not effectively prevented by the low sPEG concentration, a higher concentration of 27 wt% was investigated. While this resulted in a fibrous network when using DMSO-containing solvent (Fig. 2c) good quality fibers were generated using NMP-based solvent (Fig. 2d). Although the amount of these two solvents was small, their properties influenced the electrospinning process significantly. The values for boiling point, vapor pressure, and surface tension of the solvents have been presented in table 1. Low boiling temperatures and high vapor pressures induce fast solvent evaporation and therefore were expected to result in high quality fibers. Slow evaporation of the solvent may prevent its complete removal during the electrospinning process, resulting in the deposition of "wet" fibers which may form meshes of fused fibers on the target [19]. As NMP has a higher boiling point and a lower vapor pressure than DMSO, the results observed in this study appear to contradict this interpretation. However, the surface tension of the solvent also plays an important role. The influence of the surface tension of a solvent is a controversial subject in the literature: some authors argued that reducing surface tension may result in higher fiber quality [20] while others reported that increased surface tension generated higher quality fibers [21]. Our findings confirm former results [22] that for each polymer composition specific electrospinning conditions have to be evaluated empirically, and that knowing the polymer and solvent parameters is not enough to assess the ability of electrospinning.

Based on the data obtained from the P-PEG type fibers, the best solvent combination (acetone/DCM/NMP) was used for the PPol-PEG fiber system. This system also included the additional step of using THF to pre-dissolve the sPEG. With this solvent mixture, good oriented electrospun fibers could be generated using a variety of concentrations (Fig. 3). However, increasing the sPEG concentration above 30 wt% resulted in a solution that was too viscous and which could not be electrospun. Therefore, the mixtures with 27 and 29.5 wt% sPEG were chosen for further experiments to maximally exploit the protein repellent properties of sPEG. Inclusion of the peptide sequence GRGDS or the protein fibronectin did not affect the ability to electrospin high quality fibers (Figs. 4, 5). The calculation of volume porosity confirmed the preparation of single fibers which were separated from each other. The area which was covered with fibers was between 6 and 19% for the different fibers materials (table 5). This high variation between the different samples may be the consequence of different fiber collection times during the electrospinning process. Higher collection times resulted in higher fiber densities.

The electrospun fibers from both systems were analyzed in several ways. The contact angles of PPol-PEG3 and PPol-PEG4 fibers could not be measured because the water droplet was fully adsorbed after its deposition on the fibers. This indicated that hydrophobic PCL was gathered inside the fibers while sPEG was at the surface, resulting in highly hydrophilic fibers. PPol-PEG3 fibers were the only ones which showed reduced protein adsorption before and after incubation in water, in comparison to the PCL fibers. In P-PEG2 the sPEG content noticeably reduced protein adsorption compared to PCL fibers. In P-PEG4 fibers, the

protein repelling effect of sPEG appeared to be only partial because sPEG was washed out by incubation in water. With PPol-PEG3 fibers the protein adsorption was reduced most as visualized by fluorescence microscopy (Fig. 6). This effect was stable during incubation with water. It is known from literature that PCL fibers degrade very slowly. Within 6 month incubation in Ringer solution only very little loss of molecular weight was observed [23]. Long-term degradation assays with electrospun poly(D,L-lactide-co-glycolide)/sPEG (PLGA/sPEG) fibers showed only a minor accelerated degradation in comparison to pure PLGA fibers [18]. These results give an indication about the stability of the PCL/sPEG fibers that should be similar to pure PCL. As an indication, microscopy images of the incubated fibers did not demonstrate structural changes of the fibers within the time of incubation. However, this conclusion has to be proved in in vitro experiments.

In XPS measurements only PPol-PEG3 and PPol-PEG4 fibers demonstrated nitrogen at their surfaces (table 6). Here, the nitrogen content was interpreted as an indicator for the existence of sPEG on the surface of the fibers due to the formation of urethane groups. Interestingly, the nitrogen content of PPol-PEG3 fibers increased slightly after incubation in water, which might be the result of enrichment of sPEG molecules at the fiber surface in an aqueous environment due to the higher hydrophilicity of the sPEG chains compared with PCL polymer chains. Some fibers were contaminated with silicon which is a common impurity caused by grease from the environment in chemical laboratories.

The surface analysis of the different fibers showed that a high amount of sPEG in the electrospinning solution was necessary to overcome the protein adsorption effects of PCL. The PPol-PEG system seems to be more effective in binding sPEG to PCL, possibly due to the much higher amount of hydroxyl groups in this system. As PPol-PEG3 was the most successful in fulfilling the desired properties, in vitro assays were performed to investigate cell-substrate interaction using these fibers, as well as parallel in vitro experiments using PPol-PEG4 + Fn fibers.

4.2 Cell experiments

Cell experiments were performed on PCL, PPol-PEG3, PPol-PEG3 + RGD, and PPol-PEG4 + Fn fibers. DRG explants were placed onto the fibers and incubated for 4 DIV. Measurements of the orientation distribution of these fibers demonstrated that only around 50% of the PPol-PEG3 fibers and 63% of the PPol-PEG4 fibers were exactly parallel to each other (table 5). This resulted in intersections of the electrospun fibers. When this occurred neurites were observed to switch between fibers (Fig. 7). Neurites followed the fibers, irrespectively from their orientation which may result in the reduction of total speed of neurite growing in comparison with totally parallel aligned fibers. Thus, further research is required to increase the parallelism of the fibers.

Protein adsorption data suggested that PPol-PEG3 fibers might reduce inflammatory reactions or encapsulation in vivo [8, 9]. In addition, preventing adhesion of proteins that inhibit axonal growth, e.g., components of CNS myelin [24, 25] may be important for axonal regeneration. Non-adhesive substrates, such as PEG-containing microfibers, might on the other hand not be well suited as substrates for glia cells and axons. The tests with DRG explants confirmed the expected properties of PPol-PEG3 fibers: although DRG adhered to these substrates, only 20% of the explants demonstrated cell migration and axonal outgrowth (Fig. 7c). This property was overcome by the incorporation of integrin activating molecules to the fibers, GRGDS peptide as well as fibronectin (Figs. 7a, 9). In vitro experiments with electrospun fibers of PEO-b-PCL demonstrated similar results as described before. These fibers showed cell repellent properties due to the hydrophilicity of the PEO block at the fiber surface. By functionlization with the peptide sequence GRGDS this property was overcome and cell attached nicely to the fibers. However, in that study, human dermal fibroblasts were used on random fibers without investigating guidance properties of the fibers [16]. Therefore, a direct comparison of the results in both studies is not possible, due to the use of different cell types and different fiber orientation.

Stimulation of Schwann cells proliferation supports neurite outgrowth and axon regeneration after injuries [26, 27]. Therefore, a bridging substrate for peripheral nerve repair should possess properties that encourage the growth promoting physiology of Schwann cells. Other groups have included peptide sequences from laminin [28, 29] or growth factors [30] into biomaterial to achieve this effect. We show here that isocyanate groups of the sPEG offer the possibility to insert such functional molecules into electrospun fibers without overcoming their protein repellence. The inclusion of an ECM derived peptide, GRGDS, or a whole protein, fibronectin, to the PCL/fibers stimulated axon growth and Schwann cell migration. These two methods offer specific advantages. Peptide sequences are small molecules which are relatively easy to handle. They are often more easily solubilized than proteins and are more resistant to hydrolysis and temperature induced degradation. On the other hand, proteins mimic the natural state of the ECM more closely than peptides. They are often involved in a wide range of cellular processes and offer several binding sites for a number of cell types. Fibronectin has two major heparin-binding sites, a collagen-binding domain, and more than one cell attachment binding site for integrin receptors. In addition to the

well-known RGD sequence, a second, distinct region that acts synergistically with RGD has been identified. It is the peptide sequence Pro-His-Ser-Arg-Asn (PHSRN). Both are important for cell adhesion and migration that is mediated by integrin receptors. The alternatively spliced IIICS segment acts independently from both former mentioned sequences and contains another cell adhesive region. It is primarily recognized by cells of neural crest origin [31, 32]. Surfaces patterned with fibronectin or its binding-domain peptide (RGD) have been shown to enhance neuronal outgrowth in comparison to controls lacking such molecules [33].

Although the cellular response towards the PPol-PEG3 + RGD and PPol-PEG4 + Fn fibers did not induce Schwann cell migration to a significantly larger extent than simple PCL fibers, the first two mentioned fiber types posses two main advantages over pure PCL. They minimize unspecific protein adsorption, and they can be further modified by the incorporation of other molecules with specific biofunctionalities. Other examples of fiber functionalization has been the generation of blended PCL/collagen fibers [2] or poly(L-lactide) functionalized with basic fibroblast growth factor [30]. Such approaches demonstrated greater cell migration and axonal outgrowth, than observed in the present investigation (using GRGDS- or fibronectin-functionalized fibers), however, previous publications did not address the possible problems associated with unspecific protein adsorption.

The beneficial effects of biofunctionalization of the PCL/sPEG fibers might be improved by increasing the concentration of such bioactive molecules. In the present study, GRGDS was bound to approximately 20% of the sPEG molecules. Groll and colleagues demonstrated with GRGDS-functionalized sPEG surfaces that the binding of 0.5–1 GRGDS molecule per sPEG molecule is very effective for adhesion of SaOS cells (human epithelial-like osteosarcoma cells) [17]. Future studies will have to show whether increasing the GRGDS concentration enhances the biological activity of functionalized PPol-PEG fibers with respect to neuronal cells. Fibronectin was added with a ration of sPEG to Fn to be 2 to 1. In comparison with the molecular weight of fibronectin ($\approx$400 kDa), the active sequences such as RGD and its synergistic region were included in a very small amount. Higher protein concentrations will be tested in the future and comparisons with a range of different RGD concentrations and other peptides and the potency of this approach will be assessed further.

Another important step will be the development of three-dimensional scaffolds that incorporate biofunctionalized fibers. Simple nerve conduits consisting of hollow tubes have already implanted several hundreds patients with peripheral nerve injuries [34].With this approach a length of about 30 mm is the longest gap that can be bridged. However, animal experiments indicate that regeneration can be improved when nerve conduits contain longitudinal guiding structures [35, 36]. Some investigations have already incorporated electrospun fibers or similar filaments in a 3D configuration in nerve conduits [37–39]. Therefore, we anticipate that artificial nerve implants that can substitute autologous nerve transplantation will have to combine topographical cues, such as electrospun fibers in a 3D matrix, with molecular signals, such as integrin activating peptides.

5 Conclusion

The electrospinning technique was used to produce orientated microfibers of PCL/sPEG that could be functionalized with bioactive peptides or proteins from the ECM. A preparation consisting of 27 wt% sPEG, PCL, and PCL-diol (PPol-PEG3) minimized protein adsorption and resulted in the presence of sPEG at the surface of aligned fibers. In experiments with DRG explants, non-functionalized PPol-PEG3 fibers supported little cellular migration and slow axonal outgrowth. However, functionalization of PPol-PEG3 with the peptide sequence GRGDS strongly supported these cellular interactions. Experiments with fibronectin containing fibers showed an advantage over PPol-PEG3 only with respect to Schwann cell migration. Thus, the PPol-PEG3 + RGD fibers demonstrated their potential usefulness for promoting functional repair by supporting more specific cell-substrate interactions than would be expected if non-protein repellent materials had been chosen as the substrate.

Acknowledgments We thank Marie Pradella for help with evaluating the cell experiments. Kristin Michael and Bernd Hoffmann kindly allowed us to use their equipment for making composite photographs of DRG explants. This work was supported by a grant from the Interdisciplinary Centre for Clinical Research "BIOMAT" within the faculty of Medicine at Aachen University (RWTH) (TV B111) and by DFG-Graduiertenkolleg 1035 "Biointerface". Julia Bockelmann was supported by a Marie-Curie EST grant from the EU (EURON).

References

1. Lietz M, Dreesmann L, Hoss M, Oberhoffner S, Schlosshauer B. Neuro tissue engineering of glial nerve guides and the impact of different cell types. Biomaterials. 2006;27:1425–36.
2. Schnell E, Klinkhammer K, Balzer S, Brook G, Klee D, Dalton P, Mey J. Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly-ε-caprolactone and a collagen/poly-ε-caprolactone blend. Biomaterials. 2007;28(19):3012–25.
3. Smeal RM, Rabbitt R, Biran R, Tresco PA. Substrate curvature influences the direction of nerve outgrowth. Ann Biomed Eng. 2005;33(3):376–82.

4. Li D, Xia YN. Electrospinning of nanofibers: reinventing the wheel? Adv Mater. 2004;16(14):1151–70.
5. Huang ZM, Zhang YZ, Kotaki M, Ramakrishna S. A review on polymer nanofibers by electrospinning and their applications in nanocomposites. Compos Sci Technol. 2003;63(15):2223–53.
6. Klinkhammer K, Seiler N, Grafahrend D, Gerardo-Nava J, Mey J, Brook GA, Möller M, Dalton PD, Klee D. Deposition of electrospun fibers on reactive substrates for in vitro investigations. Tissue Eng. 2009;15(1):77–85.
7. Horbett TA. The role of adsorbed proteins in tissue response to biomaterials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials science: an introduction to materials in medicine. San Diego: Elsevier; 2004. p. 237–40.
8. Tang L, Jennings TA, Eaton JW. Mast cells mediate acute inflammatory responses to implanted biomaterials. PNAS. 1998;95:8841–6.
9. Johnson R, Harrison D, Tucci M, Tsao A, Lemos M, Puckett A, Hughes JL, Benghuzzi H. Fibrous capsule formation in response to ultrahigh molecular weight polyethylene treated with peptides that influence adhesion. Biomed Sci Instrum. 1997;34:47–52.
10. Jeon SI, Lee JH, Andrade JD, De Gennes PG. Protein-surface interactions in the presence of polyethylene oxide: I. Simplified theory. J Colloid Interface Sci. 1991;142:149–58.
11. Sharma S, Johnson RW, Desai TA. Evaluation of the stability of nonfouling ultrathin poly(ethylene glycol) films for silicon-based microdevices. Langmuir. 2004;20(2):348–56.
12. Grafahrend D, Calvet JL, Klinkhammer K, Salber J, Dalton PD, Moller M, Klee D. Control of protein adsorption on functionalized electrospun fibers. Biotechnol Bioeng. 2008;101(3):609–21.
13. Gasteier P, Reska A, Schulte P, Salber J, Offenhausser A, Moeller M, Groll J. Surface grafting of PEO-based star-shaped molecules for bioanalytical and biomedical applications. Macromol Biosci. 2007;7(8):1010–23.
14. Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials. 2003;24:4385–415.
15. Goetz H, Beginn U, Bartelink CF, Grunbauer HJM, Möller M. Preparation of isophorone diisocyanate terminated star polyethers. Macromol Mater Eng. 2002;287(4):223–30.
16. Grafahrend D, Calvet JL, Salber J, Dalton PD, Moeller M, Klee D. Biofunctionalized poly(ethylene glycol)-block-poly(epsilon-caprolactone) nanofibers for tissue engineering. J Mater Sci Mater Med. 2008;19(4):1479–84.
17. Groll J, Fiedler J, Engelhard E, Ameringer T, Tugulu S, Klok HA, Brenner RE, Moeller M. A novel star PEG-derived surface coating for specific cell adhesion. J Biomed Mater Res Part A. 2005;74A(4):607–17.
18. Grafahrend D, Gasteier P, Heffels K-H, Beer M, Dalton PD, Moeller M, Groll J. Transforming the surface chemistry of degradable polymer scaffolds with a reactive hydrophilic monomer additive. Submitted.
19. Hsu C-M, Shivkumar S. Nano-sized beads and porous fiber constructs of poly(ε-caprolactone) produced by electrospinning. J Mater Sci. 2004;39(9):3003–13.
20. Doshi J, Reneker DH. Electrospinning process and applications of electrospun fibers. J Electrost. 1995;35(2–3):151–60.
21. Liu HQ, Hsieh YL. Ultrafine fibrous cellulose membranes from electrospinning of cellulose acetate. J Polym Sci Part B Polym Phys. 2002;40:2119–29.
22. Andrady AL. Science and technology of polymer nanofibers. Hoboken: Wiley; 2008. p. 94.
23. Bölgen N, Menceloglu YZ, Acatay K, Vargel I, Piskin E. In vitro and in vivo degradation of non-woven materials made of poly(ε-caprolactone) and nanofibers prepared by electrospinning under different conditions. J Biomater Sci Polym Ed. 2005;16(12):1537–55.
24. Park JB, Yiu G, Kaneko S, Wang J, Chang J, He Z. A TNF receptor family member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. Neuron. 2005;45:345–51.
25. Vyas AA, Blixt O, Paulson JC, Schnaar RL. Potent glycan inhibitors of myelin-associated glycoprotein enhance axon outgrowth in vitro. J Biol Chem. 2005;280(16):16305–10.
26. Filbin MT, Qiu J, Cai D. Glial influences on axonal regeneration. In: Jessen KR, Richardson WD, editors. Glial cell development-basic principles and clinical relevance. Oxford: Oxford University Press; 2001. p. 279–98.
27. Lisak RP, Skundric D, Bealmear B, Ragheb S. The role of cytokines in Schwann cell damage, protection, and repair. J Infect Dis. 1997;176:S173–9.
28. Schense JC, Bloch J, Aebischer P, Hubbell JA. Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension. Nat Biotechnol. 2000;18:415–9.
29. Tong YW, Shoichet MS. Enhancing the neuronal interaction on fluoropolymer surfaces with mixed peptides or spacer group linkers. Biomaterials. 2001;22:1029–34.
30. Patel S, Kurpinski K, Quigley R, Gao H, Hsiao BS, Poo M-M, Li S. Bioactive nanofibers: synergistic effects of nanotopography and chemical signaling on cell guidance. Nano Lett. 2007;7(7):2122–8.
31. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci. 2002;115:3861–3.
32. Akiyama SK, LaFlamme SE. Bioadhesion and cell behavior. Colloids Surf B Biointerfaces. 1994;2:241–50.
33. Zhang Z, Yoo R, Wells M, Beebe TP Jr, Biran R, Tresco P. Neurite outgrowth on well-characterized surfaces: preparation and characterization of chemically and spatially controlled fibronectin and RGD substrates with good bioactivity. Biomaterials. 2005;26:47–61.
34. Schlosshauer B, Dreesmann L, Schaller H-E, Sinis N. Synthetic nerve guide implants in humans: a comprehensive survey. Neurosurgery. 2006;59:470–8.
35. Möllers S, Heschel I, Damink LH, Schugner F, Deumens R, Muller B, Bozkurt A, Nava JG, Noth J, Brook GA. Cytocompatibility of a novel, longitudinally microstructured collagen scaffold intended for the nerve tissue repair. Tissue Eng Part A. 2009;15:461–72.
36. Yu TT, Shoicht MS. Guided cell adhesion and outgrowth in peptide-modified channels for tissue engineering. Biomaterials. 2005;26:1507–14.
37. Kim YT, Haftel VK, Kumar S, Bellamkonda RV. The role of aligned polymer fiber-based constructs in the bridging of long peripheral nerve gaps. Biomaterials. 2008;29:3117–27.
38. Lundborg G, Dahlin L, Dohi D, Kanje M, Terada N. A new type of "bioartificial" nerve graft for bridging extended defects in nerves. J Hand Surg (British and European Volume). 1997;22B:299–303.
39. Matsumoto K, Ohnishi K, Kiyotani T, Sekine T, Ueda H, Nakamura T, Endo Y, Shimizu Y. Peripheral nerve regeneration across an 80-mm gap bridged by a polyglycolic acid (PGA)-collagen tube filled with laminin-coated collagen fibers: a histological and electrophysical evaluation of regenerated nerves. Brain Res. 2000;868:315–28.