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A new PLLA/PCL copolymer for nerve regeneration

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The aim of this study is to evaluate the functional and cell biological applicability of a two-ply nerve guide constructed of a PLLA/PCL (i.e. poly-L-lactide and poly-ε-caprolactone) copolymer. To do so, we performed a cytotoxicity test, a subcutaneous biodegradation test and an *in situ* implantation study in the sciatic nerve of the rat. The nerve guide copolymer was found to be non-toxic, according to ISO/EN standards, and it showed a mild foreign body reaction and complete fibrous encapsulation after implantation. Onset of biodegradation of the inner layer was seen after one month of implantation. After 18 months of implantation complete fragmentation was observed, as well as a secondary inflammatory response characterized by foreign body giant cell activity and phagocytosis of polymer debris. Recovery of both motor and sensory nerve function was observed in all nerve guides.

1. Introduction

In clinical reconstructive surgery the use of autologous nerve grafts remains the major choice for reconstruction of a nerve gap. However, both experimentally and clinically, there is increasing evidence that the use of polymeric nerve guides will also result in recovery of nerve function [1]. The use of a polymeric nerve guide will eliminate the necessity of obtaining an autologous nerve graft, and therefore prevent loss of donor nerve function and neuroma formation.

Tubular nerve guides constructed of various materials, such as silicone rubber [2–6], acrylic polymer [7], polyethylene [8, 9], elastomer hydrogel [10] and porous stainless steel [11] have already been used for this purpose. These nerve guides, however, are synthesized of durable material, which remains *in situ* as a foreign body, and may therefore limit recovery of nerve function. In a case report, described by Lundborg *et al.*, motor and sensory recovery was excellent after nerve repair with a silicone rubber chamber, but 3 years later the patient was complaining of irritation around the silicone tube, necessitating removal of the conduit.

Biodegradable polymeric nerve guides, such as copolymers of poly-L-lactide and polyurethane [12], poly-glycolic acid [10, 13–15], polylactides [5], poly-DL-lactide [14, 16], triethylcitrate [14, 16], glycolide trimethylene carbonate [17] and polyglactin [18, 19] have recently been used to repair nerve gaps and provide a successful alternative. After functioning as a scaffold, they gradually degrade in the human body. Most of the nerve guides mentioned above demonstrated very high patency rates. Robinson *et al.* reported a significantly better histological quality of the regenerated nerve in the nerve guide compared to autografts.

It is the aim of this study to evaluate whether a degradable nerve guide composed of poly-L-lactide and poly-ε-caprolactone is suitable for nerve reconstruction.

A cytotoxicity test, a subcutaneous biodegradation study and an *in situ* pilot study with this new nerve guide have proven its suitability.

2. Materials and methods

2.1. Preparation of nerve guides

The preparation of the nerve guides has been described in detail by Grijpma *et al.* [20] and Hoppen *et al.* [21].

Briefly, the biodegradable nerve guide (Fig. 1) is composed of 2 layers. The inner layer is dense and composed of a copolymer of PLLA/PCL, dip-coated on a glass mandrel (diameter 1.2–1.3 mm). The outer layer is porous and also composed of PLLA/PCL. Porosity was obtained by mixing sugar crystals into the polymer solution. After preparation, the nerve

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guides were left in distilled water for several days and dried overnight at 30 °C.

2.2. Cytotoxicity test

The poly-L-lactide and poly-ε-caprolactone copolymer was tested for cytotoxic leachables using an extract test according to ISO/EN standards. Briefly, a sample of the copolymer was sterilized in ethanol and degassed for 48 h. 60 cm^2 of the material was extracted at 37 °C for 24 h in 20 ml minimal essential medium (RPMI, tissue culture medium with 10% foetal calf serum added). Blank solutions (extracts without the test material) were prepared in a similar manner. We used ultra high molecular weight polyethylene as a negative control and latex as a positive control.

Subsequently, a monolayer of human skin fibroblasts (PK-84) was grown to 80–100% confluency and challenged with the extract of the test material. After exposure to the extract for 24, 48 and 72 h, the cells were examined microscopically for cytotoxic effects: the remaining percentage of monolayer, inhibition of cell proliferation (as established by cell count on the last observation day), intracellular granulation, cellular morphology and the percentage of cellularlysis were recorded. All procedures were performed under sterile conditions.

2.3. Subcutaneous biodegradation study

Poly-L-lactide-co-poly-ε-caprolactone nerve guides, measuring 1 cm in length and 1.5 mm in diameter (Fig. 1) were subcutaneously implanted on both sides of the back of male Wistar rats ($n = 15$), weighing approximately 200 grams. The animals had access to standard rat food and water *ad libitum*.

The nerve guides were harvested at 1 week, 1 month and 18 months after implantation. The specimens were fixed in 2% glutaraldehyde (0.1 M phosphate-buffered) for 2 h. Subsequently, the specimens were washed and dehydrated in an ethanol series. Embedding was performed in glycol methacrylate (Technovit) for light microscopic evaluation. Staining was done with alkaline fuchsin and toluidine blue. The specimens were evaluated for degradation of the biomaterial and for the degree of foreign body reaction.

2.4. *In situ* pilot study

A pilot *in situ* experiment was set up to elucidate the functional capacities of the PLLA/PCL nerve guides. For this purpose, 6 prostheses were implanted in a 1 cm gap created in the sciatic nerve of Wistar rats.

Male Wistar rats ($n = 6$) weighing approximately 200 grams were premedicated with atropine (0.25 $mg\,kg^{-1}$ body weight) and anaesthetized with 1% halothane (Fluothane R). The left sciatic nerve was exposed through a gluteal muscle splitting incision. A 6 mm nerve segment was then resected, leaving a gap of about 1 cm after retraction of the ends, and continuity was re-established by a 1 cm nerve guide. When implanting the nerve guide, both the proximal and the distal cut ends of the sciatic nerve were telescoped into the ends of the guide and fixed with a single 10-0 nylon suture (Ethylon R, BV-4 needle).

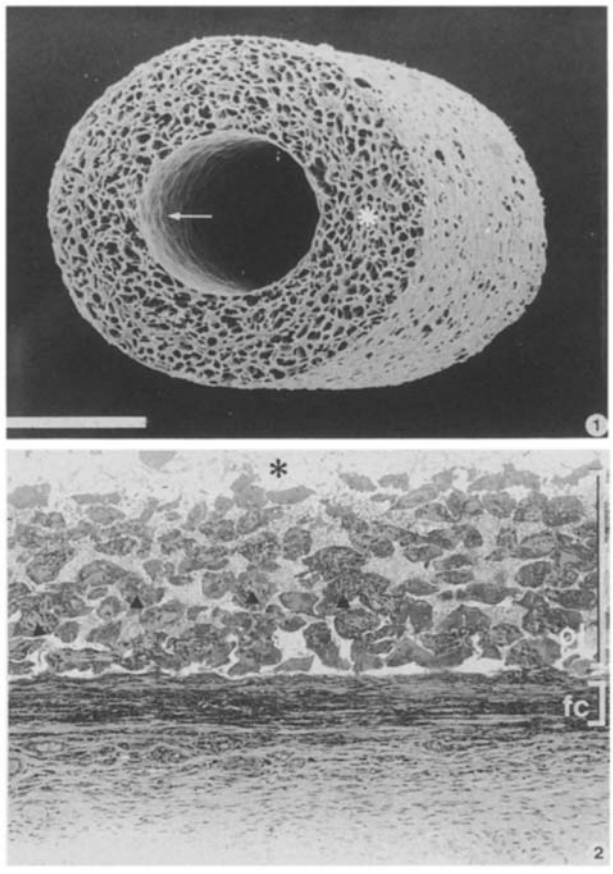


Figure 1 Scanning electron micrograph of a nerve guide used in the *in situ* pilot study. The nerve guide consists of a dense inner layer (arrow) and a porous outer layer (*). The bar represents 1 mm.
Figure 2 Light micrograph of a subcutaneously implanted nerve guide, harvested after 4 weeks. The nerve guide is surrounded by a fibrous capsule (FC) and ingrowing macrophages and fibroblasts (arrowheads) in the porous outer layer (OL) are seen, indicating a foreign body reaction. The luminal side of the nerve guide is indicated by an asterisk (*). The bar represents 250 μm.

The nerve guides were harvested at 18 months after implantation, and fixed in 2% glutaraldehyde (0.1 M phosphate-buffered) for 2 h. The specimens were washed and dehydrated routinely and the embedding was performed in glycol methacrylate (Technovit). Staining was done with either Sudan Black or a combination of alkaline fuchsin and toluidine blue.

The samples were evaluated for physiological and microscopical regeneration of nerve tissue and the foreign body reaction to the biomaterial.

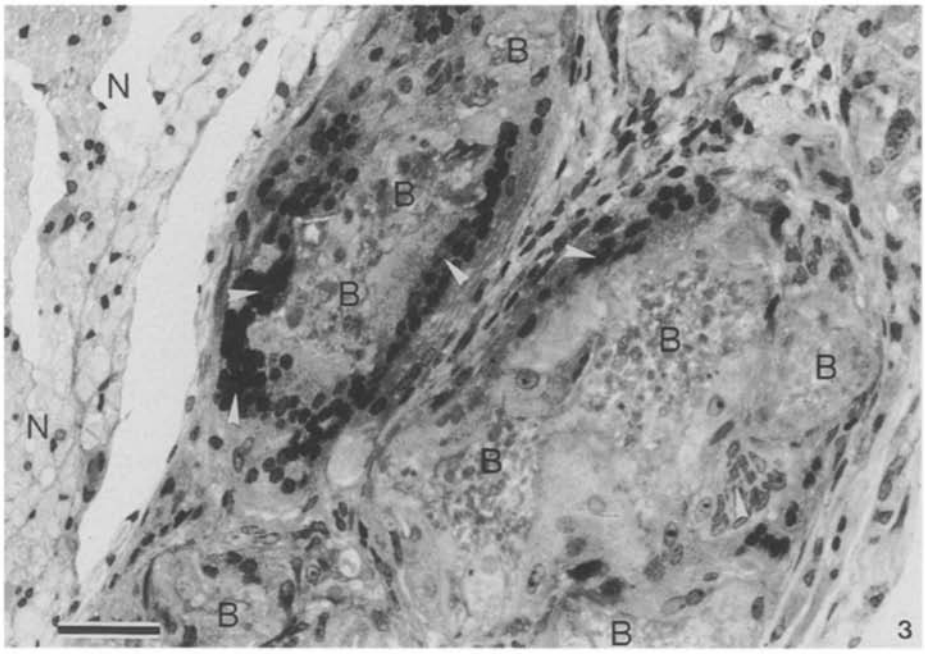
3. Results

3.1. Cytotoxicity

The cytotoxicity test demonstrated that no significant difference could be found in growth inhibition or cell death when compared with the negative controls. No intracellular granulation was observed, and only a minor inhibition in cell proliferation was seen. In total, less than 20% of the cells in contact with the extract were affected. Conforming with ISO/EN standards, we therefore consider the material to be non-toxic.

3.2. Subcutaneous biodegradation study

We evaluated the degree of biomaterial degradation and foreign body reaction after 3 periods of implantation, 1 week, 1 month and 18 months. The degree of degradation of this copolymer could be monitored by staining the specimens with Sudan Black. After a period of 1 week, the nerve guide tube was still intact. After 1 month (Fig. 2), however, the dense inner layer started to fragment, but the porous outer layer was still in place. Eighteen months after implantation there were only a few pieces of biomaterial left (Figs 3 and 4). In general, the tissue reaction was mild. One week after implantation macrophages, fibroblasts and a few granulocytes were observed around the tube. After a period of 1 month, macrophages and fibroblasts had grown into the porous outer layer of the tube. The tissue surrounding the tube was richly vascularized. Fibroblasts had encapsulated the nerve guide and had produced a collagenous capsule (Fig. 2). At 18 months, giant cells could be observed phagocytosing small pieces of biomaterial fragmented from the nerve guide (Fig. 3). This macrophage/giant cell-mediated response can be called the secondary foreign body response.



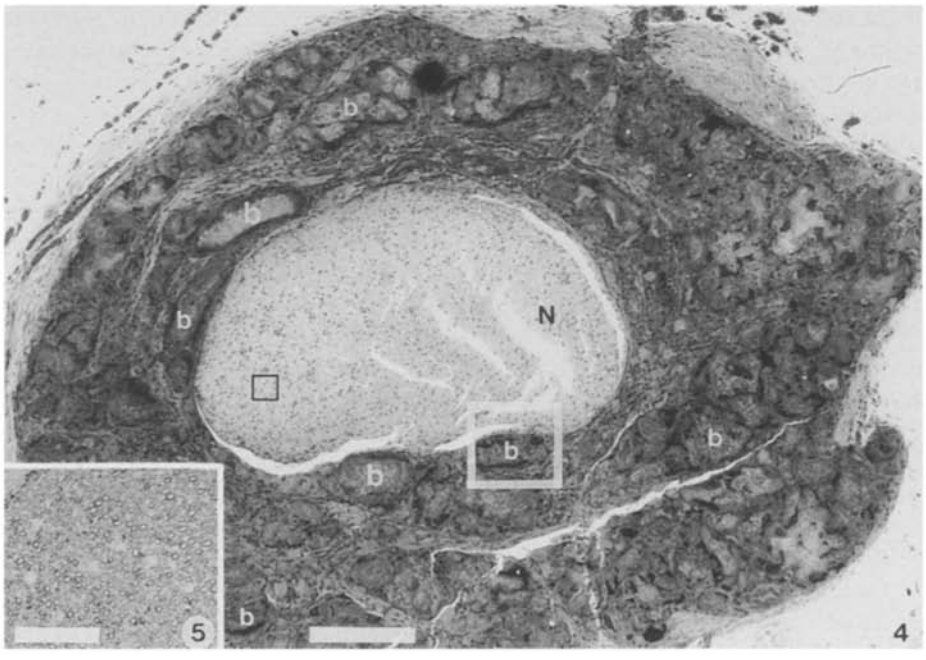


Figure 3 Light micrograph of an *in situ* implanted nerve guide, harvested after 18 months. Some regenerated nerve tissue is seen in the left top corner. Some small fragments of biomaterial debris (B) are still present, surrounded by giant cells (arrowheads). This figure is a detail from Fig. 4 (white box). The bar represents 25 μm.

Figure 4 Light micrograph showing a regenerated nerve (N) 18 months after implantation. Small fragments of biomaterial (b) are still observed in the inflammatory tissue surrounding the regenerated nerve. The white box refers to Fig. 3, the black box refers to Fig. 5 (insert). The bar represents 250 μm.

Figure 5 Detail of the regenerated nerve from Fig. 4 (black box) Sudan Black staining was used to show the myelinated nerve fibres. The bar represents 125 μm.

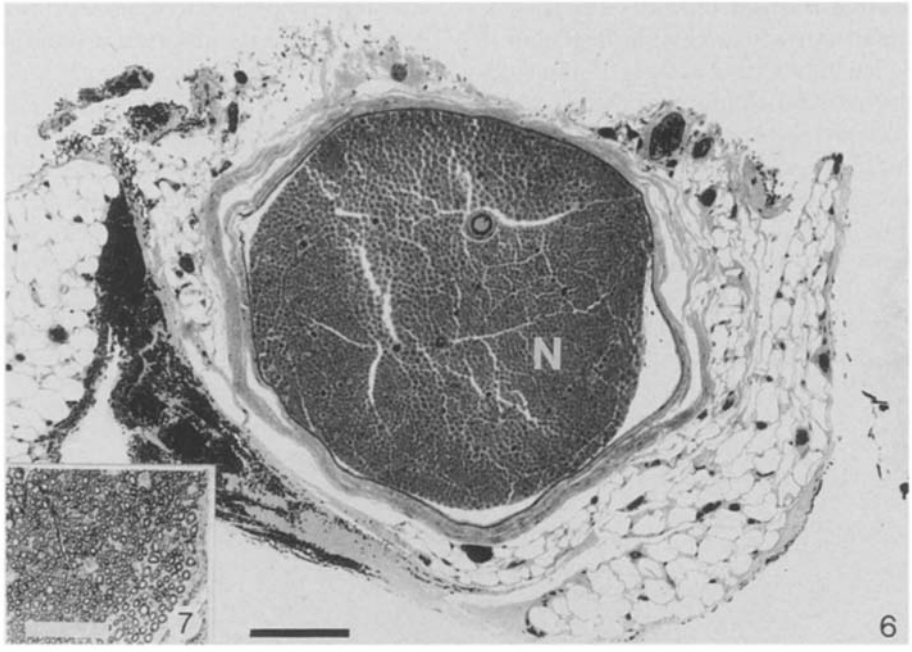


Figure 6 Light micrograph showing a normal sciatic nerve (N) of the rat. The bar represents 250 μm.
Figure 7 Detail of a normal sciatic nerve, stained with Sudan black. The bar represents 125 μm.

3.3. *In situ* pilot study

After 18 months of implantation the nerve guides were harvested. The physiological nerve response was good: the rats were able to actively plantar flex the feet, indicating return of motor function, and a mechanical stimulus always resulted in a motor reflex, indicating return of sensory function. Macroscopically, a mature nerve was always observed. However, microscopically, we found that only 1 of the 6 nerves had entirely grown through the nerve guide (Fig. 4). The other nerves had regenerated through the guides but also over the outside of the guides (i.e. they failed to function as a conduit). However, there was always a resultant recovery of both motor and sensory function.

4. Discussion

An ideal nerve guide should have a low cytotoxicity and it should protect the regenerating nerve long enough to assure full recovery of nerve function. After recovery of nerve function the nerve guide should disappear.

This study shows that this new copolymer of poly-L-lactide and poly-ε-caprolactone can be successfully used as a biomaterial for nerve repair: the copolymer shows a low cytotoxicity and a minor foreign body reaction. No intracellular granulation and only a minor inhibition in cell proliferation was observed. Macrophages and fibroblasts had grown into the outer layer of the tube. The tissue surrounding the tube was richly vascularized. After a period of 18 months, a secondary foreign body reaction was observed. Giant cells and macrophages had encapsulated the remnants of the nerve guide and were phagocytosing the polymer debris. The nerve guide conduits were at an advanced stage of degradation.

Functional nerve recovery can already be seen after a period of 16 weeks [12]. At that time the number of myelinated axons per square cross-sectional area was significantly higher when compared to a normal nerve. The mean fibre diameter was significantly lower.

We started our microscopical evaluation of the *in situ* pilot study 18 months after implantation. In light microscopic evaluation the regenerated nerves closely resembled mature nerves (Figs 4–7): the density of myelinated fibres in regenerated nerves and controls were similar. There was also a normal range in fibre diameter and there was only slightly more collagen produced.

It is possible that nerves are still regenerating after a period of 18 months, becoming more mature. In following studies we will therefore perform morphometric analysis of specimens retained for longer periods of time *in vivo*.

Some guides failed to function as conduits. It is possible that the inner layer of the guide was made too thin. If it breaks down too quickly, macrophages and fibroblasts can grow into the lumen of the tube and produce scar tissue which causes contraction and/or hampers the regeneration. As is known, a regenerating nerve grows across a 1 cm gap within 6 weeks [16], and the inner layer should remain intact for a long enough period to allow complete regeneration. The length of this period is not yet known and is the subject of further research.

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