Title: Suppression of scarring in peripheral nerve implants by drug elution

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Abstract:

Medical implants made of materials such as metal and polymers provoke a chronic inflammatory response in the adjacent tissue which results in the deposition of a collagenous scar tissue layer on their surface, that gradually thickens over time. This is a critical problem for neural interfaces. Scar build-up on electrodes results in a progressive decline in signal level because the scar tissue gradually separates axons away from the recording contacts. In regenerative sieves and microchannel electrodes progressive scar deposition will constrict and may eventually choke off the sieve hole or channel lumen. All interface designs need to address this issue if they are to be fit for long term use. This paper describes a method of inhibiting the formation and thickening of the fibrous scar. Research to date has mainly focused on methods of preventing stimulation of the foreign body response by implant surface modification. In this paper a pharmacological approach using drug elution to suppress chronic inflammation is introduced. Microchannel implants made of silicone doped with the steroid drug dexamethasone were implanted in the rat sciatic nerve for periods of up to a year. Tissue from within the microchannels was compared to that from control devices that did not release any drug. In the drug eluting implants the scar layer was significantly thinner at all timepoints, and unlike the controls it did not grow after 6 months. Control implants supported axon regeneration well initially, but axon counts fell rapidly at later timepoints as scar thickened. Axon counts in drug eluting devices were initially much lower, but increased rather than declined and by one year were significantly higher than in controls. Drug elution offers a potential long term solution to the problem of performance degradation due to scarring around neural implants.

Introduction

Almost all medical implants made of non-biological materials provoke a chronic inflammatory response in the adjacent tissue which results in the deposition of a collagenous scar tissue layer on their surface (Anderson *et al.*, 2008). Foreign body giant cells, arising from the fusion of multiple macrophages, are one of the hallmarks of this response and are present at the implant/tissue boundary throughout the lifetime of the device. These cells release inflammatory cytokines which engender the gradual thickening of the scar.

Most implantable neural interface devices in mainstream clinical use at present are stimulators that target whole nerves, such as vagus nerve stimulators for epilepsy, and scarring is not a major issue for such devices. However it may be a critical problem for interfaces designed primarily for recording, in order for example to derive motor efferent information to drive a prosthetic limb. Such interfaces typically feature multiple small contact sites in order to try to pick up high resolution information. Neural signals are weak, and close positioning of axon and electrode is important. Scar build-up on electrodes results in a progressive decline in signal level because the scar tissue gradually separates axons away from the recording contacts. In sieves (Edell, 1986; Kovacs *et al.*, 1992; Lago *et al.*, 2005) and microchannel electrodes (FitzGerald *et al.*, 2012), there is another problem: progressive scar deposition will constrict and may eventually choke off the sieve hole or channel lumen.

Various means of reducing the foreign body response (FBR) around neural electrodes have been explored. Nonspecific serum protein adsorption onto hydrophobic electrode surfaces is a trigger of inflammation (Wilson *et al.*, 2005). One strategy to deal with this is to coat the surface with a protein that is benign (in the sense of not being pro-inflammatory), an example being the extracellular matrix constituent laminin (He *et al.*, 2006). Alternatively, the implant surface can be rendered hydrophilic and thus protein-repellant by coating with substances such as polyethylene glycol (PEG) (Rao *et al.*, 2012). Another more recently recognised problem is a mismatch of compliance between neural tissue, one of the softest tissues in the body with a Young's modulus usually below 100 kiloPascals, and implant materials which are typically in the MegaPascal range for elastomers or GigaPascals for plastics (Lacour *et al.*, 2010). High surface stiffness stimulates macrophages, and reducing biomaterial stiffness can reduce macrophage activation leading to a less severe foreign body reaction (Blakney *et al.*, 2012). Hydrogel coatings can reduce the apparent stiffness of foreign bodies by several orders of magnitude, and modification, for example by the inclusion of certain oligopeptides in the hydrogel, can further reduce the FBR (Swartzlander *et al.*, 2015). Much of the research into reducing the FBR that deals specifically with neural electrodes has been done on electrodes designed for insertion into the brain rather than the peripheral nervous system, and results cannot necessarily be generalised from one setting

to the other because the mechanism of inflammation in the two sites is fundamentally different.

These methods all aim to reduce the degree to which chronic inflammatory processes are stimulated. A different approach that is examined in this paper is to accept that the implant material will trigger a chronic inflammatory response, and pharmacologically suppress it. When biological processes must be modified only in the immediate vicinity of the surface of a medical implant, drug elution, i.e. the slow release of pharmacological substances from the device itself, is an elegant solution. Applications have included the suppression of cell proliferation and re-stenosis in coronary artery stents (Rensing *et al.*, 2001; Sousa *et al.*, 2005), and the delivery of antibiotics to prevent infection of cerebrospinal fluid shunts (Bayston *et al.*, 1989). Most pertinently here, drug elution has long been used in cardiac pacemaker lead tips (Mond *et al.*, 2014) to suppress inflammation. Pacemaker leads are passed through large veins to reach the heart where their tip makes contact with the wall of one of the heart chambers. Electrical pulses are delivered through the lead to its tip to activate the muscle that it contacts. In older designs of pacing lead, the "pacing threshold", i.e. the voltage required to trigger muscle contraction, would increase significantly after implantation, partly due to the deposition of scar tissue around the lead tip at the point where it made contact, because of the same foreign body response that affects the neural interface. This reduced reliability and shortened pacemaker battery life.

In the 1980s, leads were introduced containing a tiny quantity of the steroid drug dexamethasone, the drug being held in a silicone plug next to the tip which acted as a slow release device such that it was eluted over years. This was a revolutionary change in design: pacing thresholds stayed at baseline values for over a decade (Mond and Stokes, 1996). Although dexamethasone is a powerful drug, the release rate is so low (the total quantity of dexamethasone in the lead tip is less than a tenth of the dose used orally or intravenously in a single day for some clinical indications) that there are only any significant effects in the immediate area of the lead tip and no systemic side effects whatsoever.

Dexamethasone release has been shown to reduce inflammation around electrode probes in the brain for periods of up to four weeks (Zhong and Bellamkonda, 2007). However, these results cannot be straightforwardly extrapolated to the setting of a chronic peripheral nerve interface, because we are ultimately seeking scar suppression for a period hundreds of times longer, and also because the inflammatory response in brain tissue is qualitatively different from that seen in most of the rest of the body including the peripheral nerves, characteristically featuring astrogliosis rather than a fibrotic foreign body response.

Adapting the approach used in pacemaker leads for use in microchannel arrays for peripheral nerve is straightforward in principle because they have previously been constructed from silicone (FitzGerald *et al.*, 2012), and so the substrate of the device can double as a slow release drug reservoir. The design of a prototype device is shown in figure 1 (end view of microchannel array). Within the silicone substrate of the device are areas where the silicone has been doped with very small quantities of dexamethasone. Devices of this type have been implanted in the rat sciatic nerve for periods of up to a year and this paper details evidence of the scar-suppressing effects of this technique.

Materials and methods

Implant construction

2.5 mm long microchannel arrays were constructed using a variant of the technique previously described for constructing arrays in silicone (FitzGerald *et al.*, 2012). Briefly, 150μm diameter steel pins were passed through two 100-mesh electron microscopy grids (type G100, Gilder grids, UK) to hold them in a coherent array, then this array was cast in silicone (Sylgard 184, Dow Corning). The pins were then pulled longitudinally out of the silicone leaving the array of empty microchannels. 20 randomly chosen pins were measured with a micrometer, demonstrating a high level of uniformity (mean diameter 150 μm, standard deviation 1.2 μm).

For the implants in this study the channel array was fabricated in seven sections as shown in figure 1A. Four channel-containing sections were made in the fashion described above, two containing ten channels in rows of six and four, and two containing two channels. Dexamethasone acetate powder (Sigma Aldrich no. D1881) was mixed into uncured silicone at a concentration of 10 mg/ml and the resulting suspension was cured in a 50μm thick layer. The implant comprising four channel-containing sections and three interposed steroid-doped silicone layers was assembled by applying a thin film of uncured silicone to adjoining surfaces and then curing to bond them together. In this way an array with six layers of channels in a 2-4-6-6-4-2 pattern was obtained with each channel 25 μm from a steroid depot (figure 1B and C). The steroid doped layers extended along the entire length of the array; their width was 1.4 mm for the middle layer and 0.9 mm for the outer layers, giving a total volume of steroid-doped silicone in the device of 0.40 μl. The total quantity of dexamethasone in each device was therefore 4.0 μg. The array was then inserted into a section of silicone tubing, the length of which exceeded that of the array by 2mm at either end so as to form a cuff to suture the nerve stumps into.

Control implants were made in exactly the same way with the exception that the "doped" layer contained no drug.

Implantation

Adult male Lewis rats were implanted. The right sciatic nerve was exposed at mid-thigh and divided, and the stumps were passed into the cuff at either end of the channel array so that the cut nerve ends were touching the ends of the array (and were thus 2.5 mm apart). The

stumps were secured in this position using 9/0 nylon sutures (Ethicon) to attach the epineurium to the cuff.

Implants were left in situ for 3, 6, or 12 months. For each timepoint, 6 control and 6 steroid-loaded devices were implanted.

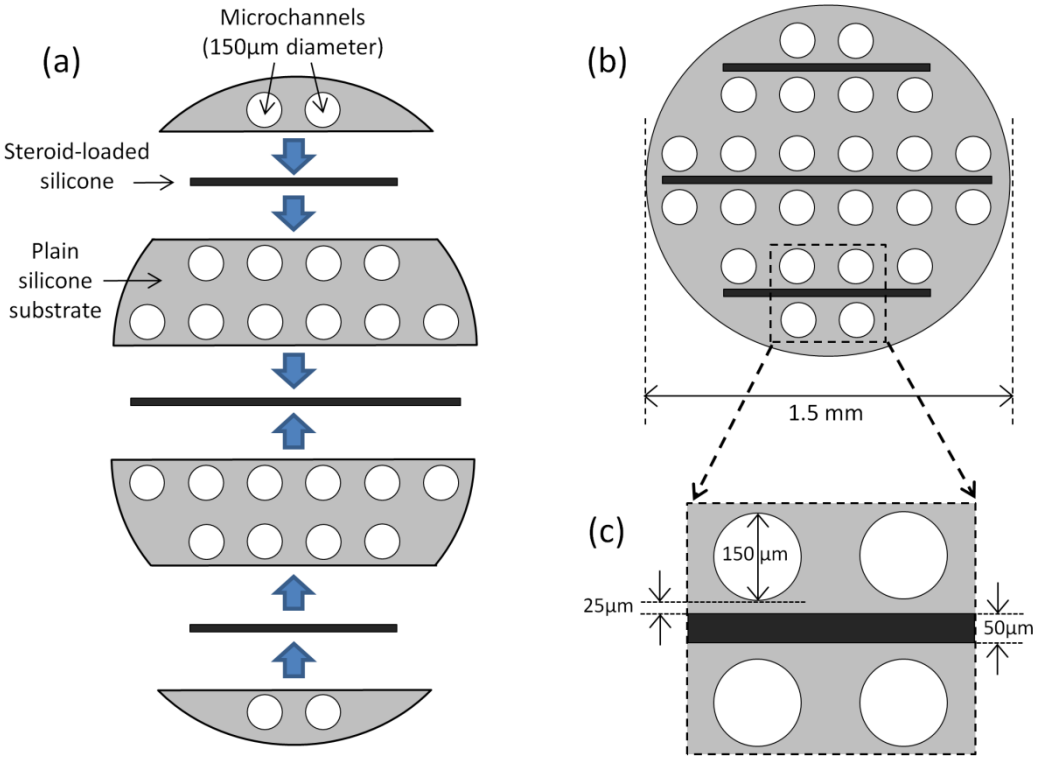

Figure 1. Construction of steroid eluting implants (end view). (a) The seven layers that make up the channel array. Sheets of silicone loaded with dexamethasone acetate powder are shown in black and the undoped silicone substrate containing cast microchannels is in grey. Assembly is in the direction of the arrows using small quantities of silicone for bonding. In control implants construction was identical but the dexamethasone was not added to the silicone when making the 'doped' sheets. (b) Assembled channel array. (c) Close-up of part of panel (b) showing details of channel and drug layer dimensions.

Histology

At the designated timepoint, after perfusion fixation with 4% paraformaldehyde the implants were removed. They were cut transversely at their midpoint with a razor blade and the stumps gently pulled away, drawing the fixed tissue out of the microchannels. Tissue was

further treated with 1% glutaraldehyde and then 1% osmium tetroxide, and embedded in resin. 1μm thick sections were cut on an ultramicrotome, oriented transversely to the nerve so as to contain cross sections of the tissue from all the microchannels. Sections were stained with toluidine blue and examined using brightfield microscopy.

The tissue from within the microchannels featured a peripheral connective tissue sheath with a central region populated with regenerated axons, as previously described (FitzGerald *et al.*, 2012) and shown in figure 2. To evaluate the scarring problem and the effect of steroids the thickness of the peripheral connective tissue layer from all channels in all implants was measured. In each channel the thickness at 4 points around the channel periphery (3, 6, 9, and 12 o'clock) was determined and the average of these taken as the measurement for that channel.

In order to evaluate the effect of steroid elution on axon regeneration and survival the number of axons in each microchannel of each device was counted.

Drug elution assay

Four drug eluting devices were used to characterise the drug elution rate. In order to match physiological conditions as closely as possible, the devices were placed in an incubator at 37^{o}C and a multichannel syringe driver (Harvard Apparatus) was used to continuously pump Hank's Balanced Salt Solution (HBSS) through each device at a rate of 1ml/24h. Samples of the fluid were collected at intervals and assayed using a dexamethasone Enzyme Linked Immunosorbent Assay (ELISA) kit (cat. no 1112-03, Bioo Scientific Corporation, Austin, USA). Two control devices were perfused similarly; fluid from them gave identical results to the negative control solution provided with the kit.

Drug elution modelling

A finite element model of drug release was constructed using Comsol Multiphysics (Comsol Ltd, Cambridge). The cross section of the steroid eluting devices (see figure 1(b)) can be considered to be made up of repeating square units 250 μm on each side, each containing one channel at its centre and half of the width of one of the steroid depots along one edge. This unit was modelled in two dimensions, using an initial concentration of 10 mg/ml of dexamethasone in the doped region and 0 mg/ml elsewhere, and assuming a perfect sink for dexamethasone within the channel. The diffusion coefficient of dexamethasone in the PDMS used here was determined empirically by fitting the model to the observed data.

Statistics

Data distributions were tested for normality using a Shapiro-Wilk test. Groups of normally distributed data were compared using parametric two-way analysis of variance (ANOVA), the independent variables being timepoint and the presence or not of steroids. Post-hoc analysis was performed using a Tukey test. Non-normally distributed data were analysed using an aligned rank transform followed by two-way ANOVA, with subsequent nonparametric pairwise testing using a Mann-Whitney U test.

Results

Chronic inflammatory response

The effects of steroid elution on scar tissue thickness are shown in figure 2. Panels (a) and (b) show cross sections of typical microchannel contents from control and steroid eluting channels respectively at 12 months: the difference in scar tissue (ST) thickness is readily apparent. Panel (c) shows the scar thickness in both control and drug eluting implants at each time point. Scar thicknesses in each group were normally distributed (Shapiro-Wilk test) and were therefore analysed using two-way analysis of variance. Both the presence of steroids and the duration of implantation were significant factors in determining scar thickness ($p < 0.0001$ for both). There was a significant interaction between these two factors ($p = 0.0053$) indicating that the scar thickness behaved differently over time in the steroid eluting implants than in the control implants. Post hoc analysis using Tukey's test showed that mean scar thicknesses were significantly greater in the control implants than in the drug eluting implants at all three timepoints ($p < 0.01$). In the untreated implants, the scar layer on the inside of the microchannel walls progressively increased with time, the thickness being 23μm at 3 months, 28μm at 6 months, and 34μm at 1 year. The increases from 3 to 6 months and from 6 months to 1 year were both significant ($p < 0.01$). Scar thickness in the drug eluting implants was 14μm at 3 months and 19μm at 6 months, a significant increase ($p < 0.01$). However, there was no significant increase from 6 months to 12 months (12 month thickness 20μm), suggesting that scar thickening had either slowed dramatically or stopped.

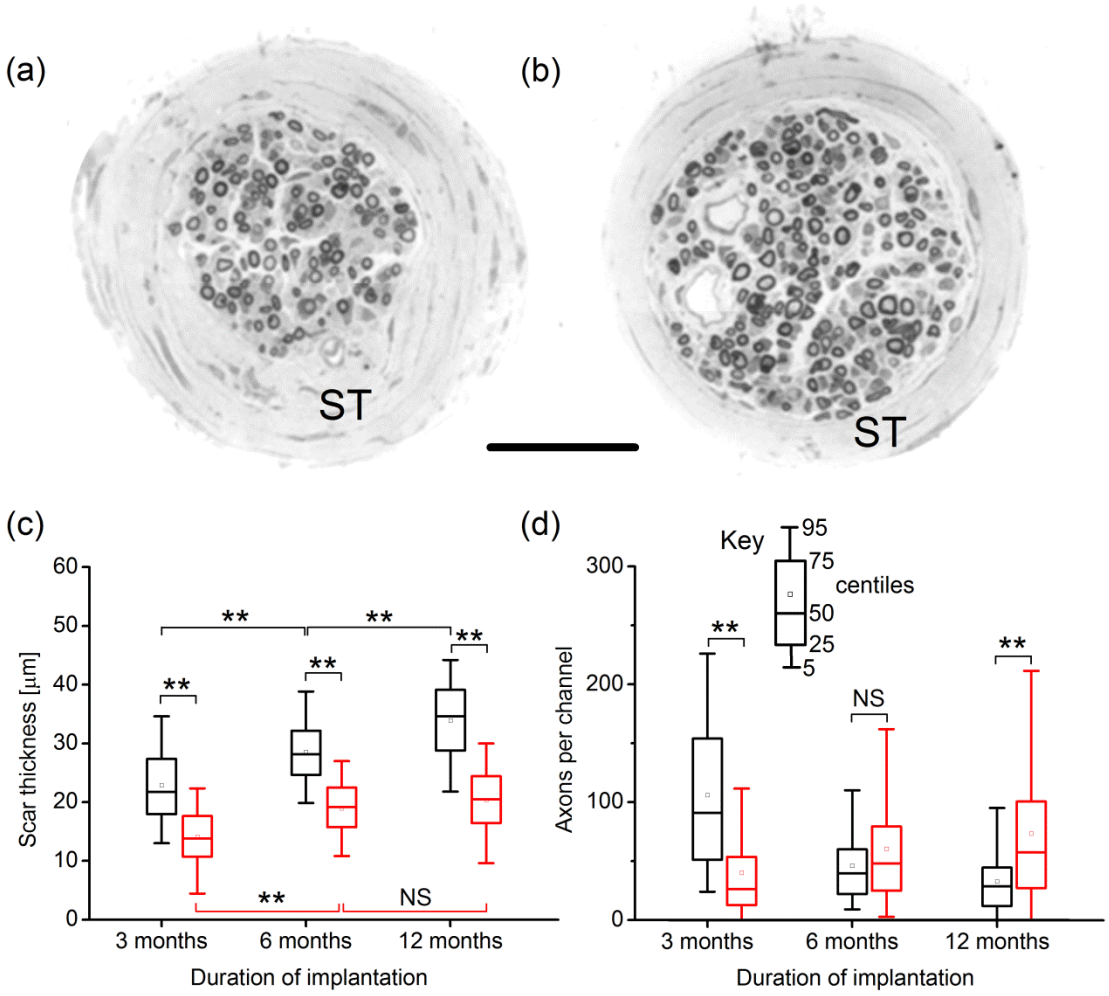

*Figure 2. Effect of steroid elution on scar layer thickness and axon regeneration in microchannels. (a) and (b) Cross sections of typical channel contents from control (a) and steroid eluting (b) implant at 12 months. The channel contents comprise a central core containing axons and peripheral scar tissue (labelled "ST"), the outer border of which is adjacent to the channel wall. The scar tissue thickness is clearly reduced in the steroid eluting implant. Bar = 50 µm. (c) Pooled data for each group of 6 implants at each timepoint in control (black) and steroid eluting (red) implants, showing the distribution of scar thicknesses. (d) Pooled data for each group of 6 implants at each timepoint in control (black) and steroid eluting (red) implants, showing the distribution of the numbers of myelinated axons at the midpoint of each microchannel. ** denotes $p < 0.01$; NS = non significant.*

Group	Control devices			Steroid eluting devices		
	3 mths	6 mths	12 mths	3 mths	6 mths	12 mths
Proportion of channels reinnervated [%]	80	81	86	73	81	74
Mean myelinated axons per implant - proximal	6120	6649	7445	5230	5436	5812
Mean myelinated axons per implant - mid device	2016	899	724	751	1198	1341
Mean myelinated axons per implant - distal	1869	763	552	449	968	1133
Mean myelinated axons in contralateral nerve	7888	7970	7923	7876	7915	8054

Table 1. Axon counts.

Axon regeneration

Approximately 80% of microchannels contained axons when analysed histologically. The proportions for each device type and timepoint are given in table 1. Counts of regenerated axons within the microchannels are shown in figure 3. Axon counts were not normally distributed (Shapiro-Wilk test) and therefore a nonparametric ANOVA was used, as described in the methods section. Both the presence of steroids and the duration of implantation were significant factors in determining axon counts ($p = 0.013$ and $p < 0.0001$ respectively). There was a significant interaction between these two factors ($p < 0.0001$) indicating that the axon counts behaved differently over time in the steroid eluting implants than in the control implants. At the 3 month timepoint, the number of axons per channel in the drug eluting devices was substantially and significantly lower than the untreated devices, suggesting that the steroid may have an inhibitory effect on axon growth through the device. There was a mean of 105.2 axons per innervated microchannel in the untreated devices compared to 40.6 in the steroid eluting devices ($p < 0.0001$). However, whereas the axon counts in the untreated devices declined over time, those in the drug eluting devices did not. At 6 months there was no significant difference in axon counts between doped and undoped devices (steroid eluting 60.9 axons per microchannel, control 45.7, $p = 0.14$), and by 12 months the original situation had been reversed, with counts in the treated devices

approximately double those in the control devices (steroid eluting 73.8 axons per microchannel, control 32.9, $p < 0.0001$). For the control devices, the decreases in axon numbers from 3 to 6 months, and from 6 to 12 months, were both statistically significant ($p < 0.0001$ and $p = 0.0001$ respectively). For the drug eluting devices the increase in axon numbers from 3 to 6 months was significant ($p = 0.0003$) but there was no significant increase from 6 to 12 months ($p = 0.18$) suggesting that, as with scar thickness, the axon count in steroid eluting devices stabilises after 6 months.

Drug elution assay and modelling

The results of the drug elution rate assay are shown in figure 3. The release rates for the four devices separately are shown in figure 3(a) and the mean release rate is shown as the solid line in figure 3(b). Measured drug release peaked on day 30 at a mean of 22 ng/24h. It then progressively declined but at 12 months the devices continued to release dexamethasone at a mean rate of 3.6 ng/24h. Figure 3(b) also shows the elution rate predicted from the finite element model (dashed line). The model gave a good fit with the elution profile when a value of 1.5×10^{-16} m^2/s was used for the diffusion coefficient of dexamethasone in PDMS at 37^{o}C. Figure 4(c) shows the changes over time in the elution rate and the concentration of drug within the device

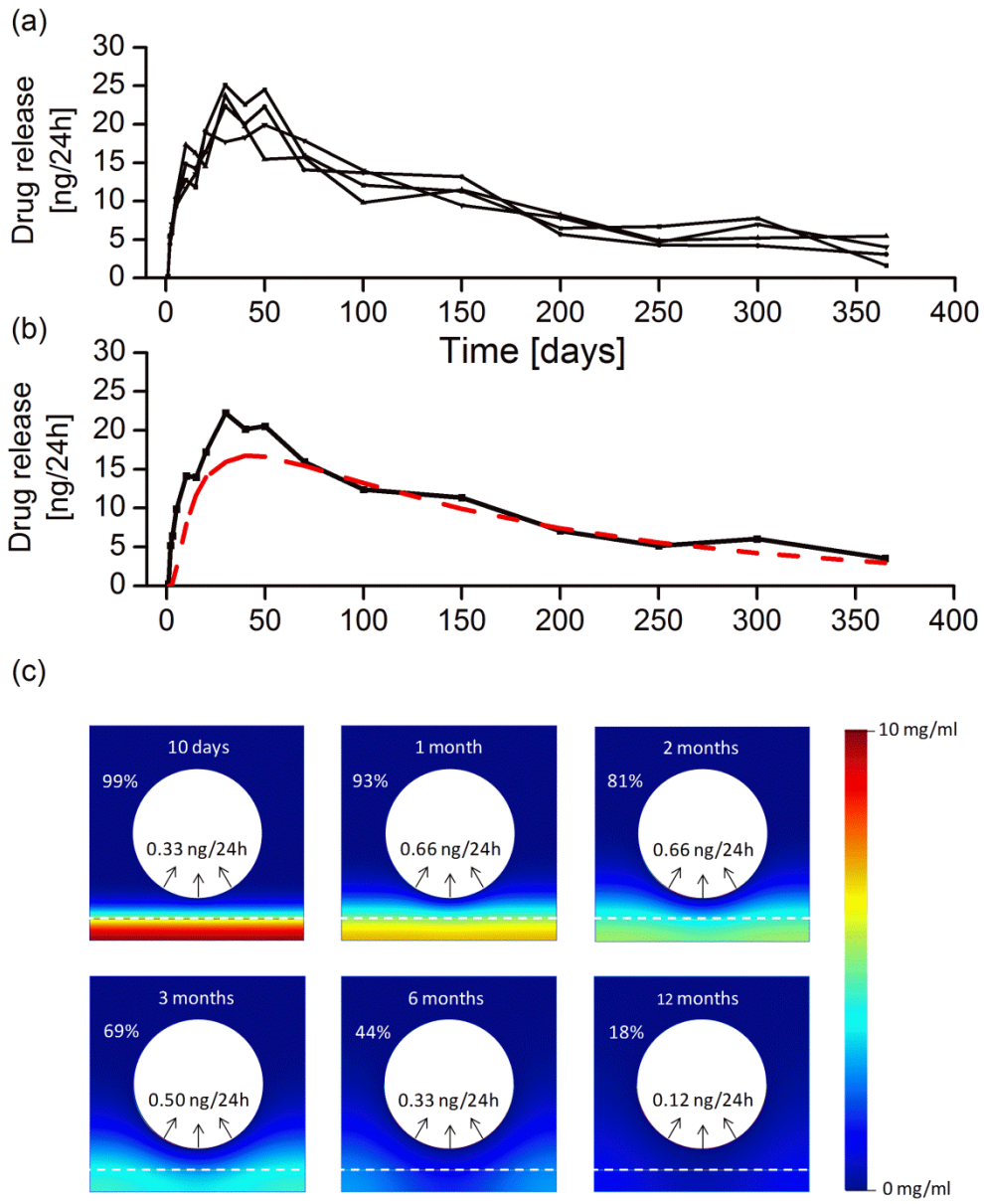

Figure 3. Drug elution rates. (a) Elution rate for 4 implants measured over 12 months. (b) Mean measured elution rate (solid line) together with elution rate from finite element model (dashed line). (c) Finite Element Model of the concentration of drug in the device and rate of release into the microchannel at several timepoints. The percentage figure shows the proportion of the starting amount of the drug that is left in the device at the timepoint shown, and the figure in the channel shows the elution rate into that channel (note there are 24 channels in the whole device). The dashed white line shows the boundary of the initially doped area (below the line).

Discussion

This study demonstrates that dexamethasone elution is effective in reducing the fibrous connective tissue deposition seen in the chronic inflammatory response to artificial materials in peripheral nerve. This is an important development in interface design because progressive scarring will impair the long term function of all designs of peripheral nerve interface.

Miniscule quantities of drug can be used that elute over very long periods of time. The steroid eluting implants used in this study each contained 0.4 µl of silicone doped with 10 mg/ml dexamethasone, equating to 4 µg of the drug per device. Drug elution rate peaked at 22 ng/24h but dexamethasone was still being released after 12 months at a mean rate of 3.6 ng/24h.

It remains to be clarified exactly what rate and profile of drug release is optimal for scar suppression, but the results here give some clues. The fact that scar thickness shows no significant increase between 6 and 12 months shows that the lower elution rate in the later part of the experiment, down to below 4 ng/24h at 12 months, may be enough to suppress scarring in the long term. Paradoxically, when drug release rates are at their highest in the interval between implantation and the first timepoint examined histologically at 3 months, the greatest growth of scar tissue is seen, although well below that in control devices. This indicates an initially higher release rate is desirable, and the type of release profile seen here with a high initial release rate and subsequent drop to much lower levels chronically appears generally appropriate. However the "tail" of the release must be made much longer if the design is to be appropriate to protect against scarring for many years. It is quite possible to engineer steroid eluting silicone devices with a chronic release phase lasting much longer: in one design of dexamethasone-eluting cardiac pacemaker lead tip, 20% of the initial dexamethasone remained in the silicone reservoir after 10 years (Mond and Stokes, 1996), during which time scarring was effectively suppressed (compared to approximately 20% remaining at one year here). Options that could be explored for lengthening the elution profile include altering the diffusion constant of dexamethasone in PDMS by changing the type of PDMS used, or adding a layer of less pervious material between the doped layer and the channels. Testing the device over a period of decades is of course impractical, and the best solution is likely to be in vitro and in vivo tests of the elution profile over a reasonable period, alongside mathematical models similar to that described here, capable of extrapolating their results far into the future. Although its action will initially be local, the drug released by the device will eventually find its way into the systemic circulation and the potential for systemic side effects is an important consideration with any drug-releasing

device. The risk of systemic side effects from the steroid released by this device is negligible. A device scaled up for use in a human median or ulnar nerve (typical diameter 3 mm) might contain approximately ten times more drug, i.e. ~ 40 µg, giving a peak release rate of some 200 ng/24h. By comparison, the human body's normal production of its endogenous steroid, cortisol, in health is 5 to 28 mg per day (Cope and Black, 1958). Taking into account that dexamethasone is some 25 times more potent than cortisol, the daily release from the implant of 200 ng of dexamethasone is equivalent to a release of 5 µg of cortisol. Thus the peak amount eluted per day would have a potency equivalent to approximately one thousandth that of the lower limit of the normal range of the body's endogenous steroid production.

At the earliest timepoint examined here, axon numbers were much lower in the drug eluting implants than in the control implants. The presence of the steroid does not prevent axon regeneration: axons grow into the drug eluting devices, and their numbers increase with time despite continued drug release. However regeneration clearly proceeds much more slowly in the presence of steroids. This is understandable because while macrophages and their inflammatory cytokines are a problem in the chronic phase, acutely they are an important component of the initial part of the process of nerve regeneration through tubes (Williams *et al.*, 1983). Selection of a different drug with more specific actions that impact on chronic but not acute inflammation may be one way to address this in future. For non-regenerative interfaces such as needle arrays, retardation of axon growth is of course not an issue.

At later timepoints, in the steroid eluting devices axon numbers are preserved, whilst in control implants numbers dropped between 6 and 12 months. By one year, the drug eluting implants contained more axons than controls. The loss of axons in the control implants is likely to be due to scarring and progressive lumenal occlusion. Axon loss between 6 and 12 months has also been seen in sieve electrodes (Lago *et al.*, 2005), which like microchannel arrays contain small orifices through which axons must pass, where they are therefore vulnerable to encroaching scar tissue. The preservation of axons in the steroid eluting implants is likely to be due to the reduction in scarring and consequent axonal strangulation.

Axon numbers in control implants at 3 months are similar to previously published data on the same type of PDMS microchannel array (FitzGerald *et al.*, 2012). One of the problems with this array is its low transparency (the percentage of the cross section of the device that is open space, available for regeneration, as opposed to occupied by the device substrate). The transparency of the device used here was less than 30%. Switching to a higher transparency device using a different construction technique can greatly increase axon counts; in a previous study using microchannel devices with a 62% transparency the mean

count at the same timepoint was 6244 (FitzGerald *et al.*, 2012). One of the important tasks ahead is to integrate the drug eluting capability into a higher transparency array.

At 12 months the scar thickness in the steroid eluting implants is reduced by 41% compared to controls, but the most important measure of scarring in regenerative implants is its effect on the cross sectional area of the aperture through which axons must pass. In the control implants in this study the typical scar layer at 12 months was 34 μm. A layer of this thickness around the periphery of the 150 μm diameter microchannel leaves a central scar-free area available for axons that is 82 μm in diameter and 5,300 μm^2 in cross section; the scar tissue has effectively reduced microchannel cross section by 70%. In the steroid eluting implants at 12 months the scar layer is 20 μm thick, leaving a central scar-free area 110 μm in diameter and 9,500 μm^2 in cross section; the scar tissue has effectively reduced microchannel cross section by 46%. Although reduced and stabilised by drug elution, the loss of space for axons to pass through remains large, and this will limit the proportion of axons in the proximal nerve stump that can gain entry to the device, potentially sacrificing valuable control information for a prosthesis. Even if drug elution stops axons from being lost over time, those for which there was no room in the first place can never be recorded from or stimulated. Thus it will be important in future work to target further reduction in scar thickness as well as simply its stabilisation. Steroid elution has resulted in speed of regeneration being sacrificed in exchange for long term stability of scar thickness and consequently sustainability of the axons in the interface and therefore its function. The output of an interface incorporating steroid elution is likely to be changeable until axonal regeneration through it is complete. There was a substantial difference in axon numbers between 3 and 6 months, but much less change between 6 and 12 months, suggesting that it may take up to 6 months for axon regeneration, and therefore device output, to become stable. Devices will need to remain functional for decades in young patients, and a delay of even six months after implantation before the device can be used is likely to be an acceptable price to pay for a system that is long lasting.

The potential for neuropathic pain is always of concern in nerve injury. No specific tests of sensation were done in this study, but none of the animals showed any sign of discomfort or distress at any point, making the presence of significant neuropathic pain unlikely.

Finally, it should be emphasised that this method of scar prevention need not be seen as an alternative to other approaches but rather complementary to them. There is nothing to prevent it being used alongside other methods and it is hoped that a dual approach, e.g. surface chemistry modification together with drug elution, might yield results superior to either method alone.

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