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Neuronal and glial responses to siRNA-coated nerve guide implants *in vitro*

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ABSTRACT

The manipulation of gene expression by RNA interference could play a key role in future neurotherapies, for example in the development of biohydrid implants to bridge nerve and spinal cord lesion gaps. Such resorbable biomaterial prostheses could serve as growth substrates together with specific siRNA to foster neuronal regeneration. To the best of our knowledge, we are the first to biofunctionalize neuronal prostheses with siRNA. We analyzed neuronal and Schwann cell responses to scrambled siRNA coated polydioxanone polymer filaments designed to imitate pro-regenerative bands of Büngner for oriented axonal regrowth. With a view to future clinical applications we were especially interested in potentially detrimental side effects. We employed a variety of *in vitro* methods, including a novel impedance electrode microchamber assay, fluorescence and scanning electron microscopy, metabolic labeling and RT-PCR. We found that the application of chitosan/siRNA nanoparticles (1) did not affect glial cell motility or (2) axonal growth in contrast to other formulations, (3) only slightly reduced proliferation, and (4) did not induce inflammatory responses that might hamper axonal regeneration. The data suggest that chitosan/siRNA nanoparticle-coated polymer filaments are suitable for use in biohybrid implants with no significant side effects on neuronal and glial cells.

Once RNA interference was recognized as a highly selective and powerful native mechanism of posttranscriptional gene regulation, the application of small interfering RNA (siRNA) rapidly found its place in the methodological toolkit of experimental medicine. Therapeutically active siRNA specimens have already been employed in clinical trials, for example counteracting exuberant angiogenesis via vascular endothelial growth factor suppression in macula degeneration of the retina, respiratory syncytial virus infection via nucleocapsid "N" gene silencing, or liver cancer targeting the kinesin spindle protein that is instrumental in cell proliferation [14]. Furthermore, preclinal investigations to suppress huntingtin in the central nervous system [5] or to halt amyotrophic lateral sclerosis [10] have been published. We have combined materials science and bioscience to develop biohybrid implants based on the combination of resorbable biomaterial microstructures and instructive biomolecules. In order to bridge lesion gaps in peripheral nerves or cysts in traumatized spinal cord, tubular implants were produced from polycaprolactone trimethylene carbonate and implanted in the spinal cord and nervus medianus [4,12]. In addition, hundreds of polymer filaments with longitudinal microgrooves have been successfully introduced into lesioned sciatic nerves in order to induce artificial Schwann cell strands (bands of Büngner) and thus, imitate the native microarchitecture of regenerating nerves [2,8]. In compressed nerves, thousands of such bands of Büngner provide highly oriented guidance cues and metabolically stimulate axon regeneration. Since purely material-based implants are able to overcome physical restrictions such as lesion gaps, but cannot counteract biological inhibition based on inhibitory/repulsive myelin and matrix components [9], we have started to biologically functionalize neuro-implants with nanoparticles composed of siRNA and chitosan, the deacetylated polysaccharide chitin. However, it is important to rule out potential detrimental side effects. Double stranded siRNA could stimulate Toll-like receptors and cause failure in axonal outgrowth, inflammatory responses or a deregulation of cell proliferation and migration, all of which are crucial for the formation of bands of Büngner. For future clinical approval we have adapted our research strategy to simultaneously investigate therapeutic- and side effects.

As described recently, endless synthetic filaments (diameter 28 μm) were produced of resorbable poly-p-dioxanone (PDO) with six longitudinal grooves (groove depth 9 μm) by melt extrusion [8]. PDO filaments were coated with 50 μg PDL/ml (30 min, 37 °C).

Chitosan 140 (molecular weight (MW) 140 kDa) and 250 (250 kDa) (Protosan UP B 80/20 and 80/200 Novamatrix, Norway)

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were deacetylated to 98%. They were dissolved in 300 mM sodium acetate buffer (pH 4.5) at 1 mg/ml concentration, as described previously. After the complete dissolution, the pH was adjusted to 5.5. Chitosan 78 (MW 78 kDa, degree of deacetylation 88%) was kindly provided by A. P. Pêgo (INEB, Portugal) [7].

Chitosan/siRNA particles were prepared by adding mismatch siRNA (scr, Dharmacon, DY547-AGUUAGUAGACCAUGGAGAGUUU) containing a fluorescence DY547-labeled strand to a chitosan solution either in 0.3 M or 5 mM acetate buffer (pH 5.5) with N:P ratios of 10 (Chitosan 250), 50 (Chitosan 78) or 60 (Chitosan 140). (N:P ratio: number of moles of primary amine groups of chitosan (N) and the number of moles of phosphate groups (P) of siRNA). Chitosan 140/250 nanoparticles were formed by mixing for 1 h at RT. Chitosan 78 nanoparticles were formed by mixing 10 s at RT and complexes were allowed to form for 30 min at RT before further use. All chitosan/siRNA particles were used for cell transfection with a final siRNA concentration of 200 nM. For immobilization on polymer filaments, particle suspensions containing 10% sucrose were plated on polymer filaments and frozen at −20 °C before lyophilization overnight at ≤0.2 mbar using a Freeze Dryer Alpha 2-4 (Christ, Osterode, Germany).

siRNA-lipofectamine particles were made according to the manufacturer's instructions. Briefly, diluted lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) and diluted siRNA (both in cell culture medium without antibiotics) were mixed gently and incubated for 30 min at RT to form lipoplexes. Lipofectamine micelles were used for cell transfection at a final siRNA concentration of 5 and 200 nM. Cell culture medium was replaced 6 h after transfection.

(A) Schwann cells were purified from the sciatic nerves of adult Lewis rats described earlier [1]. (B) Axon outgrowth and growth cone collapse of cultured dorsal root ganglia (DRG) were quantified as described elsewhere [8]. (C) 3D-glia-neuron co-cultures were established by seeding Schwann cells onto PDL coated polymer filaments for 24 h. In some cases intact DRG explants were then positioned on the Schwann cell-coated polymer filaments. Co-culture was continued for another 48 h before fixation. (D) Cell migration assays were performed in a novel microelectrode device (ECIS™ – Electrical Cell substrate Impedance Sensing; Applied Biophysics, USA). In chamber slides with integrated substrate electrodes, confluent Schwann cell monolayers were selectively lesioned in a spatially controlled fashion with a short-term, high-frequency current (30,000 HZ, 3000 pA, 30 s), and the subsequent cell migration onto and repopulation of the electrode was monitored (www.biophysics.com). Impedance was monitored online every 60 min at 2000 Hz over a 1-week period under physiological conditions (10% CO_2, 37 °C) without any interruption of cell culturing. (E) The proliferation rate of the cells was analyzed by metabolic labeling with 5-bromo-2′-deoxy-uridine (BrdU; 5 h, 37 °C, 10% CO_2) 24 h after transfection, antibody- and DNA staining (DAPI: 4,6-diamidino-2-phenylindol).

Cell type-specific immunostaining was performed with paraformaldehyde-fixed specimens using primary antibodies specific for S100 (1:300; Sigma–Aldrich, USA) and neurofilament (1:5000; SMI 31 Sternberger Monoclonals, USA), plus fluorescence-labeled secondary antisera (Alexa488- and Cy3-labeled goat anti-mouse and anti-rabbit (Jackson ImmunoResearch, USA). Ultrastructural analysis of PDO filaments was carried out by scanning electron microscopy (Zeiss Germany DSM 950) after sputtering with palladium/gold (20/80).

Schwann cells were exposed to chitosan/siRNA nanoparticles, lipofectamine/siRNA or lipofectamine/polyinosinic-polycytidylic acid (poly(I:C) = PIC); Invivogen, USA) The expression levels of the target (iNOS) and a housekeeping gene (GAPDH) were determined using the TaqMan® Gene Expression Cells-to-CT™ kit (Applied Biosystems, Darmstadt, Germany). Amplification and quantification was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Statistical analysis was based on at least three independent series of experiments with each series run in duplicate, using one-way ANOVA followed by a Tukey HSD posttest comparison. Data (mean ± s.d.) were considered to be statistically significant when the p-value was <0.05.

In order to directly bridge lesion gaps and/or provide multiple growth substrata in hollow nerve guide tube implants *in vivo*, we produced polydioxanone (PDO) filaments of 28 μm diameter. Oriented regrowth of cells was fostered by integrating six longitudinal grooves (9 μm depth) into the filament design (Fig. 1A). As intended, the topography of the filaments directly affected the attached cells. After 1–2 days *in vitro* essentially all Schwann cells added onto the polymer filaments displayed an elongated morphology (Fig. 1B) resembling *in vivo*-like bands of Büngner. In addition, the cationized polymer surface also allowed axonal outgrowth. As depicted in Fig. 1C and D axons of dorsal root ganglia (green profiles) extended in a highly oriented manner, especially when polymer filaments were precoated with Schwann cells (blue profiles). In addition, PDO filaments were successfully coated with (red-labeled) siRNA, which was shown to be taken up by the cells growing on the filament surface (Fig. 1E, encircled cell).

Since it has been shown that axonal outgrowth can be hampered by double-stranded RNA, we set out to determine whether the novel siRNA nanoparticle formulation that we used for PDO filament coating would influence axon extension. Microscopic analysis *in vitro* after βIII-tubulin immunostaining revealed intact lamellopodia-rich growth cone morphologies following chitosan/siRNA application (Fig. 2A). This is in contrast to lipofectamine/siRNA and synthetic dsRNA analogue poly:IC (Fig. 2B). Quantification substantiated the qualitative data, as indicated by highly significant differences (Fig. 2C). Similar results were obtained with regard to axonal outgrowth length (Fig. 2D–F). In summary, the data suggest that our chitosan/siRNA nanoparticle formulation has little effect on axonal outgrowth, in contrast to other formulations.

Migratory behavior was analyzed with a novel micro-electrode chamber which measured changes in the electrical impedance of a cell layer grown on electrodes used as substrate (Fig. 3A). When confluent Schwann cell monolayers were exposed to a short, destructive current pulse, impedance declined instantaneously (arrow in Fig. 3B). Online monitoring indicated that in control cultures impedance recovered over the next 2 days, indicating repopulation of the electrode surface. Chitosan/siRNA nanoparticle-transfected cells recovered somewhat slower but finally reached a similar high plateau as controls (Fig. 3B, red and black lines). In contrast to controls, lipofectamine/siRNA-transfected Schwann cells displayed diminished impedance values at all time points (Fig. 3B). Like cell migration, cell proliferation is crucial for the formation of bands of Büngner. BrdU labeling (Fig. 3C, green cells) and subsequent microscopic image analysis revealed that chitosan/siRNA nanoparticles caused some reduction of Schwann cell proliferation in the case of both of the chitosan fractions that we analyzed (Fig. 3D). Lipofectamine transfection resulted in a higher mitotic index. Anti-mitotic cisplatin was used as a negative control.

The release of inflammatory factors from stimulated Schwann cells could hamper subsequent neuronal regeneration. Since inducible nitric oxide synthase (iNOS) is considered to be an inflammatory key element and therefore indicative of inflammatory cell responses, we analyzed the expression levels of iNOS. GAPDH served as an internal control (Fig. 4A). Quantification revealed that none of the transfection protocols employing siRNA induced NOS expression, in contrast to the nearly fivefold iNOS induction by the synthetic double-stranded RNA analogue poly(I:C) (LF/PIC) used as positive control (Fig. 4B).