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Microstructured polydimethylsiloxane membranes for peripheral nerve regeneration

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ABSTRACT

We engineered anisotropic microstructured membranes for nerve repair applications by exploiting electron beam lithography on silicon substrates and soft lithography techniques. Our substrates were patterned with polydimethylsiloxane (PDMS) gratings (alternating lines of grooves and ridges) of varying ridge/groove width and depth. These gratings were used as scaffolds to study rat Schwann cell contact interaction for cell migration. We observed that cell motion was affected by the presence of the grating and by its periodicity, while cells on flat substrates showed random spatial migration. Our results allow the identification of specific topographical elements that may be exploited for the production of new devices for enhancing nerve regeneration by promoting Schwann cell invasion, proliferation, and terminal differentiation.

1. Introduction

Peripheral nerve injury is a common form of trauma with up to 300,000 cases each year in Europe. Nerve lesions can significantly complicate the clinical course and outcome of injured patients and are present in about 5% of all open wounds in the extremities caused by sports or traffic accidents.

Nerve regeneration is a complex biological phenomenon [1]. In the peripheral nervous system, nerves regenerate spontaneously when injuries are minor. In the most severe lesions, in which there is distortion of the endo-neural tubes with or without peri-neural disruption (Sunderland grade III or grade IV), prognosis for spontaneous regrowth is diminished and surgical repair is often required. In complete nerve lesions (Sunderland grade V) axonal regrowth will not usually occur unless the nerve endings are free from scar tissue and surgically reapproximated. Although advances in surgical techniques have brought significant improvements, the functional recovery is often suboptimal. The choice of the surgical protocol is dependent on the size of the nerve gap between the proximal and distal stumps [2]. Short gaps can be repaired directly by mobilization of the proximal and distal stumps with end-to-end coaptation and epi-neural suturing. Long nerve gaps (>2 cm) require additional material to bridge the defect, which further reduces the functional outcome [3]. The current standard repair method is based on the use of autologous nerve grafts (autografts), which provide the regenerating axons with a natural guidance channel populated with functioning Schwann cells surrounded by their basal lamina [4]. Nerve autografting, however, is far from being an optimal treatment, and even after optimal surgical repair the functional outcome is disappointingly poor, especially for sensory recovery [5]. This poor outcome is largely due to the death of primary sensory neurons, but also to the lack of fiber regeneration over the gap that leads to target-organ denervation.

A promising alternative to nerve autografting is the use of artificial scaffolds. They are typically composed of an active biomaterial embedded in a supporting conduit. This approach has several advantages over autografts. First of all it does not require the extraction of healthy tissue for the patient. Then, the scaffold typically provides a guidance channel and mechanical support, and reduces scar formation by limiting the invasion of the connective tissue into the lesion [6]. Moreover, it can be engineered to provide the optimal chemical and physical microenvironment for nerve functional recovery [7–8]. Basic research and clinical results have shown that bio-absorbable devices can induce comparable or even superior nerve reconstruction than nerve autografts [4]. Nevertheless, the nerve functional recovery is still not satisfying and their use is limited to rather small nerve gaps (<5 cm in humans and 1.5 cm in rats).

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Here, we present anisotropic microstructured polydimethylsiloxane (PDMS) membranes for nerve repair applications. These scaffolds were patterned with gratings (alternating lines of grooves and ridges) having periodicities between 2 and 10 μm. We evaluated their performance *in vitro* by studying the guidance of primary rat Schwann cells (SC). Single SC migration was recorded with time-lapse microscopy, and compared with flat PDMS surfaces in terms of velocity, and directionality.

2. Material and methods

2.1. Mold fabrication

Micropatterned silica molds were used in this work (see Fig. 1a). The structure was patterned by electron beam lithography (EBL) on a thin polymer layer (SU-8 2000, MicroChem) which was spin-coated on 500 μm thick *p*-doped silicon (SYLTRONIX, France). The samples were developed in SU-8 Developer (MicroChem) and hard baked at 200 °C for 20 min. The molds were inspected by scanning-electron microscopy and finally silanized by a vapour deposition of chloro trimethylsilane (Sigma Aldrich, Italy) to reduce surface energy and ease master/replica detachment.

Ridges and grooves with different periods, p, and depths, h, defined the structures used for this research. All the structures had a ridge width equal to the groove width: in particular we used a T4 pattern (characterized by $p = 4$ μm and $h = 0.85$ μm) and a T20 pattern (characterized by $p = 20$ μm and $h = 2.5$ μm). Flat silicon wafers were used to produce the standard control membrane.

2.2. Polydimethylsiloxane membrane production

Polydimethylsiloxane (PDMS) membranes were fabricated by a process of replica molding. The prepolymer was mixed with the curing agent at a ratio of 10:1 and spin-coated onto the micro-structured mold. In order to obtain a film of 170 ± 20 μm thickness, the spin-coating was carried out for 4 min at a speed of 300 rpm. The uncured membrane was left resting for 10 min in order to reduce surface inhomogeneities, then it was baked in an oven for 10 min at 80 °C. After thermal curing, the replica was removed from the master using a scalpel and tweezers and placed onto a standard WilCo dish (Fig. 1b and c). Static water contact angle measurements were performed using a CAM 101(KSV Instruments Ltd., Finland) contact angle meter. The samples were finally sterilized by treatment with ethanol and then carefully rinsed with phosphate buffered solution (PBS) and conditioned with complete cell culture medium.

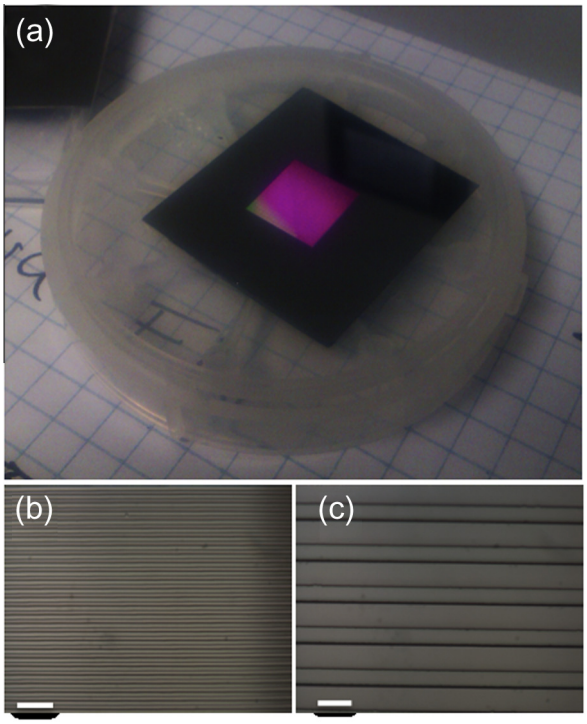

Fig. 1. Substrates. (a) Representative images of the micropatterned molds. The structure was created by electron beam lithography on a thin layer of SU8 resist. The patterned area is 1 cm^2. (b, c) Representative images of T4 and T20 PDMS membranes acquired by bright field optical microscopy. Samples were fabricated by replica molding. Scale bar = 16 μm.

2.3. Schwan cell extraction, selection and culture

Primary Schwann cell culture was established from sciatic nerves of adult Wistar rats. Nerves were removed and incubated in culture for 2 weeks: then the tissues were dissociated and cultured in the presence of glial growth factor (63 ng/ml) and Forskolin (10 μM) in DMEM supplemented with 10% FBS, 4 mM L-glutamine, and antibiotics. Cells were routinely immunodepleted by anti-rat Thy1.1 antibody to enrich the culture in Schwann cells and reduce the presence of fibroblasts. Schwann cells were maintained in standard tissue culture plates functionalized by Poly-D-Lysine (PDL) (100 μg/ml). The PDMS membranes, however, required Poly-L-Lysine (0.01%, at room temperature for 30 min) and Laminin coating (50 μg/ml, at 37° for 30 min) for proper cell adhesion.

Schwan cells were seeded on the functionalized PDMS membranes to perform single cell migration experiments at concentrations of 80 * 10^3 cells/cm^2.

2.4. Single cell migration experiments

Three independent time-lapse experiments were performed in epifluorescence using a 20× air Nikon objective, N.A. 0.45, PlanFluor and an Eclipse Ti inverted microscope (Nikon, Japan) equipped with a perfect focus systems, an incubating chamber (Okolab, Italy) and a CCD ORCA R2 (Hamamatzu, Japan). Images were acquired for 17 h with sampling every 15 min. Movies were analyzed with the ImageJ manual tracking plugin. The coordinates of single cells as a function of time were extracted and analyzed by a custom-made algorithm written in Matlab. The following parameters were measured: cell displacement (R; distance from the origin after 17 h), total path covered in 17 h (S), migration step (**dS**; corresponding to the cell motion calculated after 15 min) and the average speed (V). **dS** was analyzed along two directions: parallel and perpendicular to the pattern directionality ($\boldsymbol{ds}_{||}$ and $\boldsymbol{ds}_{\perp}$, respectively). Hence we classified **dS** in two populations: parallel and perpendicular steps (see Fig. 2b). **dS** was considered parallel if the angle between the step and the reference direction was between 0° and 15°, while it is considered perpendicular if the angle was between 75° and 90°. Note that bold characters indicate vectors.

2.5. Statistical analysis

The experiments were independently repeated at least three times for each reported dataset and all showed consistent results. Data are reported as means of the mean value for each experiment ± the standard error of the mean. Data were statistically analyzed using the commercial software OriginLab. They were first tested for normality (Shapiro–Wilk normality test). Then One-Way Anova with Tukey's post-test analysis was used. Unless otherwise stated, statistical significance is shown as *, **, ***, ****, for to $P < 0.05$, $P < 0.01$, $P < 0.005$, $P < 0.001$, respectively.