Adhesion and Patterning of Cortical Neurons on Polyethylenimine- and Fluorocarbon-Coated Surfaces

Teun G. Ruardij*, Martijn H. Goedbloed, and Wim L. C. Rutten

***Abstract*—Adhesion and patterning of cortical neurons was investigated on isolated islands of neuron-adhesive polyethylenimine (PEI) surrounded by a neuron-repellent fluorocarbon (FC) layer. In addition, the development of fasciculated neurites between the PEI-coated areas was studied over a time period of fifteen days. The patterns consisted of PEI-coated wells (diameter 150 μm, depth 0.5 μm) which were etched in a coating of fluorocarbon (FC) on top of polyimide (PI) coated glass. The separation distance between the PEI-coated wells were varied between 10 and 90 μm. This paper shows that chemical patterns of PEI and FC result in highly compliant patterns of adhering cortical neurons after one day *in vitro*. Interconnecting neurite fascicles between PEI-coated wells were especially present on patterns with a separation distance of 10 μm after eight days *in vitro*. A significant lower number of interconnecting neurite fascicles was observed on 20 μm separated patterns. Effective isolation of neurons into PEI-coated wells was achieved on patterns with a separation distance of 80 μm as no interconnecting neurite fascicles were observed.**

***Index Terms*—Adhesion, cortical neurons, cultured neuron probe, fluorocarbon, patterning, polyethylenimine.**

I. Introduction

PATTERNING of cells on material surfaces has become a subject of biomaterial research in the last decade. Selective surface modification at micrometer-scale is the necessary step to direct cellular adhesion and growth into patterns, evoked by chemical surface modifications [1], [2], topographical surface modifications [3], [4] or combinations of both [5], [6]. Both microlithography and micro-contact printing methods have been used as tools to produce topographical and chemical patterns respectively [6], [7].

The factors that are believed to play an essential role in cell adhesion mechanisms are the specific surface chemistry [8], surface hydrophobicity [9], surface topography [10], surface charge [11], and specific protein interactions [12]. Basically two different trends can be found in literature that exploit at least one of the factors mentioned. One group of researchers emphasize the importance of specific protein interactions [12] while the other group focuses more on nonspecific factors like surface hydrophobicity or surface charge [9], [10]. Despite this apparent contradictory approach, the general observation is that both specific and nonspecific methods facilitate the preparation of cellular patterns on material surfaces. A restriction is that cellular patterns were usually studied within a limited period of time (<14 days) [13]. A prerequisite for long-term patterning of cells (>14 days) is the stability of the underlying chemical pattern. In view of this, several methods of chemical patterning of surfaces will be discussed.

A frequently reported nonspecific approach is the chemical surface modification of silica-like materials with silanes. Typically, authors have reported the use of alkylsilanes and amino-rich alkoxysilanes to produce nonadhesive and adhesive areas respectively [14]. Unfortunately a generally acknowledged problem of silane modified surfaces is the hydrolysis of the Si–O–Si bonds under physiological conditions [15]. A sustainable silane-based system to maintain cellular patterns over longer periods of time is therefore unlikely and has not been reported to our knowledge yet. Another method of wet chemical modification is the preparation of thiolate-based monolayers on gold. Desorption of self-assembled monolayers of alkanethiols is not expected within 48 hours [13] and is reported to be stable over even longer periods of time [7]. However, Scotchford *et al.* [13] observed a decreasing clarity of patterned osteoblasts on gold after six days and suggested the possibility of erosion of the alkanethiol-coated surfaces.

Biomolecules present another means to establish cellular patterns on surfaces. Extracellular matrix (ECM) components like fibronectin or laminin [16], [17] are very effective as cell-adhesive substances and were exploited in detail by synthetic preparation of functional domains of the above mentioned ECM components [18]. In contrast, albumin is an effective nonadhesive biological coating on hydrophobic surfaces due to the irreversible strong adsorption and conformational change [19], [20] and therefore suppresses the well-known phenomenon of protein displacement and exchange [21]. Biomolecules are usually polypeptides and their drawback under physiological circumstances is the hydrolysis of amide linkages in the peptide chain [22]. The hydrolysis of ECM proteins is enzymatically catalyzed by proteases [23] and threatens the durability of ECM proteins as adhesive or nonadhesive coatings on surfaces.

Our group is interested in the preparation of isolated islands of neurons on single electrodes of a so-called neuron probe [24], [25]. These islands eventually have to form a natural interface between individual nerve fibers and single electrodes which is desirable for a highly selective stimulation of nerve fibers and improved muscle control. A critical distance between the islands is necessary to prevent electrical signaling between electrodes through neuronal tissue. A prerequisite of desirable long-term maintenance of these patterned islands is a stable and chemically inert combination of adhesive and nonadhesive materials

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*T. G. Ruardij is with the Faculty of Electrical Engineering, Institute for Biomedical Technology, University of Twente, P.O. Box 217, 7500 AE Twente, The Netherlands (e-mail: T.G.Ruardi@el.utwente.nlj.

M. H. Goedbloed and W. L. C. Rutten are with the Department of Electrical Engineering, University of Twente, 7500 AE Twente, The Netherlands.

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on the surface of the probe. A promising solution for the nonadhesive part of such a system was described by Makohliso *et al.* [26] who investigated spin-coated fluorocarbon (FC) layers as nonadhesive materials for neurons. The properties of fluorocarbon coatings resemble the properties of polytetrafluoroethylene [27], [28], a very hydrophobic biocompatible material with nonadhesive properties to cells due to irreversible adsorption of albumin [19], [29].

Neuron adhesive coatings usually will not maintain their surface properties in a physiological environment. Despite this disadvantage, choice for many coatings is driven by the supposed electrostatic interaction between positively charged aminogroups and negatively charged phospholipids in the cell membrane. An interesting alternative that exploits this advantage and simultaneously avoids the presence of peptide-like amide linkages in the polymer backbone is polyethylenimine (PEI) [30]–[32]. The electrostatic properties and chemical stability promote a stable binding between neuron membranes and negatively charged surfaces like mica or silicondioxide [33]–[35].

The aim of this paper is to study the adhesion and patterning of cortical neurons on isolated islands of neuron-adhesive polyethylenimine (PEI) surrounded by neuron-repellent fluorocarbon (FC). Two different types of fluorocarbon (FC) layers i.e. spin-coated FC and plasma-deposited FC will be evaluated. In addition, the influence of the separation distance between PEI-coated areas on neurite development and fasciculation of interconnecting neurites is studied over a time span of fifteen days.

II. Materials and Methods

A. Experimental Setup

The cellular studies on the materials surfaces were divided in two distinct groups. Initially the adhesion of cortical neurons on different combinations of neurophilic and neurophobic coatings was investigated in a pilot study. Based on this pilot study, combinations of neurophilic and neurophobic coatings could be selected and used for the preparation of highly compliant neuronal patterns on surfaces. Glass plates (Glaverbel, Mol, Belgium) with a size of 48 $\times$ 48 $\times$ 1 mm were used as basic substrates onto which different types of coatings were deposited and were cut into pieces with smaller dimensions (16 $\times$ 16 $\times$ 1 mm) afterwards.

B. Cortical Neuron Isolation and Procedures:

Cerebral cortex from one day old newborn rats was dissected out under sterile circumstances and cut into pieces of approximately 1 mm^3. After collection, the tissue was trypsinized (0.25% Trypsin/EDTA, Gibco) for 45 min in an incubator at 37 °C at 5% CO_2 and subsequently treated with Soybean Trypsin Inhibitor (STI, 1 mg/ml) and Desoxyribonuclease I (DNAse I, 1.1 unit/ml). The dissociated tissue was spun down at 1200 rpm during 5 min and resuspended in chemically defined medium R12 (DMEM /Hams F12, Gibco) without serum [36]. Trypan blue stain (0.4%) was used to discriminate and count living neurons in a Bürker chamber, prior to the sedimentation of the neurons onto the surfaces. Neurons were seeded onto the patterned structures with a plating density of 5000 living cells/mm^2 in pilot experiments and 1500 living cells/mm^2 in patterning experiments. Cells were allowed to adhere onto the surfaces during a time period of 4 hours. Samples were rinsed with a 0.9% NaCl solution to remove nonadherent cells.

C. Neurophobic Material Deposition:

1) Spin-Coated Fluorocarbon (Spin-FC): The polymer solution Fluorad FC-722® (3M, St. Paul, MN) was dissolved in Fluorinert FC-40® (3M, St. Paul, MN) according to a volume ratio of 1 : 1. A spinning speed of 2000 rpm was chosen as standard. After spinning, the samples underwent a post-bake procedure at 120 °C during 5 min to remove some residual solvent.

2) Plasma-Coated Fluorocarbon (Plasma-FC): FC-coatings have been deposited in a reactive ion etching (RIE) system via a plasma-polymerization of carbonhydrotrifluoride (CHF_3). The total FC-coating was formed by an initial treatment in CHF_3 plasma (25 sccm CHF_3, 150 mtorr, $4.2*10^{-2}$ W/cm^2) for 10.5 min and a second low-energy treatment in CHF_3 plasma (25 sccm CHF_3, 150 mtorr, $2.1*10^{-2}$ W/cm^2) for 1.5 min.

D. Neurophilic Material Deposition:

1) Polyethyleneimine (PEI): A stock solution of PEI in water (Fluka Chemie AG, Buchs, Switzerland) was diluted to reach a final concentration of 10 μg/ml. The molecular weight of PEI varied between $0.6*10^6$ and $1.0*10^6$ according to product specifications. Samples for pilot studies and patterning studies were immersed into this solution under sterile conditions.

E. Preparation Procedures of Coated Surfaces

1) Pilot Studies: Initially the FC-coatings were deposited (see conditions above) and covered with rectangular silicon strips. The second step was the selective removal of the uncovered FC-coating in the reactive ion etching (RIE) system with an CHF_3/O_2 plasma (25 sccm CHF_3, 5 sccm O_2, 10 mtorr and $3.1*10^{-1}$ W/cm^2). The result were samples with a region of deposited FC (Spin-FC or Plasma-FC) and a region of etched glass. After cutting, samples were sterilized in an autoclave (126 °C, 30 min). A selected number of samples were backfilled with PEI in aqueous solution (see conditions above) to obtain combinations of PEI-coated glass/PEI-coated FC, either of the spin-coated or plasma-coated type.

2) Patterning Studies: Insufficient adhesion between glass and FC was circumvented by deposition of a sticky spin-coated (4000 rpm, 30 s) polyimide (PI, Probimide 7510®, Arch Chemicals N.V., Zwijndrecht, Belgium) layer [28]. PI was diluted in n-methyl pyrolidon (1 : 1 v/v), dried on a hot plate (120 °C, 5 min), exposed to UV-light, and baked (300 °C, 90 min).

Samples were subsequently treated with an etching CHF_3/O_2 plasma (25 sccm CHF_3, 5 sccm O_2, 150 mtorr and $2.1*10^{-1}$ W/cm^2) and a final depositing CHF_3 treatment as described above. The Plasma-FC coated samples were spin-coated with a protective layer of positive photoresist (OiR 907/17, Arch Chemicals N.V) at 4000 rpm (20 s). Photoresist was selectively developed and removed from the surface (Developer OPD 4262, Arch Chemicals N.V.) after UV light exposure through a chromium mask. The underlying FC and PI-layers were

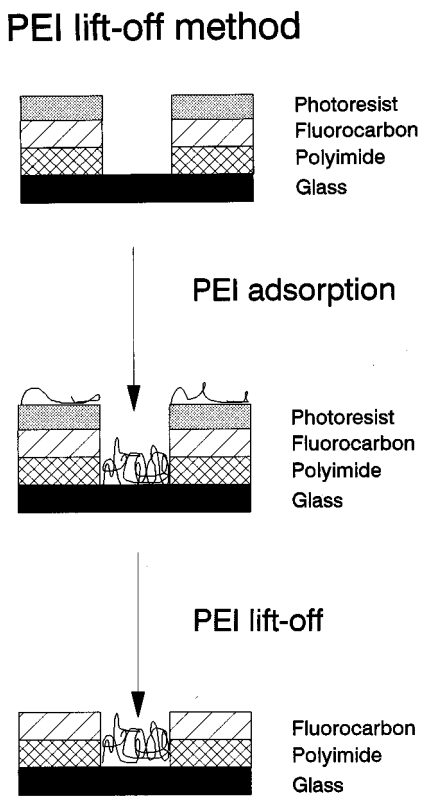


Fig. 1. Schematic illustration of the applied method to prepare chemical patterns with a combination of polyethylenimine (PEI) and fluorocarbon (FC).

removed with an etching CHF_3/O_2 plasma (25 sccm, 5 sccm O_2, 10 mtorr and $3.1*10^{-1}$ Wkm^2). One day before application in cell experiments, samples were pretreated with an O_2 plasma (150 °C, 2 mbar O_2 and $5.0*10^{-1}$ Wkm^2) for 1 min. Finally the adsorption of PEI on the surface was done with a so-called PEI lift-off method (Fig. 1). Samples were immersed in the PEI-solution for 60 min. After the PEI-treatment, photoresist with adsorbed PEI was removed by ultrasonic cleaning (40 s) and rinsing (1 min) in a 1.0 M NaOH solution, followed by immersion and rinsing in Milli-Q water (1 min).

The patterns consisted of PEI-coated wells (diameter of 150 μm, depth 0.5 μm) which were arranged on nine separate subsections. Separation distances between the wells were varied from 10 μm up to 90 μm on the nine different subsections of the pattern (Fig. 2).

F. Evaluation of Adhesion and Morphological Development

1) Pilot Studies: Three digital images were grabbed in the glass and FC areas using a CCD/RGB camera (Type DXC-151P, Sony, Japan) attached to an inverted phase contrast microscope (Nikon Diaphot-TMD, Tokyo, Japan). Two different samples were evaluated after one day *in vitro*. The adhering cells, which were individually identifiable, were counted manually and the corresponding cell adhesion density was calculated.

2) Patterning Studies: Microphotographs using a 35-mm photocamera (Nikon-FE, Tokyo, Japan) were taken on the nine separate subsections of the pattern after one, four, eight, and fifteen days. Each time-lapse procedure was done on six different samples. Adhesion was evaluated after one day *in vitro* with a manual counting procedure of individually identifiable neurons. Furthermore the development of fasciculated neurites across neurophobic FC, as a function of the separation distance, was quantified by calculation of the average number of surrounding PEI-coated wells, connected to a single PEI-coated well through neurite fascicles. Wells at the edges of the patterns were disregarded to keep number of surrounding wells at a constant number of 6. Neurite development was studied on day four, eight, and fifteen.

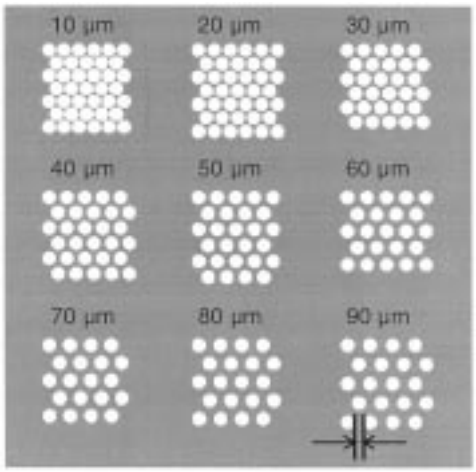


Fig. 2. Overview of nine patterns used for the experiments. Dark areas represent the Plasma-FC coated part of the surface. Light areas represent the PEI-coated part of the surface. The numbers refer to the separation distances between the PEI-coated wells. Diameter of wells is 150 μm.

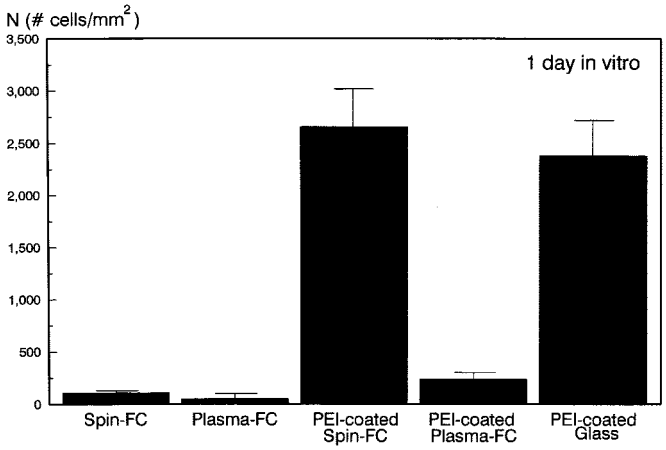


Fig. 3. Histogram of the number N of adhering cortical neurons on five surfaces. Bars represent the standard deviation calculated over six images from two independent experiments.

III. Results

A. Pilot Studies

Fig. 3 presents the adhesion of cortical neurons on the pilot samples after one day *in vitro*. Neuronal adhesion was almost negligible on the FC-coated surfaces of the spin-coated and plasma-coated type. The adsorption of PEI onto Plasma-FC enhanced the adhesion to a moderate extent. PEI significantly enhanced the adhesion of the cortical neurons onto Spin-FC. In addition the results on PEI-coated glass and PEI-coated

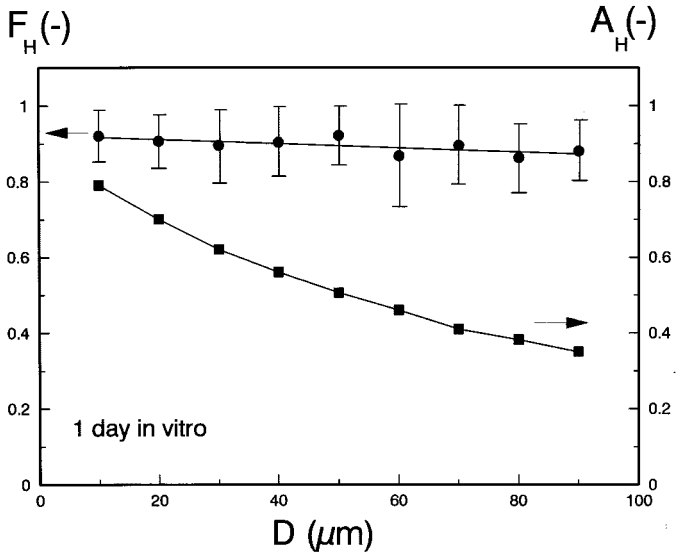


Fig. 4. The amount of adhering cortical neurons in PEI-coated wells calculated as the fraction F_H of the total number of adhering cortical neurons (•, left axis) and fraction A_H of the surface covered with PEI-coated wells (■, right axis) as a function of the separation distance D between PEI-coated wells. Bars indicate the standard deviation calculated over six images from independent experiments.

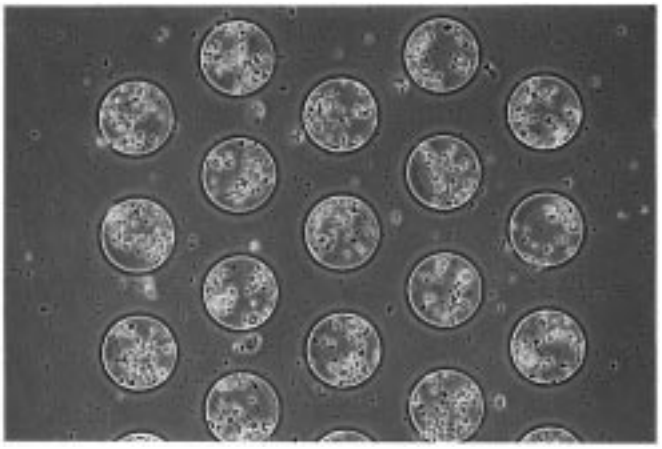


Fig. 5. Examples of preferential adhesion of neurons onto PEI-coated wells surrounded by Plasma-FC. Diameter of the wells is 150 μm. Separation distance is 90 μm.

Spin-FC were comparable. Adhesion of separate neurons onto glass was not evaluated because neurons could not be identified individually due to aggregation of the cells.

B. Patterning Studies

Based on the results of the pilot studies, combinations of 1) PEI-coated glass and Plasma-FC and 2) PEI-coated glass and Spin-FC were evaluated as candidates to prepare neuronal patterns (Fig. 1). Unfortunately the nonwettable properties of the Spin-FC surface (water contact angle of 120°) appeared to be unsuitable for photoresist-based microlithography in contrast with the surface properties of Plasma-FC (water contact angle between 95 and 105°) [28]. Fig. 5 presents a characteristic result of cortical neuron adhesion onto this patterned combination

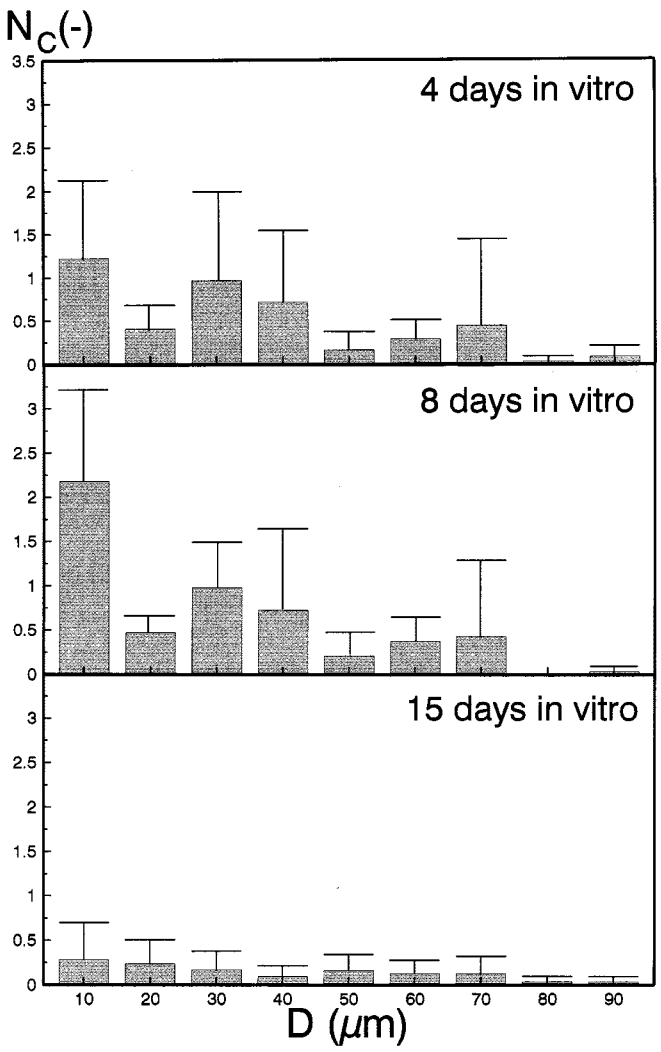
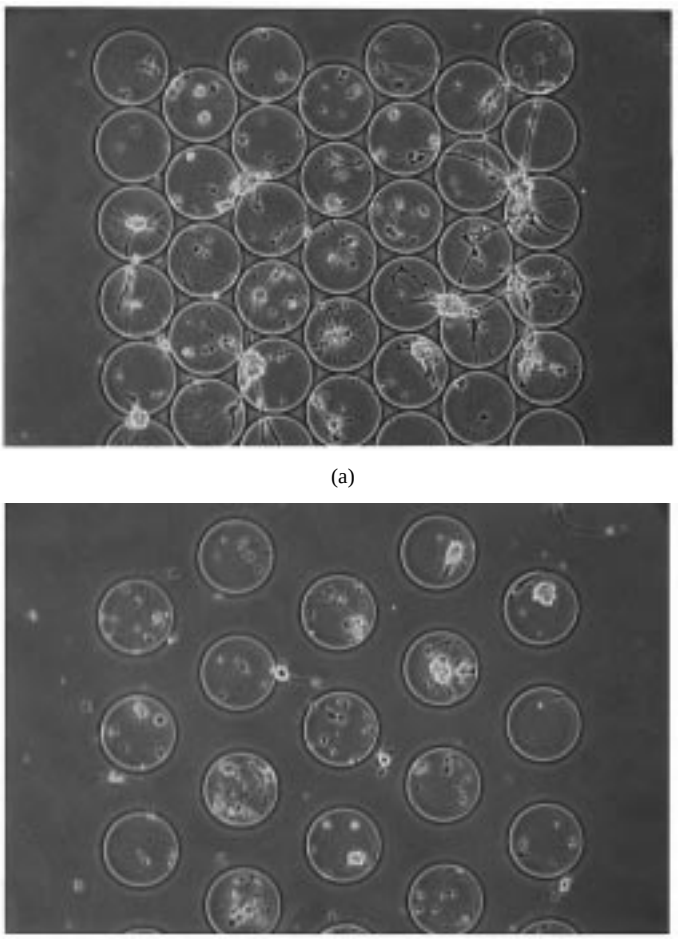


Fig. 6. The number of observed interconnecting neurite fascicles N_c between a PEI-coated well and the surrounding wells vs. the separation distance D between the wells. Bars represents the standard deviation calculated over five images from independent experiments.

after one day *in vitro*. The quantitative result, demonstrating the preferential adhesion in the PEI-coated wells on all nine patterns, was shown in Fig. 4.

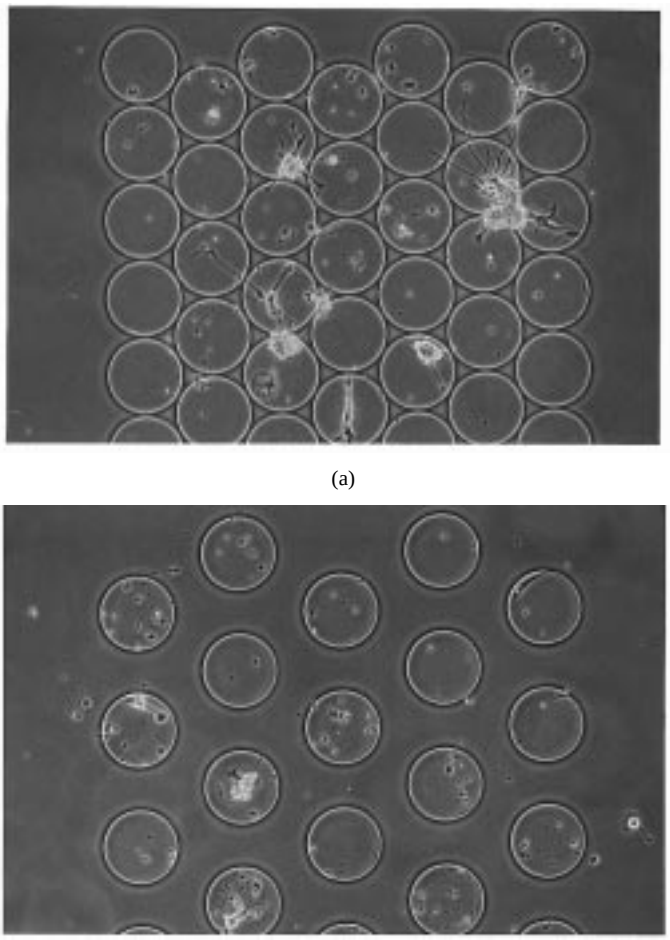
Fig. 6 presents the number of interconnecting neurite fascicles between PEI-coated wells as a function of the separation distance between the PEI-coated wells. An inversely proportional relation between the number of interconnecting neurite fascicles N_c and the separation distance between the wells exists after four and eight days *in vitro*. The average number of interconnecting neurite fascicles N_c around a PEI-coated well reached the highest value on patterns with a minimal separation distance of 10 μm after four and eight days *in vitro*. On patterns with a separation distance of 80 μm, no interconnecting neurite fascicles between PEI-coated wells were found. Corresponding photomicrographs after eight days *in vitro* are shown (Fig. 7). Contrary to the situation after four and eight days, neurite fascicles between PEI-coated wells were hardly observed after fifteen days *in vitro* and aggregates of neurons appeared to be detached in the time span between eight and fifteen days



(a)

(b)

Fig. 7. Examples of interconnecting neurite fascicles between PEI-coated wells after eight days *in vitro* on patterned surfaces with a separation distance of (a) 10 μm and (b) 90 μm.



(a)

(b)

Fig. 8. Examples of interconnecting neurite fascicles between PEI-coated wells after fifteen days *in vitro* on patterned surfaces with a separation distance of (a) 10 μm and (b) 90 μm.

(Fig. 8). The separation distance was rather irrelevant after fifteen days and interconnection of neurite fascicles was present on a more-or-less constant level.

IV. Discussion

In the pilot study, a PEI backfill procedure was applied on FC/Glass samples with the aim to obtain selective neuronal adhesion on PEI coated glass due to selective adsorption and/or coupling of neuron-adhesive PEI onto Glass [34]. Such a procedure is especially advantageous in the case where samples have to be recycled and selectively coated with PEI without the repeated need for clean-room facilities. It was demonstrated that neuronal adhesion on PEI coated Plasma-FC was indeed lower compared to PEI coated glass (Fig. 3). However, the data also indicate that this was only the case on Plasma-FC and that uncoated FC layers inhibit the adhesion even more. The Plasma-FC and Spin-FC layers from this study were characterized in detail by Jansen *et al.* [28] showing the presence of negatively charged carbonyl or hydroxyl groups ($<$10% Oxygen) which can interact with PEI in an electrostatic way [33] or through covalent bonding. Although the concept of PEI backfilling contained definite advantages, the adsorption and coupling of PEI onto FC, associated with the presence of oxygen-rich groups in the FC surface, encouraged us to use the PEI lift-off method (Fig. 1) as the gateway toward high contrast patterning of individual islands of neurons.

Authors have investigated neurite outgrowth and guidance on topographical micropatterns [3], [4], [37], chemical micropatterns [1], [2], [38], [39], and combinations of both [5], [40]. Results on topographical micropatterns demonstrated that outgrowth and guidance of neurites appeared to be unaffected on patterns with groove depths smaller than 1 μm which indicates that ultrafine topography at the submicron level can not account for neurite guidance [37]. Despite the fact that differences in the experimental setup complicate the comparison of studies, the general conclusions from these topographical studies suggest that the entrapment of neurons and behavior of neurites in our study was not caused by the depth (0.5 μm) of the wells. Studies on combined topographical and chemical patterns confirm that surface chemistry is a much stronger cue than topography [41]. Several authors investigated neurite guidance on chemical micropatterns, prepared with adhesive laminin and a nonadhesive counter surface and showed that the number of neurites, bridging over the nonadhesive gap, was inversely proportional to the separation distance between the laminin-coated

areas [38], [39]. Models describing growth cone dynamics of filopodia showed reasonable agreement with these experimental data [40]. A minimal nonadhesive gap of 50 μm [39] or even 40 μm [41] was reported to be essential to prevent the bridging of neurites over nonadhesive gaps.

Our approach tackled the problem of neuron patterning differently in two ways. From a materials point of view, biomolecules as the neuron-adhesive part of the surface were avoided and replaced by PEI. Second, we focused on the preparation of chemical patterns that can actually lead to isolated neuronal networks, cultured on planar surfaces instead of multiple parallel tracks of alternating neuron-adhesive and nonadhesive surfaces [39], [40]. Our results do show an inversely proportional relation between interconnection of PEI-coated wells through neurite fascicles and the separation distance between the PEI-coated wells. However, a minimal separation distance that clearly marks the critical size of the neurophobic gap can not be given. Nevertheless, a significant difference in the number of interconnecting neurite fascicles was already found between 10- and 20-μm separated patterns after eight days *in vitro*. Complete isolation of neuronal islands was obtained on patterns with separation distance of 80 μm or higher.

The aggregation of cortical neurons eventually leads to the detachment of the aggregates and interconnecting neurites fascicles between eight and fifteen days *in vitro*. These results might suggest that the underlying PEI and FC coatings are affected but it was reported by several authors that neutral pH renders PEI-coatings on silica-like surfaces to be stable [34], [35]. Furthermore, the adhesion of the FC-layer, as part of the overall stability, was effectively improved by deposition of a sticky polyimide layer [28]. After fifteen days of culturing, visual inspection of cultured samples revealed that restructuring of the Plasma-FC surface, a general phenomenon on plasma deposited layers, did not change the hydrophobic and resulting neuron-repellent character of Plasma-FC.

Willems *et al*. [43] showed that aggregation of cortical neurons is inhibited on homogeneously PEI-coated multielectrode arrays and our own observations (not shown here) on homogeneously PEI-coated control areas, located at the borders of the pattern, indicate the same. Thus cortical neurons form a relatively adhesive sheet of connected tissue on nonpatterned surfaces in contrast with the observations of a disconnected layer of tissue on our patterned surfaces.

This paper points out that highly compliant patterns of adhering cortical neurons can be prepared with a chemically inert combination of PEI and FC. Furthermore, an increase in the separation distance between PEI-coated wells up to 90 μm, facilitated the preparation of isolated neuronal networks. Unfortunately, the geometrical characteristics of the applied pattern were not optimal for long-term survival. Nevertheless, for the development of a cultured neuron probe [25], [44], a chemical pattern of PEI and FC, with appropriate geometrical properties, seems to be a promising option.

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Teun G. Ruardij was born in Workum, The Netherlands, in 1967. He received his M.Sc. degree in chemical engineering at the University of Twente, Twente, The Netherlands, in 1991. His Ph.D. work (1993–1997) at the University of Groningen, concentrated on surface modification of biomaterials for development of vascular prostheses.

He currently works as a Post-Doc in the Signals & Systems-BME group at the Department of Electrical Engineering, University of Twente. His present research interests are chemical surface modification of multielectrode arrays, neural engineering on micro-patterned surfaces, and development of cultured neuron probes.

Martijn H. Goedbloed was born in Geldrop, The Netherlands, in 1970. He received the M.Sc. degree in chemical engineering at Eindhoven University of Technology, Eindhoven, The Netherlands, in 1994.

After one year at the Philips thin-film magnetic heads division, he went to the Signals & Systems-Biomedical Engineering group at the Department of Electrical Engineering, University of Twente, Twente, The Netherlands. He works on the development of thin-film multielectrode arrays, designed for interaction with cultured neurons.

Wim L. C. Rutten was born in 1950. He was trained as an experimental physicist (receiving the Ph.D. degree in 1979) at Leiden University, Leiden, The Netherlands. Thereafter, he studied the auditory system at the ENT department of Leiden University Hospital.

Since 1985, he is with BMTI/Faculty of Electrical Engineering, University of Twente, Twente, , The Netherlands, as an Assistant and Associate Professor of Biomedical Control Systems. His present research interests are neural engineering and cellular engineering (neuro-electronic interfaces, cultured probes) signal processing and bioelectricity.