Improved nerve regeneration through piezoelectric vinylidenefluoride-trifluoroethylene copolymer guidance channels

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Piezoelectric materials generating electrical charges in response to mechanical strain may be used to stimulate axonal regeneration following nerve injury. Tubular nerve guidance channels were extruded from a vinylidenefluoride–trifluoroethylene copolymer using a melt-extrusion process. Unlike vinylidenefluoride homopolymer, the copolymer does not need mechanical stretching to achieve a dipole-containing crystal structure, enabling the fabrication of complex piezoelectric devices. Selected tubes were rendered piezoelectric in a high voltage corona poling apparatus. Crystal structure changes induced by poling were evaluated with differential scanning calorimetry. In contrast to unpoled samples, poled ones displayed a sharp endothermic peak and a greater heat of transition at the Curie temperature, indicative of an increase in crystal order and size. The piezoelectric output of poled tubes was characterized using a laser-monitored deflection system interfaced with a charge amplifier and oscilloscope. Poled tubes generated significant voltages in response to slight mechanical deformations. The magnitude of electrical output was independent of the poling polarity. Unpoled tubes showed no electrical output. Positive, negative and unpoled vinylidenefluoride–trifluoroethylene copolymer tubes were used to repair a 10 mm gap in transected sciatic nerves of adult rats. Nerves regenerated in positively poled channels had a significantly greater number of myelinated axons than those regenerated in unpoled channels 4 wk post-implantation. Negatively poled channels contained an intermediate number of myelinated axons. We concluded that piezoelectrically active vinylidenefluoride–trifluoroethylene copolymer tubes significantly enhance nerve regeneration as compared to chemically identical, unpoled tubes and that the polarity of the corona poling procedure used to fabricate piezoelectric materials may play a role in determining biological responses.

Keywords: Nerve regeneration, piezoelectric polymer, guidance channel, sciatic nerve

Mammalian peripheral nerves are capable of regeneration after transection injury. Transected nerves are repaired clinically by end-to-end approximation of the stumps using fine suture materials. When nerve injury results in a surgically irreducible gap, autologous nerve grafts are used to bridge the deficit. For both types of repair, functional return is quite variable and usually does not reach pre-injury levels. Synthetic nerve guidance channels have been used experimentally to study mechanisms underlying axonal regeneration. Guidance channels may simplify end-to-end repair and may be useful in repairing long nerve gaps. The guidance channel reduces tension at the suture line, protects the regenerating nerve from infiltrating scar tissue and directs sprouting axons toward their distal target.

The properties of the guidance channel can be modified to optimize the regeneration process. For example, releasing growth factors from the wall of the channel into the regenerating environment increases the distance over which nerves will regrow[1]. The use of a semipermeable channel supports significant outgrowth, even in the absence of distal nerve tissue[2,3]. Guidance structures with smooth surface textures enhance the number of regenerated fibres and improve the morphological patterns of regeneration[4,5]. Fluoropolymer channels producing transient[6] (i.e. piezoelectric) or static[7] (i.e. electret) surface charges have been shown to enhance the number of myelinated axons regenerating after injury significantly. In a previous study[6],

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piezoelectric tubes fabricated from polyvinylidene fluoride homopolymer (PVDF) were shown to be sensitive mechano-electrical transducers which generated significant electrical output under small deformational forces. Mechanical deflections of implanted piezoelectric tubes caused by random animal movements were believed to elicit an electrical output that stimulated the regeneration process. PVDF is a semi-crystalline polymer which can be rendered piezoelectric by a two-stage process involving mechanical stretching and electrical poling[8,9]. When PVDF cools from the melt, its polymer chains are arranged in a random non-polar configuration. When stretched, polymer chains within crystal domains extend into an all-*trans* configuration with hydrogen atoms and electronegative fluorine atoms on opposite sides of the carbon backbone. This creates a strong dipole moment in individual crystals but no net dipole moment due to the random orientation of crystal domains within the polymer bulk. The bulk dipoles can be preferentially orientated by exposing the polymer to a high voltage corona poling field. Poled PVDF displays high piezoelectric activity and dynamic deformation of the material causes displacement of orientated dipoles and a transient, compensatory surface charge[8,9]. Copolymerizing PVDF with trifluoroethylene (TrFE), causes PVDF chains to transform directly from the melt into an all-*trans* configuration due to the steric hindrance of the larger TrFE group[8]. Therefore, the vinylidene fluoride-trifluorethylene P(VDF-TrFE) copolymer does not need to be stretched before poling. This simplifies preparation techniques and, more importantly, makes it possible to fabricate porous or semipermeable channels and films whose fine structure would otherwise be destroyed by the stretching step.

Since copolymer tubes are not available commercially, they were fabricated, characterized and tested in our laboratory. Nerve guidance channels were extruded from P(VDF-TrFE) resin and exposed to a high voltage corona poling process designed to accommodate the tubes used. Piezoelectric properties were determined by direct measurement of electrical activity and by differential scanning calorimetry (DSC). These techniques were used to quantify the degree of electrical output and dipole orientation.

The purpose of this study was to determine if piezoelectric P(VDF-TrFE) channels would enhance regeneration in a transected rat sciatic nerve model. A different animal model was used to eliminate the possibility of a species-dependent effect, since previous studies were conducted using a murine model. The fact that the copolymer and homopolymer have different chemistries allowed us to test the hypothesis that enhanced regeneration in piezoelectric tubes is due to electrical activity and is not dependent on chemical composition.

MATERIALS AND METHODS

Tube fabrication

Tubes were fabricated from 70/30% P(VDF-TrFE) pellets (Atochem, Pierre-Benite, France. The copolymer pellets were heated to their melting range (135–140°C) in a holding reservoir and extruded through a stainless steel nozzle (*Figure 1*) to create dense-walled tubing with an internal diameter of 1.5 mm and an outer diameter of 2.0 mm. The internal diameter was chosen to provide a snug fit for the rat sciatic nerves to be repaired.

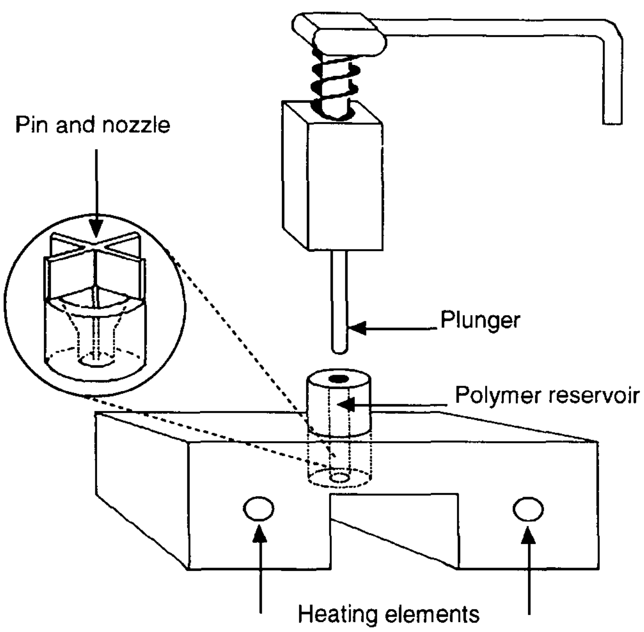

Figure 1 *Extrusion device used to prepare vinylidenefluoride-trifluoroethylene copolymer [P(VDF-TrFE)] tubes. The descending plunger forces the polymer out of the heated reservoir and through the nozzle to form a tubular device.*

Poling

Lengths of tubing, each 5 cm, were rendered piezoelectric by submitting them to a high intensity corona poling procedure. A tightly fitting conductive steel mandrel placed in the lumen of the copolymer tube served as the grounding electrode and a circumferential array of steel needles in a Teflon® housing served as the outer electrode (*Figure 2*). The poling field was achieved with a high voltage, low current d.c. power supply with reversible polarity outputs (Bertan Associates Inc., model 205-50R, Syosset, NY, USA). Voltage was increased by increments of 1 kV/h up to 15 kV.

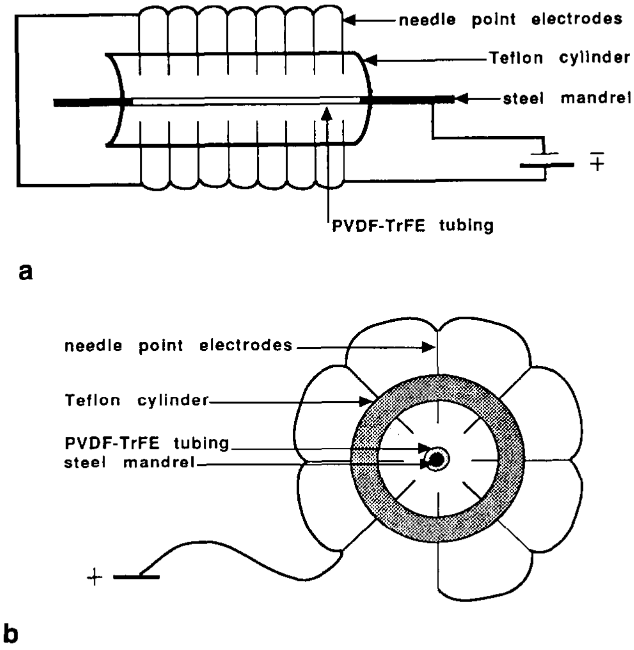

Figure 2 *Corona poling device used to render vinylidenefluoride-trifluoroethylene copolymer [P(VDF-TrFE)] tubes piezoelectric. A high voltage d.c. field applied across the wall of the channel aligns dipoles located in polymer crystals. All needle electrodes are connected in parallel to the power supply. (a) Longitudinal and (b) cross-sectional views of the poling device.*

This potential was maintained for 12 h. Selected tubes were poled positively or negatively with respect to ground and unpoled tubes served as controls. All poling procedures were performed at room temperature.

Tube characterization

Scanning electron microscopy (Amray 1000A, Bedford, MA, USA) was performed on extruded tubes to determine the uniformity of wall thickness and the surface texture of both poled and unpoled tubes.

The piezoelectric activity of copolymer tubes was determined by coating their outer surface with conductive silver ink (Emerson and Cummings Inc., system CT-5030, Woburn, MA, USA) and placing a conductive steel mandrel in the lumen of the tube. Electrode leads were attached and then connected to a charge amplifier (Kistler Instrument Corp., model 5004, Amherst, NY, USA) through a high capacitance probe (Tektronix, model P6062B, Beaverton, OR, USA). The tube ends were held securely in a clamping device while the midpoint was deflected over a range 0.05–1.0 mm by a Teflon coated impinger (*Figure 3*). The charge induced on the surface of the channel could be calculated from the amplified signal display on a storage oscilliscope (Tektronix, model 5113, Beaverton, OR, USA). The distance travelled by the head of the impinger was measured by a laser optic deflection sensor. The beam of a 20 mW helium neon laser (Edmund Scientific, model ML855, Barrington, NJ, USA) was directed toward a reflector that moved with the impinger. The intensity of the reflected beam, which varied directly with the distance travelled, was sensed with a phototransistor (Tandy Corp., Fort Worth, TX, USA). Reflector movement was proportional to the tube deflection distance. The charge induced per unit of deflection was recorded.

DSC thermograms of the raw dope, poled and unpoled samples weighing between 5 and 10 mg were obtained with a DuPont DSC 29109 (Willmington, DE, USA). The endothermic peaks upon melting (T_m) and upon transition from the ferroelectric to paraelectric structure (i.e. Curie temperature, T_c) were analysed as the samples were heated at a rate of 10°C/min.

Tube implantation

Nerve guidance channels 12 mm in length were cut from 5 cm lengths of poled and unpoled copolymer tubing. Small holes (200 μm) were drilled 1 mm in from either tube end to aid in suturing, since the semicrystalline material is not penetrable with the fine needles used. The tubes were washed and sonicated in a 1% Alconox® detergent solution and rinsed several times with double distilled water. The tubes were stored in filtered 70% ethanol for sterilization. At the time of implantation, the tubes were rinsed thoroughly in sterile physiologic saline solution.

The sciatic nerve of Nembutal® anaesthetized young male albino rats (Charles River Labs, MA, USA, 225–250 g) was exposed by an incision in the left hindlimb and retraction of the gluteus maximus muscle. A 6 mm long segment of nerve, 1–2 mm proximal to the tibial-peroneal bifurcation was resected and discarded. The nerve stumps were allowed to retract naturally, creating a gap large enough to secure the stumps 10 mm apart within a 12 mm tube using single 10-0 nylon suture stitches. The tubes were primed with saline to prevent trapping of air bubbles. Three cohorts of six animals each were implanted for 4 wk with positive, negative and unpoled copolymer channels.

At the time of explantation, rats were deeply anaesthetized with Nembutal and transcardially perfused with 100 ml of heparinized saline followed by 100 ml of 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffered saline at pH 7.4. The guidance channel with an extra 3 mm of native nerve at each end was dissected free and soaked overnight in fixative. Specimens were then cut in half and post-fixed in a 1% osmium tetroxide solution, dehydrated and embedded in Spurr resin. Semithin transverse sections taken 2.5, 5.0, 7.5 and 10.0 mm from the proximal stump were cut on Sorval MT-5000 microtome and stained for light microscopy[6]. Counts of myelinated axons were performed under ×630 magnification using an oil-immersion lens. At this magnification, myelinated axons were easily identified by their myelin sheaths. All myelinated axons present in each nerve cross-section were counted. Sections were analysed with the aid of a morphometric analysis system (Cue-2, Olympus Corp., Lake Success, NY, USA) interfaced to an IM 35 Zeiss microscope. The neoepineurial area was defined as the layer of conective tissue surrounding the regenerated neural tissue and blood vessels.

Data are presented as mean ± standard deviation. Statistical significance between various populations was assessed using a two-way variance analysis (SuperANOVA®, Abacus Concepts, Inc., Berkely, CA, USA).

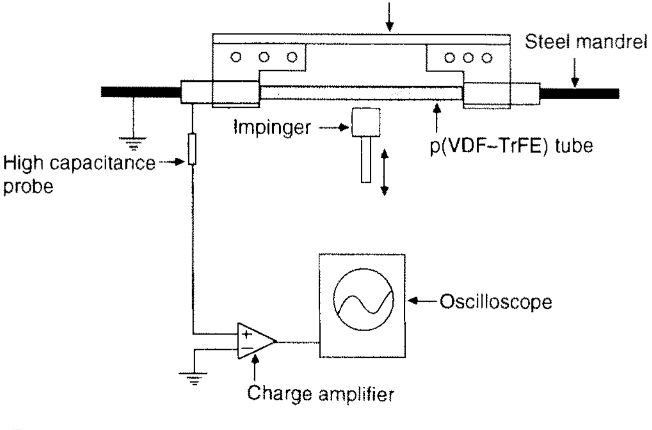

Figure 3 Set-up for measurement of piezoelectric output. The moving impinger deflects the firmly clamped piezoelectric tube. The transient surface charge generated by mechanical deformation is detected by the high capacitance probe, amplified and read out on an oscilloscope. Deflection distances were monitored with laser-based photodetector.

RESULTS

Tube characterization

Scanning electron microscopy. For both poled and unpoled tubes, electron micrographs revealed smooth external surfaces and smooth internal surfaces with sparce groove lines, probably resulting from the extrusion process. Electron micrographs of tube cross-sections showed a uniform wall thickness of about 250 μm.

Piezoelectric activity. Poled tubes were very sensitive to mechanical deformation and generated significant output, even during gentle handling. Deflections on the order of 0.2 mm at the midpoint of poled copolymer tubes induced surges in charge on the order of 150 picoCoulombs/cm^2 of tube surface area (*Figure 4*). Positive and negative poled samples showed identical charge output curves. The output

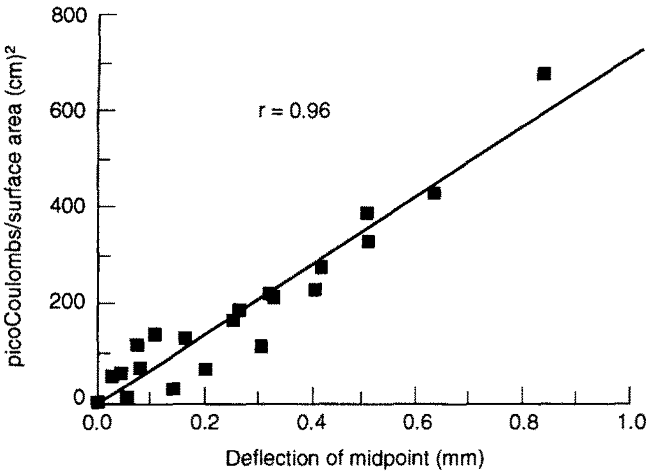

Figure 4 Piezoelectric output of positively poled extruded tubes in response to various deflection distances. The correlation coefficient (r) was calculated using linear regression analysis, r = 0.96.

increased linearly with increases in deflection distance. The correlation coefficient (r) was equal to 0.96, as calculated using linear regression analysis. Unpoled copolymer tubes showed no electrical activity even under maximal deflection.

Differential scanning calorimetry. DSC can be used to determine changes in the heat of transition at T_c where a ferroelectric to paraelectric phase change occurs in the crystal structure of the piezoelectric material[10, 11]. Thermograms were plotted with heat flow *versus* temperature for copolymer samples of positive and negative poled and unpoled samples as well as for the raw dope. For first run samples, poled samples had a sharper endothermic peak with a greater heat of transition at T_c than the raw dope and unpoled samples (*Figure 5*). This indicates an increased degree of crystallinity and crystallite size for poled tubes[10, 11]. In addition, the Curie transition shifts to a higher temperature after the material is poled. Positive and negative poled tubes displayed similar DSC profiles. Second run data, taken after first run sample meltdown, showed severely reduced crystallinity for all materials, with profiles similar to those of first run extruded unpoled material (data not shown).

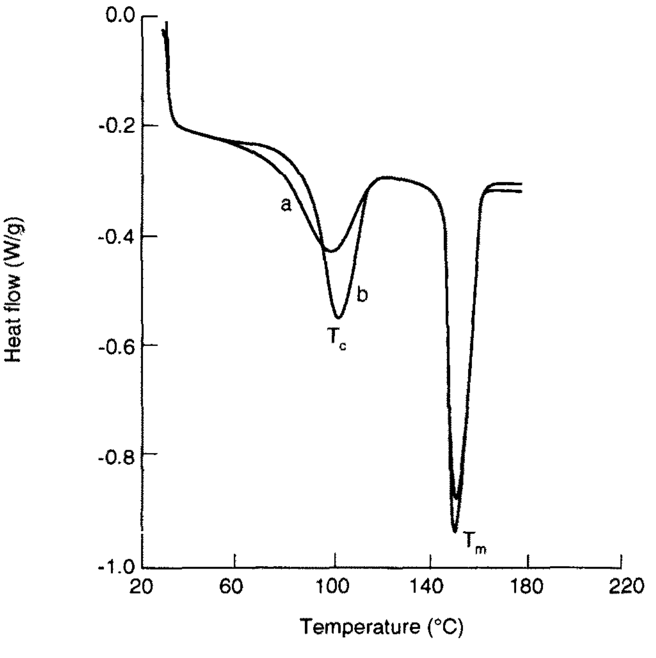

Figure 5 DSC thermograms of first run samples of (a) unpoled and (b) positive poled copolymer tubes. Note the sharper, higher Curie transition temperature (T_c) for the poled sample. Melting temperatures (T_m) are similar. The temperature was raised at a rate of 10°C/min.

Nerve regeneration studies

P(VDF-TrFE) channels elicited a minimal tissue reaction, consisting of a thin, translucent connective tissue sheath covering the tubes. Regenerated nerve cables bridged the 10 mm gap in 50% of each group of unpoled, negative and positive poled tubes, although all the nerve stumps remained sutured within the ends of the guidance channels. The regenerated cables were centrally located in an acellular gel. The cables were about the same size as the somewhat swollen native nerve stumps and tapered to a smaller diameter at the midpoint of the gap. All cables were surrounded by a thin neoepineurium and contained numerous Schwann cells and myelinated axons within microfascicles (*Figure 6*).

Nerve cables regenerated in positive, negative and unpoled channels differed with respect to the number of myelinated axons they contained. Cables regenerated in positive poled tubes had a significantly greater number of myelinated axons compared to the unpoled channels at all points along their length (*Figure 7*). Positive poled channels also contained a greater number of myelinated axons than negative poled channels, but this difference was not statistically significant.

In general, the cross-sectional area of the regenerated cables was greatest for the positive poled channels and smallest for the unpoled channels. At the midpoint, the average cross-sectional cable areas $(\times 10^3\,\mu m^2)$ in the positive, negative and unpoled tubes were 231 ± 55, 195 ± 46, 174 ± 37, respectively. The differences were not however statistically significant at any point along the cables. The percentage of the regenerated cable area comprised of neoepineurium increased towards the distal stump in all cases. For all regenerating nerves, the epineurium accounted for approximately 25% of the total cable area 2.5 mm from the proximal stump and increased to approximately 55% at the distal stump. The number of blood vessels found in the regenerated cables was similar for all tube types. Small and large blood vessels, ranging from 60 to 1000 μm^2 in area, were found scattered throughout the nerve cables.

DISCUSSION

The fabrication and electrical poling of P(VDF-TrFE) copolymer tubes for use as nerve guidance channels was accomplished by heat melt extrusion followed by a corona poling procedure. Uniform, smooth-walled tubes were reproducibly fabricated. A relatively long poling time, 12 h, was used to ensure adequate dipole orientation of the 250 μm thick tubes. The poling process resulted in a significant increase in crystal structure order and crystallite size as indicated by differences in the DSC thermograms for unpoled and poled material[10, 11]. The ferroelectric nature of poled samples was evidenced by the sharper endothermic peak and greater heat of transition at T_c. Positive and negative poled samples showed similar DSC thermograms, indicating that the degree of bulk dipole orientation was similar. Piezoelectric charge output due to controlled mechanical deflection indicated that both positive and negative poled channels were highly active and generated similar levels of piezoelectric charge. Output increased

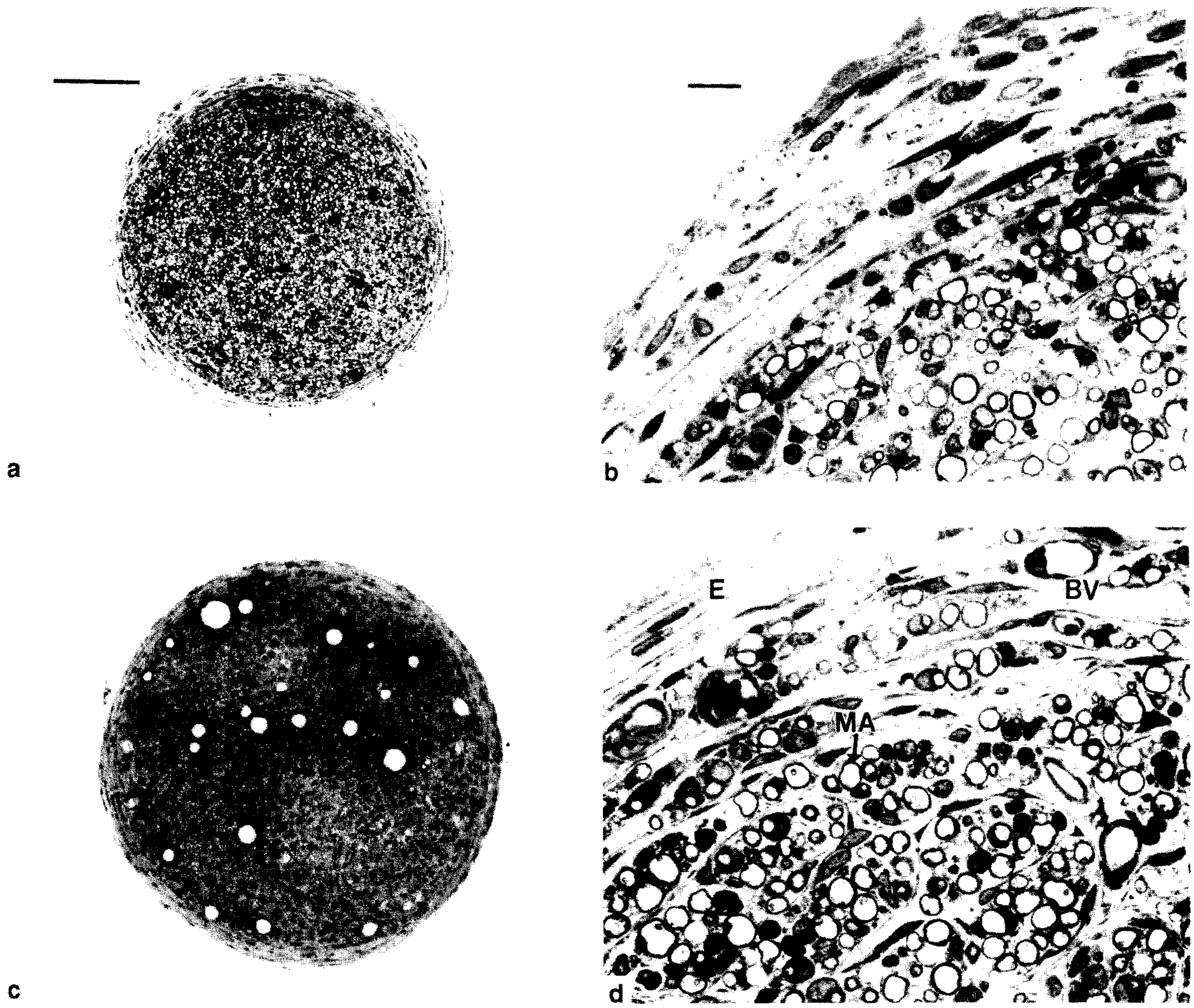

*Figure 6 Toluidine blue stained transverse sections taken at the midpoint of nerve cables regenerated through vinylidenefluoride–trifluoroethylene copolymer [P(VDF-TrFE)] tubes 4 wk post-implantation. (a) and (b) Low and high power micrographs of nerves regenerated in unpoled tubes. (c) and (d) Low and high power micrographs of nerves regenerated in positive poled tubes. Note the epineurium (**E**) surrounding numerous myelinated axons (**MA**) and blood vessels (**BV**). Scale bar in (a) and (c) 100 μm. Scale bar in (b) and (d) 10 μm.*

linearly with deformation and very small deflections resulted in sizeable charge generation.

The P(VDF-TrFE) copolymer, like other fluorinated polymers, shows excellent biocompatibility. As was seen with PVDF homopolymer tubes in mice, nerves regenerated in piezoelectric copolymer guidance channels contained a greater number of myelinated axons compared to their non-piezoelectric controls[6]. Cables regenerated in poled P(VDF-TrFE) channels were generally larger and showed thinner neoepineurium formation than their counterparts in unpoled tubes. Positively poled tubes contained more axons than negatively poled ones, even though their piezoelectric output was similar. Similar results have been observed with positively and negatively charged polytetrafluoroethylene (PTFE) tubes used to repair transected mouse sciatic nerves[7]. Differences in the mechanism of corona poling may lead to disparate charge injection profiles and dipole distributions for positive and negative poled tubes. Altered charge distributions and patterns of electrical output may exert different biological effects.

Minute deflections of the piezoelectric tubes resulted in measurable surface charge signals, suggesting that significant charges are also produced when implanted tubes are deformed by animal movement. Experimental animals displayed normal levels of activity soon after recovering from anaesthesia. Since the muscles of the upper leg are innervated by nerves other than the severed sciatic nerve, movement of the operated hindlimb was indistinguishable from that of the contralateral leg. Transient charge generation by the piezoelectric tubes is believed to enhance regeneration, since the chemical composition, surface morphology and physical structure of poled *versus* unpoled tubes are identical. Copolymer tubes produce results similar to those for homopolymer tubes in a different animal model and over a longer gap distance provide further evidence that the effects are not related to material composition or species differences. It has recently been observed that neuroblastoma cells cultured on piezoelectric PVDF and P(VDF-TrFE) substrates show enhanced levels of neuronal differentiation and process outgrowth compared to cells plated on unpoled PVDF[12]. Highly sensitive surface analysis with electron spectroscopy for chemical analysis (ESCA) and

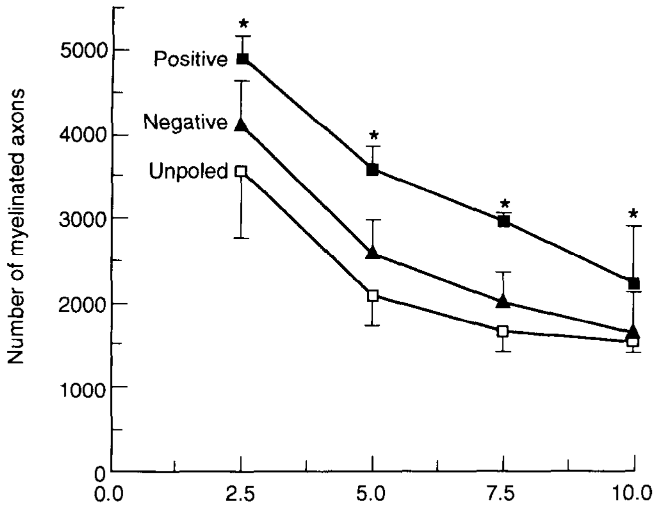

Figure 7 *Number of myelinated axons regenerated in (□) unpoled, (▲) negative and (■) positive poled vinylidenefluoride-trifluoroethylene copolymer [P(VDF-TrFE)] tubes at four points along the length of the tubes; n = 3 for each group and only includes the channels in which regeneration was successful. Data are presented as mean ±SD. Statistical significance was assessed using a two-way variance analysis;* $^{*}P < 0.005$.

comprehensive wettability measurements demonstrate that poled and unpoled surfaces are indistinguishable, meaning that enhanced outgrowth was due to charge generation[12]. This makes electrically charged fluoropolymers a unique group of biomaterials, for which bulk electrical properties rather than surface properties are most important in mediating cell/material interactions.

The mechanism by which these piezoelectric tubes enhance regeneration is not known. It has been shown that weak exogenously applied electric fields influence the direction and rate of neurite outgrowth *in vitro*[13-15] and improve regeneration *in vivo*[16-18]. Field effects may also predominate within piezoelectric tubes, since the regenerating tissue grows down the centre of the tube and does not use the luminal wall surface as a substrate.

Nerves regenerated successfully through only 50% of each copolymer tube type. Since the 10 mm nerve gap studied is known to be the greatest distance over which saline-filled impermeable tubes support outgrowth in rats[19], the lack of permeability in the rigid copolymer tubes may be a limiting factor. Permselective channels are known to support substantial axonal outgrowth, even in the absence of distal nerve tissue[2,3]. The P(VDF-TrFE) copolymer offers greater flexibility in the design of devices than the PVDF homopolymer, since it does not need to be stretched to be rendered piezoelectric. Dry-jet wet spinning techniques can be used to fabricate piezoelectric channels which are also semipermeable. Combining permeability and intrinsic charge properties may further enhance regeneration and may allow regeneration over greater gaps.

This study demonstrates that intrinsically charged guidance channels significantly enhance the regrowth of severed peripheral nerves. The flexibility and wide design opportunities afforded by the P(VDF-TrFE) copolymer make it a favourable material for a composite device which may be used to facilitate clinical repair after nerve injury.

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