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Misdirection and guidance of regenerating motor axons after experimental nerve injury and repair

Ruiter, G. de

Citation

Ruiter, G. de. (2013, May 21). *Misdirection and guidance of regenerating motor axons after experimental nerve injury and repair*. Retrieved from <https://hdl.handle.net/1887/20891>

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Author: Ruiter, Godard de

Title: Misdirection and guidance of regenerating motor axons after experimental nerve injury and repair

Issue Date: 2013-05-21

CHAPTER 6

Methods for *in vitro* characterization of multichannel nerve tubes

Godard C.W. de Ruiter ^{1,2,4}, Irene Onyeneho ¹, Ellen T. Liang ¹, Michael J. Moore ³, Andrew Knight ¹, Martijn J.A. Malessy ⁴, Robert J. Spinner ², Lichun Lu ³, Bradford L. Currier ³, Michael J. Yaszemski ³, Anthony J. Windebank ¹

¹ Laboratory for Molecular Neuroscience, Mayo Clinic, Rochester MN, USA

² Department of Neurologic Surgery, Mayo Clinic, Rochester MN, USA

³ Laboratory for Biomedical Engineering, Mayo Clinic, Rochester MN, USA

⁴ Department of Neurosurgery, Leiden University Medical Center, The Netherlands

ABSTRACT

Background Multichannel conduits have been developed for both experimental peripheral nerve and spinal cord repair. We present a series of methods to characterize multichannel nerve tubes for properties of bending, deformation, swelling, and degradation and introduce a new method to test the permeability of multichannel nerve tubes from the rate of diffusion of different-sized fluorescent dextran molecules (10, 40, and 70 kDa).

Methods First, single lumen nerve tubes made with different poly(lactic co-glycolic acid) (PLGA) ratios (50:50, 75:25 and 85:15) were compared. One ratio (75:25 PLGA) was subsequently used to compare single lumen and multichannel nerve tubes.

Results Nerve tubes made with lower ratios were found to be more flexible than nerve tubes made with a higher PLGA ratio. For lower ratios, however, swelling was also greater as a result of a faster degradation. Multichannel structure did not interfere with the permeability of the tube; the rate of diffusion into multichannel 75:25 PLGA nerve tubes appeared to be even higher than that into single lumen ones, but this was only significant for 70-kDa molecules. Also, multichannel 75:25 PLGA nerve tubes were more flexible and, at the same time, more resistant to deformation. However, swelling significantly decreased the total cross-sectional lumen area, especially in multichannel 75:25 PLGA nerve tubes.

Conclusion Permeability, bending, deformation, swelling and degradation are important properties to characterize in the development of multichannel nerve tubes. The methods presented in this study can be used as a basis for optimizing these properties for possible future clinical application.

INTRODUCTION

Biodegradable single lumen or hollow nerve tubes have been developed as an alternative for autologous nerve graft repair (for review see **Chapter 5**). The disadvantages of using an autograft include donor-site morbidity, limited availability and size mismatch with the injured nerve. In comparison, nerve tubes are available off the shelf in different sizes.

Multichannel conduits have been developed for experimental peripheral nerve [1, 2] and spinal cord repair [3-7]. The multichannel structure provides more surface area for cell attachment and local release of incorporated growth factors. Also, the multichannel structure may better support regeneration across larger gaps by stabilizing the fibrin matrix [8, 9], and may better guide regenerating axons (**Chapter 7** and **8**).

However, the extra internal structure of multichannel conduits may also interfere with important physical properties of nerve tubes, including permeability, bending and deformation properties, swelling, and degradation. Permeability of a nerve

tube has been shown to influence the results of regeneration [10-13], and is needed for the survival of cells inside the channel before the graft is vascularized. Bending properties are important because the nerve tube may be implanted into a mobile limb. The nerve tube should be flexible, but, at the same time, resistant to permanent deformation and kinking. These properties are also important if the tube is used to repair a large nerve gap. Swelling and degradation are important because swelling of the internal structure may compress regenerated nerve fibers. The rate of degradation may affect swelling properties through the formation of small degradation products that increase the osmotic pressure of the tube [14].

In this study, we compared important physical properties of single lumen and multichannel nerve tubes made from different ratios poly(lactic-co-glycolic acid) (PLGA) (50:50, 75:25, 85:15). This biomaterial is approved by the US Food and Drug Administration (FDA), is used clinically in sutures (polyglactin 910) and has been used previously to fabricate nerve tubes [2, 15]. Because the physical characteristics of PLGA may vary depending on the ratio of lactic to glycolic acid [16], we first tested single lumen nerve tubes made with different PLGA ratios (50:50, 75:25, 85:15) for the properties of bending, deformation, swelling and degradation. One ratio (75:25 PLGA) was subsequently used to compare these properties and the permeability of single lumen and multichannel nerve tubes.

MATERIALS AND METHODS

COMPARISON OF SINGLE-LUMEN NERVE TUBES MADE WITH DIFFERENT PLGA RATIOS

Single-lumen 50:50, 75:25, and 85:15 PLGA nerve tubes were compared for different properties of bending, deformation, swelling, and degradation.

Fabrication of single lumen nerve tubes

Single lumen nerve tubes were fabricated using an injection-molding-solvent-evaporation technique [4]. The polytef (Teflon) mold consisted of cylindrical space (2.1mm in diameter) with endcaps through which a single stainless steel wire (1.6mm in diameter) was inserted (Figure 1A). The dimensions of the wire were based on the diameter of a transected rat sciatic nerve (1.6mm with mushrooming effect, unpublished results). A solution of PLGA polymer (for PLGA ratios 50:50, 75:25, and 85:15; Fisher Scientific, Birmingham, AL, molecular weights 58.8, 92, and 120kDa respectively) in methylene chloride (450 μ l/300mg PLGA) was injected into the mold. The mold was placed overnight in an airtight chamber attached to a high-vacuum pump (VP 190; Savant Instruments, Holbrook, NY) to create porous nerve tubes with semi-permeable outside and inside layers[7].

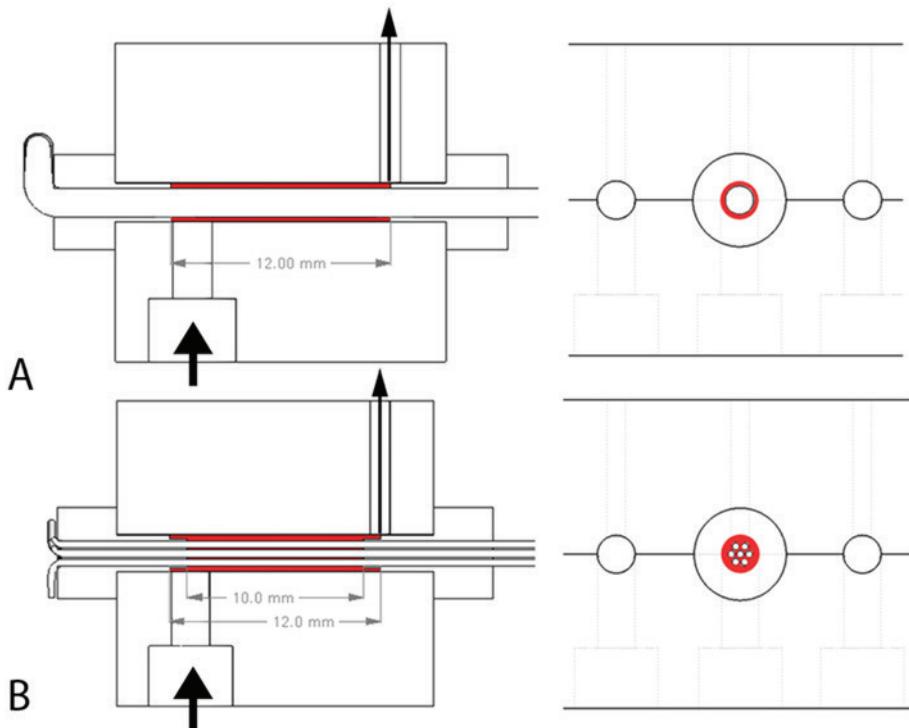
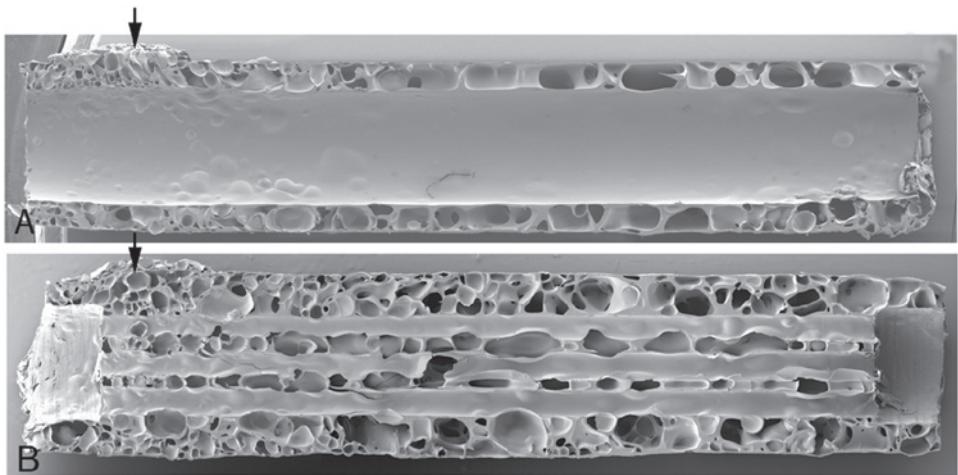


Figure 1

Side views (left) and cross-sectional views (right) of the mold with different assemblies for the fabrication of single lumen (A) and multichannel (B) nerve tubes. The mold consists of cylindrical spaces (2.1mm in diameter) with Teflon endcaps that can hold either (a) one stainless steel wire (1.6mm in diameter, Microparts) for the fabrication of single lumen nerve tubes or (B) seven 400- μ m wires for the fabrication of multichannel nerve tubes. Large arrow, polymer injection site; small arrow, polymer evaporation site.

Bending and deformation properties

The bending and deformation properties of 50:50, 75:25, and 85:15 single-lumen PLGA nerve tubes were analyzed by 3-point-bending on a dynamical mechanical analyzer (DMA, 2980, TA instruments, New Castle, DE). Intact 12-mm tubes were placed on the holder at two points 1 cm apart. The third point was lowered from above in between these two points with increasing force. The displacement was measured and displayed graphically as function of the force (Figure 3A). From these graphs, stiffness could be calculated from a straight line drawn through the bending part of the graph, as shown in Figure 3 (A). The start of deformation of the tube was analyzed at the end of the bending part, the yield point. At this point, the minimal force need to deform the tube was noted and the maximum angle of bending was calculated from the displacement and length of the tube (Figure 3B). Five tubes per group were tested.

**Figure 2**

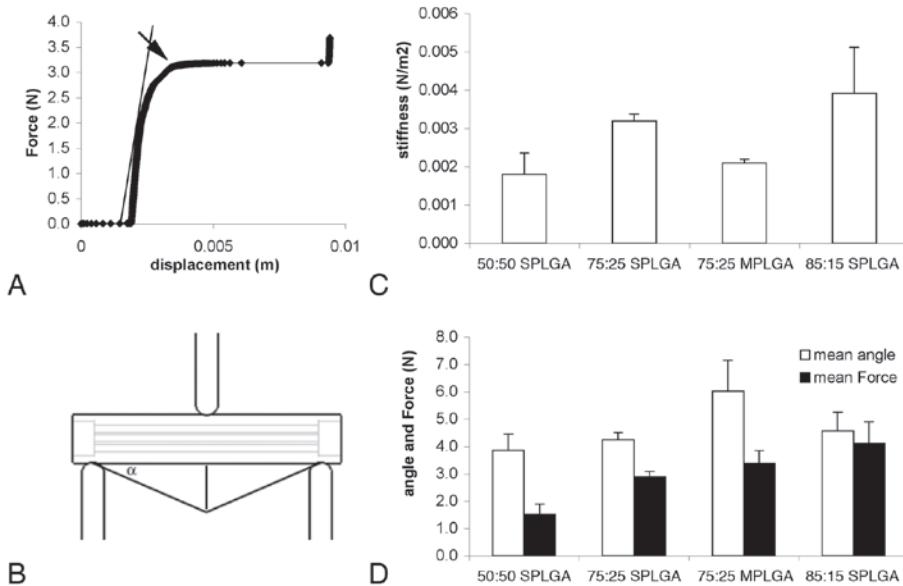
Scanning electron micrograph reconstructions (25xmagnification) of single lumen (A) and multi-channel (B) nerve tubes. Black arrows, polymer injection sites. White arrowheads, internal structure of the multichannel 75:25 nerve tube consists of smaller pores that appear to be more interconnected than the pores inside the single lumen 75:25 nerve tube.

Swelling and degradation

In vitro swelling and degradation of 50:50, 75:25, and 85:15 single lumen PLGA nerve tubes were tested in phosphate-buffered saline (PBS). Samples of a tube (3mm from the mid part) were placed in separate test tubes containing 1.5ml of PBS (Ph 7.4) with 2% sodium azide (to prevent contamination). Before placement, all sample were prehydrated in a series of ethanol concentrations (30 min in 100% ethanol, 20 min in 50% ethanol, and 20 min in 10% ethanol) and water (20 min) and weighed [17]. Test tubes were placed on a shaker in an incubator at 37°C. PBS was replaced every 3 or 4 days for the first 2 weeks and every 2 weeks thereafter to maintain a constant pH. Four samples per group were evaluated at each time point (day 0, day 3, week 1, 2, 4, 6, 8, 10, and 12). Samples were analyzed for residual weight (wet and dry), nerve tube dimensions, and mean molecular weight.

Nerve tube dimensions were determined for the total lumen cross-sectional area and total tube cross-sectional area (total cross-sectional area minus total lumen cross-sectional area). These areas were measured from digital pictures (Nikon Coolpixel 5700) taken through an inverted microscope with KS400 program (version 3.0 Zeiss) (Figure 4A-D). The mass swelling ratio was calculated from the wet and dry residual tube weights using the formula:

$$\text{Mass swelling ratio} = \frac{(W_{\text{wet}} - W_{\text{dry}})}{W_{\text{dry}}}$$

**Figure 3**

In vitro flexibility. A: An example of a 3-point-bending trial for a single lumen 75:25 PLGA tube. The line represents calculation of the stiffness from the bending trial. The arrow points at the yield point, the start of deformation. B: Diagram of 3-point bending and measurement of the angle (α) at yield point. C: Stiffness of single lumen 50:50, 75:25, 85:15 PLGA tubes (SPLGA) and multichannel 75:25 PLGA (MPLGA) tubes; single lumen nerve tubes made of lower PLGA ratios (85:15 > 75:25 > 50:50) were more flexible or less stiff; multichannel 75:25 PLGA nerve tubes were more flexible or less stiff than single lumen PLGA tubes. D: Angle and force at yield point (movement the tubes started to deform). Data on point on the graphs are the mean \pm SD for the four nerve tube samples per group.

The mean molecular weight of the residual tubes was analyzed using gel permeation chromatography (Waters 717 Plus Autosampler, Milford, MA). The results were compared with a calibration curve obtained from monodisperse polystyrene standards (Polysciences, Warrington, PA) [7].

COMPARISON OF SINGLE LUMEN AND MULTICHANNEL 75:25 PLGA NERVE TUBES

One ratio, 75:25 PLGA, was used to compare single lumen and multichannel nerve tubes for the properties of permeability, bending, deformation swelling and degradation. The same analysis as described above for single lumen nerve tubes was used for multichannel nerve tubes, except for the analysis of permeability (see later). In addition, scanning electron microscopy was performed to observe the

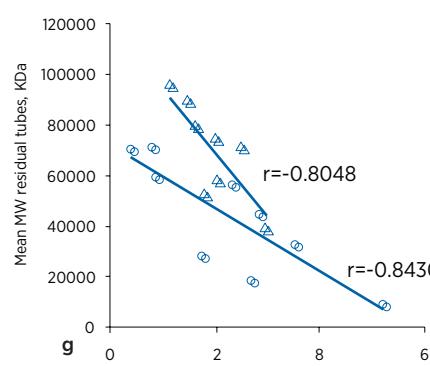
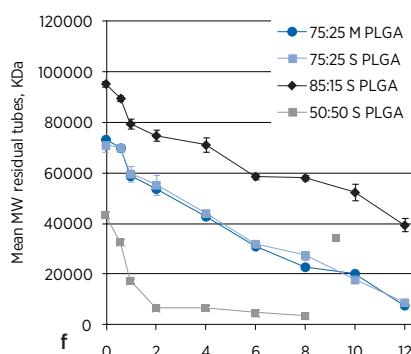
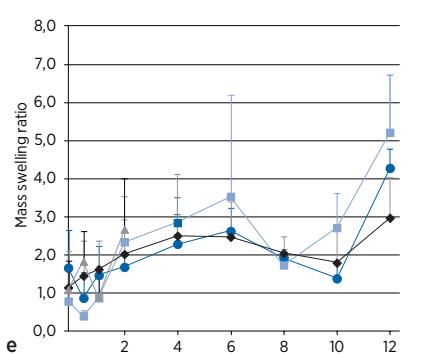
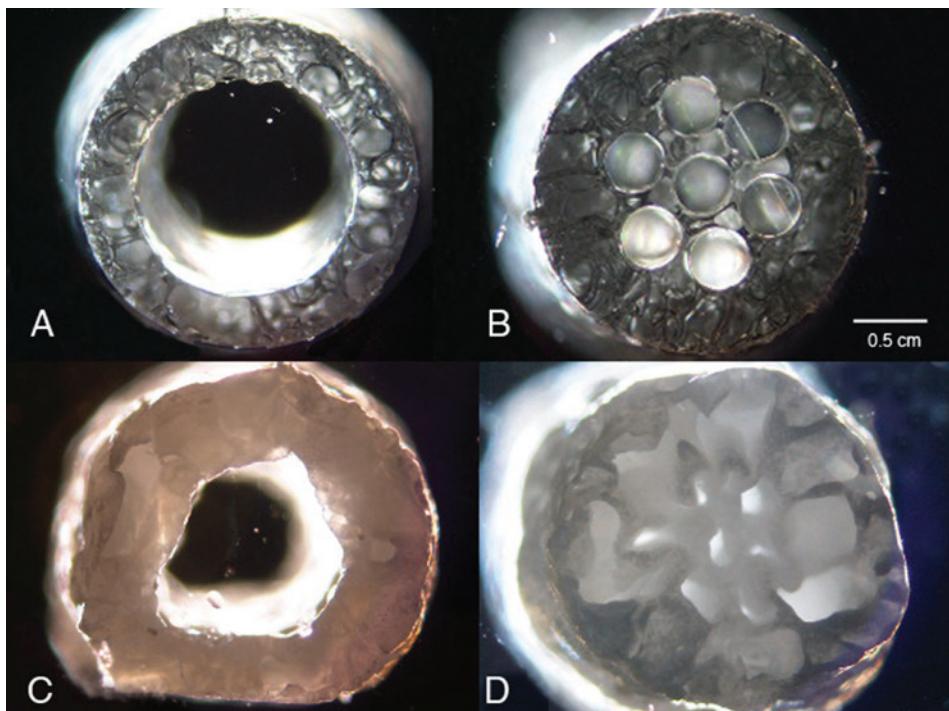


Figure 4

In vitro swelling and degradation. A: Single lumen 75:25 PLGA tube at day 0 dry. B: Multichannel 75:25 PLGA tube at day 0 dry. C: Single lumen 75:25 PLGA tube after 12 weeks in PBS. D: Multichannel 75:25 PLGA tube after 12 weeks in PBS. E: Mass swelling ratio measured for 12 weeks for single lumen 50:50, 75:25, 85:15 PLGA and 75:25 multichannel PLGA (MPLGA) tubes. Swelling ratios for 50:50 single lumen PLGA (SPLGA) tubes could not be measured after 2 weeks. F: Mean molecular weight (MW) of the residual PLGA nerve tubes analyzed over 12 weeks with gel permeation chromatography. G: Correlation between mass swelling ratio and mean molecular weight of the residual tubes for single lumen 75:25 and 85:15 PLGA.

porous structure of the tube and to analyze the surface texture of the lumen and channels.

FABRICATION TECHNIQUE OF MULTICHANNEL NERVE TUBES

Multichannel nerve tubes were fabricated with the same injection-molding technique as described earlier for the fabrication of single lumen nerve tubes. The same mold was also used, only with a different mold assembly (Figure 1). For multichannel nerve tubes, seven 0.4-mm diameter wires were inserted through endcaps. This was the optimal size and number of channels that could be fitted in the available cross-sectional lumen area of 1.6mm in diameter, with the minimal interchannel distance of 0.1mm that was needed to drill the holes in the endcaps. From the center line: 0.1mm + 0.4mm (channel) + 0.1mm + 0.4mm (channel) + 0.1mm + 0.4mm (channel) + 0.1mm = 1.6mm.

Scanning electron microscopy

Single lumen and multi-channel 75:25 PLGA tubes were cut longitudinally into halves and sputter coated (with Bio-Rad/Polaron E5400 High Resolution Sputter Coater). Pore structure was observed under a scanning electron microscope (Hitachi S-4700 Cold Field Emission Scanning Electron Microscope) at 25x magnification. Tiled pictures were taken and reconstructed in Adobe Photoshop (Adobe). The surface area of the lumen and channels was analyzed at 4,000 and 11,000x.

Permeability

The permeability of single lumen and multi-channel 75:25 PLGA tubes was tested by determining the rates at which molecules of fluorescein-isothiocyanate-dextran (FITC-D, Sigma, St. Louis, MO) with a range of molecular weights (10, 40 and 70kDa) diffused into the lumen and channels, respectively, of the tube. The rate of diffusion was determined from the fluorescence intensity inside the lumen or channels at different times.

Samples of tubes (5-mm samples from the mid-part) were placed vertically on a tissue culture dish. The bottom was sealed with a drop of paraffin. The dish was filled to a depth of 4 mm with a 10-mg/mL solution of FITC-D and placed on a modified fluorescence lighting stage (Microlite FL 1000 ultraviolet lamp, Three Rivers, MA) of a dissecting microscope (Carl Zeiss). Images were captured with a digital camera (Nikon Coolpixel 5700) through the objective of the microscope (Figure 5A). The concentration of FITC-D in the lumen or channels was determined from the fluorescent intensity. The intensity inside a channel was compared with the intensity of the known concentration outside the channel. Digital images were inverted and analyzed for color intensity in Adobe Photoshop (Figure 5B). For each tube, the measured concentration was displayed graphically as a function of time (Figure 5c). For multichannel tubes, this analysis was performed separately for

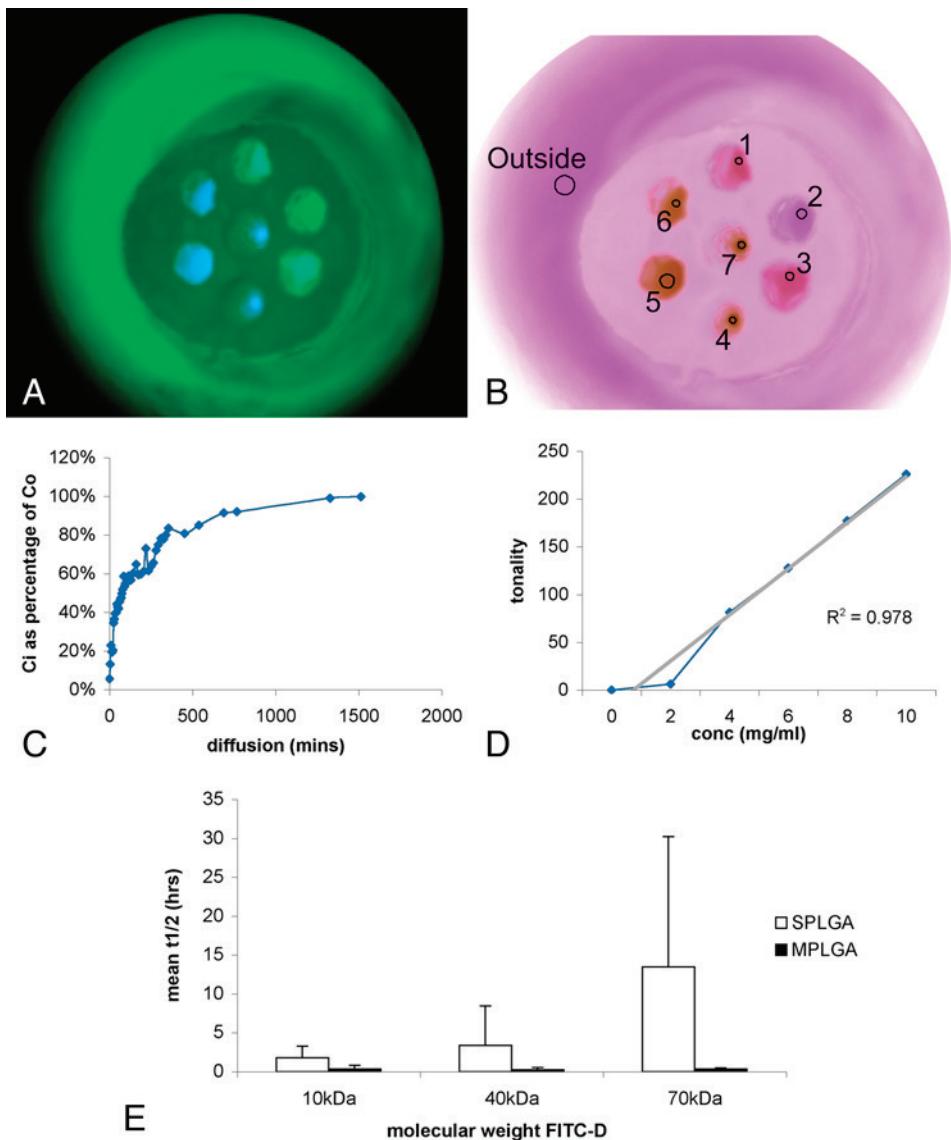


Figure 5

In vitro permeability. A: Digital image of a typical diffusion study with fluorescein isothiocyanate-dextran (FITC-D) molecules (taken through the objective of a microscope). B: Inverted images in Adobe Photoshop for measurements of color intensities: comparison of the inside of the channels with the known concentration of FITC-D outside the channels. In the electronic image, a representative sampling area was chosen outside the tube. Care was taken to avoid sampling heterogeneous color areas over the wax base. Similar areas were measured from each channel. Channels were consistently numbered 1-7. C: First-order kinetics of diffusion into a single lumen tube. $t_{1/2}$ was the time needed for the inside concentration (C_i) to be equal to 50% of the outside concentration (C_o). D: Linear correlation between known concentration and picture tonality

measured in Adobe Photoshop. The best correlation was found the blue color channel ($R^2 > 0.95$). Analysis of the intensity for longer periods also showed that there was no notable fading or change in outside concentration during the course of the experiment (unpublished data). E: Rate of diffusion for the different FITC-D molecule sizes (10, 40, and 70 kDa). Data points on the graphs are the mean \pm SD for the four nerve tube samples per group. Multichannel 75:25 PLGA nerve tubes was not significant (for all molecular weights, $P > 0.05$). For multichannel nerve tube, the mean rate of diffusion was independent of the molecular size. For single lumen nerve tubes, the mean rate of diffusion tended to decrease with molecular weight (10 < 40 < 70 kDa), but this was not significant.

each channel. The rate of diffusion was presented for $t_{1/2}$, that is, the time at which the concentration in the lumen or channel was equal to 50% of the concentration outside. Four samples of tube were used per group. Before analysis, samples were prehydrated (same procedure as described earlier). Samples from multichannel tubes were always placed with the same orientation of numbered channels to investigate the influence of channel position with respect to the mold during fabrication. Channels were numbered according to their position in the mold (Figure 5B): 12 o'clock, number 1; 2 o'clock, number 2; 4 o'clock, number 3; 6 o'clock, umber 4; 8 o'clock, number 5; 10 o'clock, number 6; and center, number 7. The method was validated for a sequence of known concentrations. Linear regressions were calculated for the different color channels (original, gray, red, green, and blue).

Statistics

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Statistical analysis was performed with the Student *t*-test (unpaired, 2-tailed *p* value) for comparison of two groups when the data were normally distributed. For comparison in which the data violated the assumptions of normality, a Mann-Whitney test was performed. For comparison of three groups or more, one-way analysis of the variance (ANOVA) was performed with a Bonferroni posttest. Linear correlations were investigated using Pearson analysis.

RESULTS

COMPARISON OF SINGLE LUMEN TUBES MADE WITH DIFFERENT PLGA RATIOS

Comparison of single lumen nerve tubes made with different PLGA ratios showed that the stiffness of the tubes was greater for higher PLGA ratios (85:15>75:25>50:50, $P < 0.05$, four samples were lost during the experiment) (Figure 3C). The difference however, was only significant for 50:50 versus 85:15 PLGA (posttest $P < 0.05$). The force needed to deform the nerve tube was also greater for higher PLGA ratios ($P < 0.05$) (Figure 3D), but again only significantly for 50:50 vs 85:15 (posttest $P < 0.001$). Swelling was greater for lower PLGA ratios (50:50 > 75:25 > 85:15) (Figure 4E). At 12 weeks, mass swelling ratios for single lumen 75:25 and 85:15 PLGA nerve

tubes were 5.2 and 3.0, respectively, but this difference was not statistically significant. The results for 50:50 single lumen PLGA nerve tubes could not be analyzed after 2 weeks because the tubes had completely lost their structural integrity. Also, the rate of degradation was greater for lower PLGA ratios (Figure 4F). There was a linear correlation between the results for the mass swelling ratio (Figure 4E) and mean molecular weight of the residual tubes in time (Figure 4F) (75:25 PLGA $r = -0.8430$, 85:15 PLGA $r = -0.8048$) (Figure 4G). The same bimodal distribution that was observed for the mass swelling ratios (Figure 4E) was also observed (although less obvious) for mean molecular weight of the residual tubes (Figure 4F). Results for the change in nerve tube dimensions showed that swelling significantly reduced the cross-sectional lumen or channel area of the tube (Figure 6).

Because of the increased swelling for the lower PLGA ratio (50:50) and decreased flexibility for higher ratio (85:15), we chose to use the 75:25 PLGA ratio, to compare single lumen and multichannel nerve tubes.

COMPARISON OF SINGLE LUMEN AND MULTICHANNEL 75:25 PLGA NERVE TUBES

Comparison of the permeability of single lumen and multichannel 75:25 PLGA nerve tubes showed that there was no difference in rate of diffusion of FITC-D molecules into lumen or channels of the tubes (Figure 5); multichannel nerve tubes appeared to be even more permeable, although this difference was only significant for 70-kDa FITC-D ($P < 0.05$) (Figure 5E). The $t_{1/2}$ for the individual channels of the multichannel tube was averaged because no difference was found for the rate of diffusion into the separate channels, including the most central one.

Scanning electron micrographs showed that both single lumen and multichannel 75:25 nerve tubes consisted of a highly porous internal structure surrounded by a continuous inside and outside polymer layer (Figure 2). At higher magnification, these layers were seen to be smooth and nonporous. The internal structure of the multichannel 75:25 PLGA nerve tube consisted of smaller pores when compared with the internal structure of single lumen 75:25 PLGA nerve tubes that appeared to be more interconnected (Figure 2, arrowheads).

Results for 3-point-bending showed that multichannel 75:25 PLGA nerve tube were less stiff or more flexible than single lumen 75:25 PLGA tubes ($P < 0.001$), and at the same time more force was needed to deform multichannel 75:25 PLGA tubes (although not significantly) with a significantly larger angle at yield point ($P < 0.05$) (Figure 3D).

Swelling was similar for single lumen and multichannel nerve tubes, but it had a significantly greater impact on the total cross-sectional channel area for multichannel 75:25 PLGA nerve tubes (43%) than on the total cross-sectional lumen area of single lumen 75:25 PLGA nerve tubes (at 10 weeks, 43 and 23%, respectively).

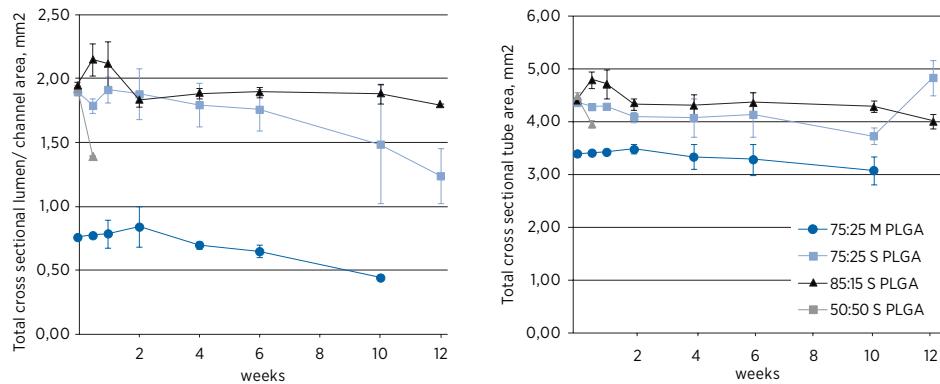


Figure 6

A: Change in tube dimensions for 12 weeks for the total cross-sectional nerve tube lumen/channel area. Dimensions for 75:25 MPLGA tubes could not be measured at 12 weeks. B: Change in tube dimensions for 12 weeks for the total cross-sectional tube area (including the total cross-sectional lumen/channel area). Data points on the graphs are the mean \pm SD for the four nerve tube samples per time point per group.

DISCUSSION

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The physical characteristics of a conduit may determine its ability to support regeneration [10-12, 18]. Therefore, *in vitro* characterization of different physical properties is important in the development of a nerve tube. Physical properties not only depend on the biomaterial that is used to fabricate the nerve tube, but also on the technique of fabrication, and different modifications to the common single lumen nerve tube [19], as in our study, multichannel nerve tube structure. In this study, we introduced a series of methods that can be used to characterize the physical properties of nerve tubes and especially that of conduits with more complex internal structures, including a novel method to test permeability.

Permeability

The fluorescence diffusion-intensity method introduced in our study was found to be a useful method to test the permeability of multichannel nerve tubes. There was a linear response with a high correlation coefficient between a sequence of known concentrations and color intensities. Multiple channels could be analyzed in time without direct sampling. Also analysis can be performed for a range of fluorescent dextran molecules comparable to the size of growth factors. Other methods can be used to measure the porosity of nerve tubes, including mercury intrusion porosimetry [15] or microcomputed tomography [20], but these methods do not necessarily correlate with effective diffusion that may also depend on the hydrophobic properties of the material and other properties.

Multichannel 75:25 PLGA tubes tended to be more permeable than single lumen 75:25 PLGA tubes. This difference can be explained by more interconnected pores in multichannel nerve tubes (Figure 2), although this was not quantified using, for example, microcomputed tomography [20]. Other techniques have been used to create porous nerve tube structures by cutting holes into the wall of the tube [11], by rolling of meshes [21, 22], by fiber spinning [23] or by adding salt or sugar crystals to a polymer suspension during fabrication and leaching them out afterward [15] or sugar [12]. The advantage of the solvent-evaporation technique is that porous nerve tubes can be created that have semipermeable inside and outside layers.

Bending and deformation properties

Testing of bending and deformation properties on a dynamic mechanical analyzer by 3-point-bending was useful in characterizing multichannel nerve tubes. Multichannel 75:25 PLGA tubes were more flexible than single lumen tubes but also more resistant to permanent deformation. Overall however, nerve tubes made from PLGA were stiff and easy to deform, with irreversible collapse of shape (with a mean angle at yield point of $\sim 4^\circ$ for single lumen 75:25 PLGA tubes and $\sim 6^\circ$ for multichannel 75:25 PLGA tubes). In addition to the tests performed in this study, dynamic mechanical analysis can be performed under different conditions, for example in PBS solution at 37°C . Repetitive bending can also be performed to test chronic wear. Although it is difficult to mimic exactly the *in vivo* conditions [24], 3-point bending is a useful test in the initial stages of the development of a nerve tube.

Swelling and degradation

As our study shows, swelling is also an important property to characterize in the development of a multichannel nerve tube. Swelling has a greater effect on the total cross-sectional lumen area of a multichannel tube than on a single lumen tube and initially is more than two times smaller (0.88 and 2.0 mm^2 , respectively, calculated on the basis of the radius channel/lumen). Swelling can be explained by the formation of small degradation products that increase the osmotic value of the tube; the degradation of PLGA is an autocatalytic process that results in a faster rate of degradation on the inside than on the outside because of the accumulation of small degradation products on the inside (the outside forms a crust) [16, 25, 26]. This heterogeneous degradation of PLGA can also explain the bimodal distribution of mass swelling ratios in time. Finally, the faster rate of degradation for lower PLGA ratios explains the increased mass swelling for these ratios.

CONCLUSIONS

We present a series of methods to analyze important nerve tube properties that can also be applied for conduits with more complex structures (e.g. multichan-

nel nerve tubes). In our study, multichannel structure did not reduce permeability, and multichannel conduits were more flexible than single lumen ones made from the same biomaterial (75:25 PLGA) using the same fabrication technique. Overall however, nerve tubes made of PLGA were stiff and easily deformed. Swelling significantly reduced the total cross-sectional lumen area, especially in multichannel nerve tubes, which might explain the limited results we found for *in vivo* regeneration across single lumen and multichannel 75:25 PLGA nerve tubes [27] (Chapter 7). For future clinical application, more flexible multichannel nerve tubes are needed: ones that are permeable and do not swell. The methods for analysis of tube properties presented in this study can be used to optimize the development of multichannel nerve tubes.

ACKNOWLEDGEMENTS

We thank Frederick M. Schultz for the design and fabrication of the molds, Mark E Zobitz and Qingshan (Frank) Chen for their advice on the flexibility studies, Scott I. Gamb for his assistance with SEM analysis, Geraldine K. Bernard and Peggy L. Chihak for the graphic design.

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