Electrospun microfiber tubes and self-assembling peptides stimulate neural regeneration in rat sciatic nerve transections

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ABSTRACT

Although many nerve prostheses have been proposed peripheral nerve injury is still a pathology that may impair patient's movements. New synthetic nerve grafts comprising electrospun tubes and tailored self-assembling peptides are used in this study to regenerate 1 cm nerve gap of rat sciatic nerve in vivo. Experimental groups comprise lesioned animals (control), lesioned animals subjected to guide conduits implantation filled with gels or saline solution. Three months after surgery in control group sciatic nerves failed to reconnect the two stumps of transected nerve. In treated animals our experiments showed a significant regeneration and functional reconnection of the stumps of lesion. Neural tracers revealed the re-establishment of functional neuronal connections between the proximal and distal stumps of the lesions in animals subjected to the complete treatment. Our approach is the first neural regenerative strategy making jointly use of different nanotechnology techniques. A similar approach can be adapted to regenerate lesions in tissues like skin and bone.

Keywords: self-assembling peptide, scaffold, electrospinning, sciatic nerve, functional motifs.

1 INTRODUCTION

Many nerve prostheses have been proposed in the last years and self-transplantation from other donor nerves is still the most effective strategy adopted in clinics in case of peripheral nerve injury. Nonetheless, in case of consistent loss of nerve tissue both nerve microsuture and transplantation are impractical, and, consequently, nerve transection is still a traumatic pathology that can impair patient's movements by interrupting his motor-sensory pathways.

Self-assembling peptides are a class of nanostructured biomaterials that spontaneously self-assemble upon exposure to body fluids or physiologic solutions. They can be functionalized with biologically active motifs at user's will to coax cells or tissues to a specific response for the desired applications. Functionalized self-assembling peptides have been recently proved to be promising substrates for 3D culturing of neural stem cells [1] and bone cells.

We here propose a preliminary study comprising composite scaffolds for nerve regeneration in lesioned rats. The supportive frame is a guide tube made of electrospun microfibers (diameter range 300nm-2um) of a PCL-PLGA blend while the inner lumen is filled with the functionalized self-assembling peptide RADA-BMHP1 [1]. Results showed promising neural reconnection of the sectioned stumps 3 months after surgery. Myelination of the regenerated fibers has been detected too.

2 MATERIALS AND METHODS

2.1 Self-assembling peptide scaffolds

The peptides used for this study were previously tested in in vitro experiments for NSCs adhesion and differentiation [1]. They were synthesized and purified by CASLO (Lingby, Denmark). Self-assembling peptides form nanofiber-made gels once exposed to physiological pH solutions. The alanines of the self-assembling sequence RADA16 providing hydrophobic interaction are on one side of the peptide, and the arginines and aspartates form complementary ionic bonds on the other [2].

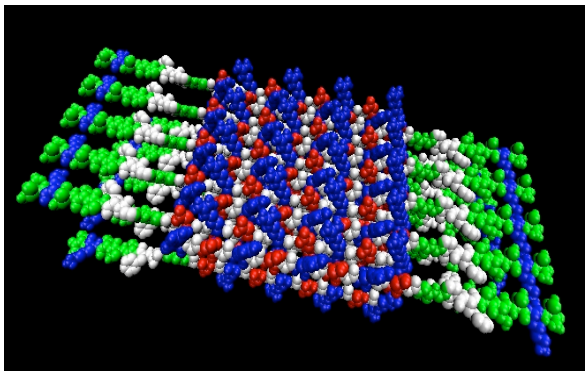

Figure 1: Molecular model of self-assembled peptide functionalized to enhance neural regeneration. The self-assembling sequence RADA16 is an alternating16-residue peptide with basic arginine (blue), hydrophobic alanine (white) and aspartic acid (red). Neutral polar residues are drawn in green.

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Once dissolved in acid solvents like distilled water an energy barrier due to electrostatic repulsion kinetically inhibits charged surfaces from assembling; but, if its amplitude is sufficiently decreased by screening the charges through the addition of salt or ions, the attraction force becomes dominant causing the surfaces to pack and self-assemble [3].

The BMHP1 motif is directly extended from RADA16 with two glycine spacers and is composed of a lysine (blue), serine and threonine (green) and different hydrophobic (white) residues. Each nanofiber is then made of a double-layered anti-parallel ß-sheets with functional motifs flagging on each sides (figure 1).

2.2 Biopolymer electrospinning and tube synthesis

Poly(DL-lactide-co-glycolide) (PLGA, 75:25, mol. wt. 66,000-107,000) and poly(ε-caprolactone) (PCL, mol wt 80,000) were purchased from Sigma-Aldrich, St. Louis, Missouri. Chloroform and methanol solvents were purchased from Mallinkrodt and Sigma-Aldrich, respectively. A solution of 5.5% PCL and 4% PLGA, by weight, was mixed in 3:1 chloroform:methanol (by volume).

The electrospinning apparatus was designed with a Gamma High Voltage Research HV power supply linked to a 16 cm-wide flat plate. A Harvard Apparatus PHD 2000 Infusion syringe pump dispersed solution at a rate of 0.02 mL/min through a 35cm Teflon tube connected to a metal needle (inner diameter: 1.06 mm) protruding through the flat plate. Nanofibers were collected on a round, flat target coated with nonstick Reynolds aluminum foil. The distance between the charged plate and grounded target was 35 cm. Current was measured using a Fluke 189 True RMS Multimeter in series with a 1 Megohm resistor. Voltage was set at 35-37 kV to achieve stable spinning.

PCL/PLGA fibers possessed a diameter of 610 ± 270 nm, with fibers spanning 290nm to 1.1μm. The fibers exhibited isolated bead-on-a-string conformation along the backbone of select fibers. Fibers were sputter coated with several nanometers of gold with a Polaron Range Sputter Coater and viewed using a JEOL JSM-6060 Scanning Electron Microscope.

To create the sciatic nerve tube implants, nanofibers were deposited on a 16-gauge copper wire (diameter: 1.29 mm) held near the grounded target. The wire was grounded and rotated during the synthesis process to assure even coating. Coating time for the wires was 90 seconds (tube wall thickness: 50 - 75μm). Samples were annealed for 24 hours at 55°C under vacuum to remove any possible residual solvent and further crystallize PCL segments for added mechanical strength [4].

2.3 Surgery and animal care

Thirty female Sprague-Dawley rats weighing 200-250 gr were randomly assigned to 3 groups: experimental groups consist of lesioned animals (L group), lesioned animals subjected to tube implantation filled with saline solution (LT group), lesioned animals with tube and RADA16-BMHP1 gel implantation (LTG group).

Rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Under aseptic conditions sciatic nerves were exposed by skin incision along the femur followed by the separation of the biceps femoris and superficial gluteal muscles. Nerves were then sharply transected at the mid-thigh level, proximal to the tibial and peroneal bifurcation.

In L group nerves were transected only. In LT and LTG group proximal and distal nerve stumps were placed for 1.5 mm into each openings of the 1.3 cm long PLGA/PCL tube (pre-soaked in sterile PBS) and sutured with 8-0 Vycril (Ethicon, Somerville). The final inter-stump gap is 10 mm. Gaps were filled with saline solution (LT group) or RADA16-BMHP1 (group LTG) (fig. 2). In all groups, muscle wound beds were sutured with 3-0 Vicryl and skin incisions were closed with surgical staples. Carprofen analgesia (5mg/kg) was administered daily for 1 week postoperatively in all groups.

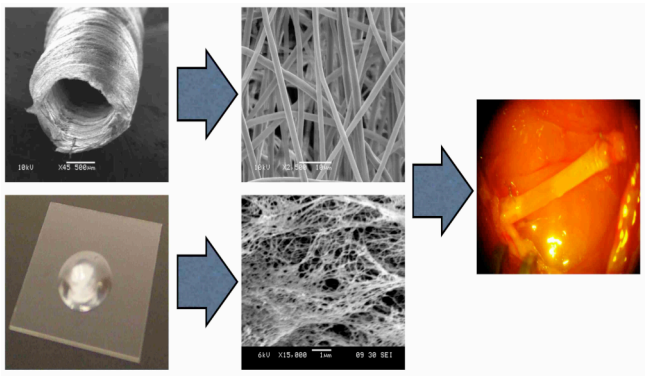

Figure 2: Experimental model. Self-assembling gels have been injected into electrospun tubes. Tubes are made of both micro and nanofibers. Self-assembling peptide are made of functionalized nanofibers only. Scaffolds are then sutured to the stumps of transected rat sciatic nerves.

After 10 weeks 3 rats of each group were anesthetized and received an injection of 3 µl of FluoroRuby (Molecular Probes, St.Louis), a retrograde neuronal tracer. FR was intraneurally injected proximally to the bifurcation of the sciatic nerve and distally to the nerve guides via an Hamilton syringe (33G needle).

2.4 Tissues Processing

Rats were deeply anesthetized with an intraperitoneal overdose of ketamine/kylazine and perfused intracardially with 4% paraformaldehyde 12 weeks after surgery. The sciatic nerve and the PLGA/PLC tube were removed, postfixed for 4h in 4% paraformaldehyde and cryoprotected with 30% sucrose overnight. Longitudinal and transversal sections of the frozen tissues were serially collected via freezing microtome (Microm, Walldorf). For hystochemical analysis, slices were stained with Hematoxylin-Eosin (H&E), Bielschowsky silver stain reaction. For fluorescence imaging cell nuclei were stained with DAPI (Molecular Probes, Eugene) and antigens were labeled with the following primary antibodies: anti-neurofilament NF200 (Sigma, Sant Louis), anti myelin MBP (Sternberger Monoclonals Incorporated, Lutherville) and anti-CNPase (Chemicon International, Temecula), anti-rat macrophage marker CD68 (Serotec, Dusseldorf), anti-prolyl 4-hydroxylase fibroblast marker (Acris Antibodies, Hiddenhausen), anti-β-tubulin (Berkeley Antibody Company, Berkeley), anti-rat Collagen IV (Cedarlene, Hornby). Primary antibodies were then stained with secondary ALEXA 488 (Molecular Probes) and CY3 (Jackson Immuno Research, West Grove). Sections were mounted with FluorSave reagent (Calbiochem, Darmstadt) and examined by an upright Nikon200 fluorescence microscope. For neural tracer imaging sections were mounted with Fluorsave and inspected.

3 RESULTS

Three months after surgery, nervous tissue did not reconnect the two stumps of transected sciatic nerves in all cases of L group. A spontaneous and random neural sprouting occurred from proximal stumps, however nervous fibers targeted muscles located by the lesion sites and the amount of occasional reinnervation connecting the distal nerve tract was negligible. Distal stumps showed macroscopic atrophy and neural degeneration. In all treated animals no significant macrophage infiltration was detected (CD68 staining) if compared with untreated nerves. Capillary neoformation has been detected via H&E staining in correspondence with regenerated nervous tissue.

In 50% of the LT and LTG animal groups tubes collapsed because of the cyclic compression given by surrounding muscle tissues during animal's walk. Closed tubes are not considered in the following results.

In all animals Collagen IV, one of the main components of the basal lamina in nervous tissues, has been found inside the tube lumens in amounts and distribution comparable to healthy nerves. Fibroblasts have been found in concentration and distribution comparable to healthy nerves (data not shown). In less than 20% LT group animals NF200 and Bielschowsky positive fibers were detected along the implanted tubes. However none of them was located in the distal stumps.

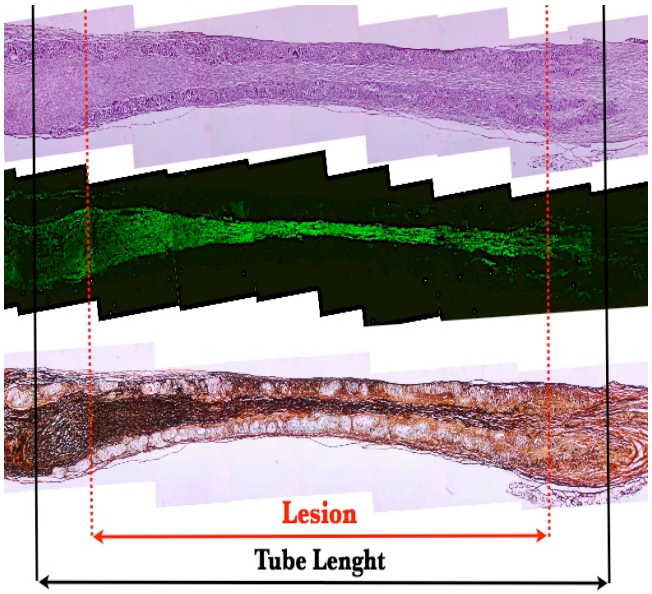

Figure 3: Longitudinal sections of the implanted tubes (LTG group). Three months after surgery nervous fibers regenerate inside the electrospun tubes and reconnect the two stumps.

More than 85% LTG group animals showed nervous fibers that infiltrated and reconnected the two stumps of sciatic nerve (see β-tubulin and Bielchowsky positive fibers in figure 3) and most of them were myelinated. Indeed MBP positive cells were found along the all tube axis as well as the distal nerve stumps.

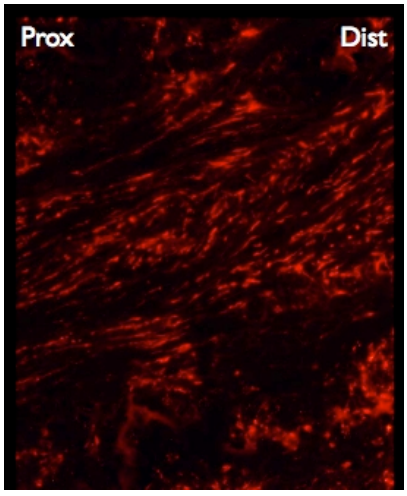

Figure 4: Fluorescence neural tracer imaging of distal portion of longitudinal section in rat sciatic nerve. 40x magnification. Fluororuby, previously injected at the proximal stump and detected at the distal one (red positive cells), demonstrates that the regenerating neural fibers extending from the proximal portion of the nerve are functionally connected to the distal counterpart.

Fluororuby positive cells, located in all tube sections and distally the lesion gap, were found in LTG group animals only. See high magnification fluorescence imaging of a nerve section distal to the lesion in figure 4.

4 DISCUSSION

Our work confirmed RADA16 derived self-assembling peptides are highly biocompatible when injected into nervous system [5] because of their intrinsic purity, natural components (amino-acids) and harmless self-assembling reaction. They closely resemble the nanostructured morphology of native extracellular matrix [1] and have been functionalized to stimulate neural cell adhesion and migration.

Control group confirmed that a strategy for nerve repairs requires effective guiding channels to stimulate nervous regeneration in case of consistent loss of tissue. However a scaffold comprising PCL/PLGA and similar biopolymers is not sufficient: in LT group nervous fibers infiltrated the tubes however they didn't reconnect to the distal stumps of the lesions. When designer nanostructured scaffolds are adopted (RADA16-BMHP1) nervous regeneration of 1 cm-long nervous gap have been demonstrated. Fluororuby revealed the re-establishment of functional neuronal connections between the proximal and distal stumps of the lesions in LTG group animals. Nervous fibers myelination, essential for conducting nervous pulses in motor-sensory tracts, has been showed in sections of the LTG animals both inside, rostrally and caudally the implanted tubes. Moreover, vascularization and basal lamina deposition similar to physiologic tissues, conditions equally essential for a successful nervous regeneration [6][7] have been detected.

Despite our promising results, our approach has to be improved by increasing the mechanical properties of electrospun tubes to prevent their occlusion and consequently limit any possible nervous regeneration.

Currently additional experiments are being conducted to corroborate our results, to evaluate animal functional recovery over time via behavioral animal tests and evoked potentials. Further ongoing experiments have been designed to evaluate possible benefits given by a combined approach with stem cell therapy and/or drug release of neurotrophic factors from these biocomposite scaffolds.

5 CONCLUSIONS

We provided evidence that our strategy can stimulate host lesioned peripheral nervous tissue to regenerate and to reestablish functional connections between the two transected stumps, giving a cytoarchitecture similar to the native tissue. Our nanotechnology based prostheses are completely reabsorbable, synthetic and highly reproducible. Our strategy can be adopted to regenerate spinal cord injury and other neural traumatic pathologies. Further functionalizations can be developed to obtain a controlled long-term drug delivery via self-assembling peptide scaffolds. This approach can be adapted to regenerate other tissues like cartilage, blood vessels or bone [8] by choosing different functional motifs and various nanofiber electrospun made frames.

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