Neurite outgrowth on microstructured surfaces functionalized by a neural adhesion protein

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Designed networks of neurons are potentially very useful to investigate neural activities. Using photolithography microgrooves suited in size for single neurons have been produced on glass chips. Two conducting gold lanes ending in each microgroove allow extracelluar stimulation of the neurons and recording of their activity. A cell adhesive surface was created by functionalization of glass with the adhesion peptide RGDC. In addition, in order to optimize the contact of the neuronal cell membrane to the electrode surface axonin-1, a specific neural adhesion protein was used. A recombinant form of axonin-1 was produced and immobilized in a correct orientation on protected gold surfaces through a C-terminal cysteine residue. Neurite outgrowth of neurons cultured on chips derivatized with RGDC or axonin-1 were compared. The developed materials and methods represent a first step towards establishing designed functionalized glass surfaces for neurophysiological investigations.

1. Introduction

Guidance of neuronal processes on artificial surfaces can be achieved by a suitable design of the topography (for a review see [1]) or by an appropriate chemical treatment [2, 3]. Patterns of micrometer dimensions have been generated by chemical surface modifications with alkanamines, peptides or proteins in order to achieve cell guidance (for a review see [4]). Furthermore, extracellular electrodes have been produced and used for multisite recording and stimulation of long-term neural networks [5]. Polylysine and RGD-analogues are usually coated or covalently immobilized on surfaces in order to promote cell adhesion. However, these treatments do not induce the intimate membrane–electrode contact required for a good quality of the detected signal [6]. This can probably be achieved using axonin-1, a cell adhesion molecule of the immunoglobulin-superfamily involved in neurite outgrowth [7], axonal pathfinding [8] and neurite fasciculation [7]. By molecular engineering we have produced a recombinant axonin-1 protein with a functional SH-group at the C-terminus (Cys-axonin-1), allowing a covalent and oriented immobilization on glass and gold surfaces.

We have developed a glass chip for culturing individual neurons at predefined positions and for addressing the neurites with electrodes. The adhesion peptide Arg-Gly-Asp-Cys (RGDC) is covalently immobilized on glass in order to induce cell adhesion and neurite outgrowth. The presence of the cell adhesion protein Cys-axonin-1 on the gold electrodes is expected to minimize the cell-electrode distance. We report here on first results of dissociated neuron cells cultured on surfaces derivatized with RGDC and Cys-axonin-1.

2. Materials and methods

2.1. Chip fabrication

For the development of the required procedures and methods a model chip was designed consisting of an array of parallel microelectrodes (625 μm^2 per electrode) on a glass substrate covered with a structured polyimide layer which confines the cells into grooves of 100 μm in length, 25 μm in width and 18 μm in depth. Fig. 1 shows the steps required for this fabrication, using three different photolithographic masks for structure definition: the gold microelectrode array of 40 nm thickness on a 15 nm Ti-adhesion layer was produced in the first step and was isolated by a 200 nm SiO_2 passivation layer structured using photolithography and oxide etching in NH_3-buffered HF. The photosensitive polyimide film (Probimide P-7020, OCG Microelectronic materials AG, Switzerland) was spin-coated on the substrate. After structuring, the polyimide was cured at 350 °C in dry N_2. The wafer was then sawed into 1 cm^2 chips. Fig. 2 shows five microgrooves for the neurons on the chip.

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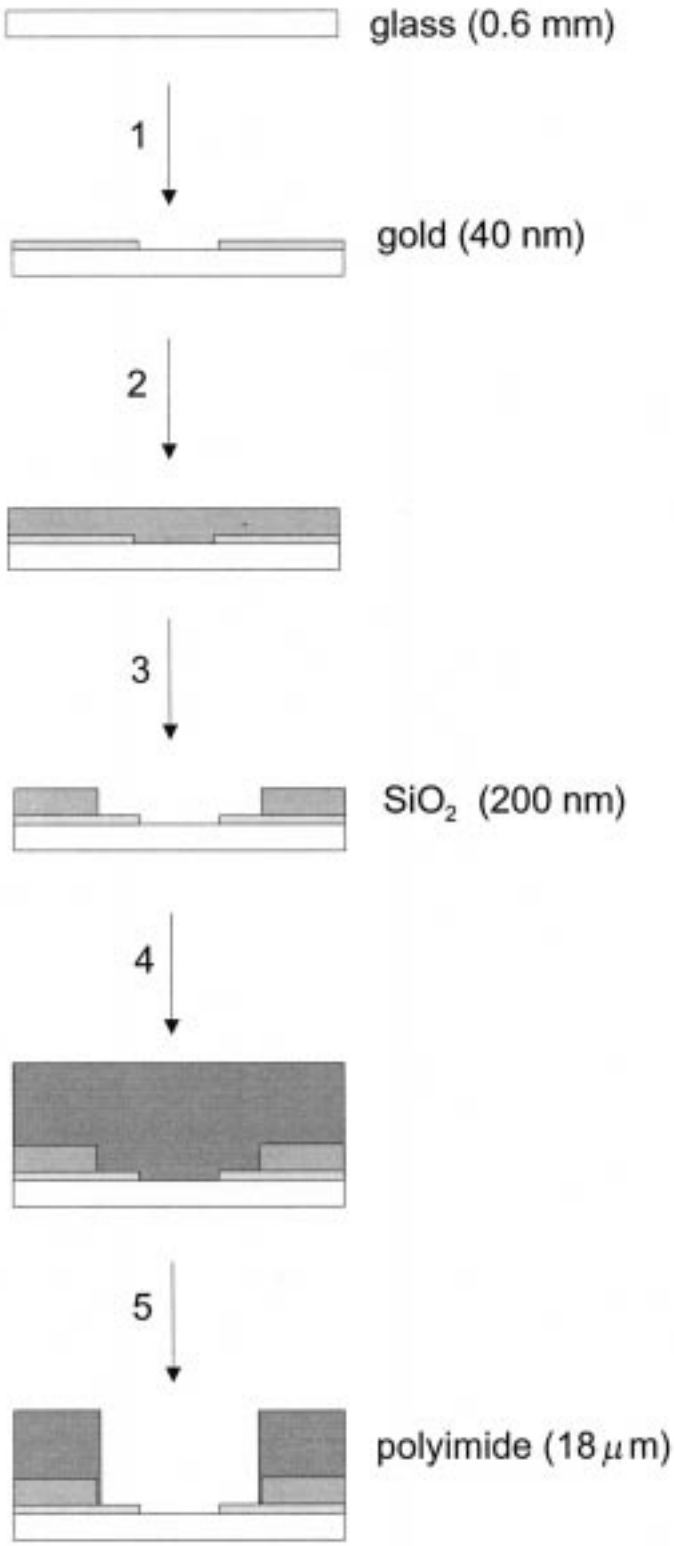

Figure 1 Scheme of the processes used for the fabrication of neurochips. Gold electrodes were produced by photolithography, gold evaporation and a lift-off process (1). A 200 nm SiO_2 passivation layer was deposited (2) and structured using photolithography and etching in NH_3-buffered HF (3). A photosensitive polyimide layer was spin-coated on the surface (4) and structured using photolithography (5).

2.2. Production of the recombinant adhesion molecule axonin-1

A sequence coding for a peptide linker (Gly-Gly-Ser-Gly-Cys-COOH) was introduced into the cDNA encoding the chicken adhesion molecule axonin-1 [9]. The recombinant protein Cys-axonin-1 was expressed in HEK293T cells after transfection with calcium phosphate. The protein was isolated from the supernatant of 4-day cultures by a series of chromatography steps (Concanavalin A column, MonoQ anion-exchanger, superose 12 gel filtration). Characterization with sodium dodecyl sulfate–polyacrylamide gel electrophor-

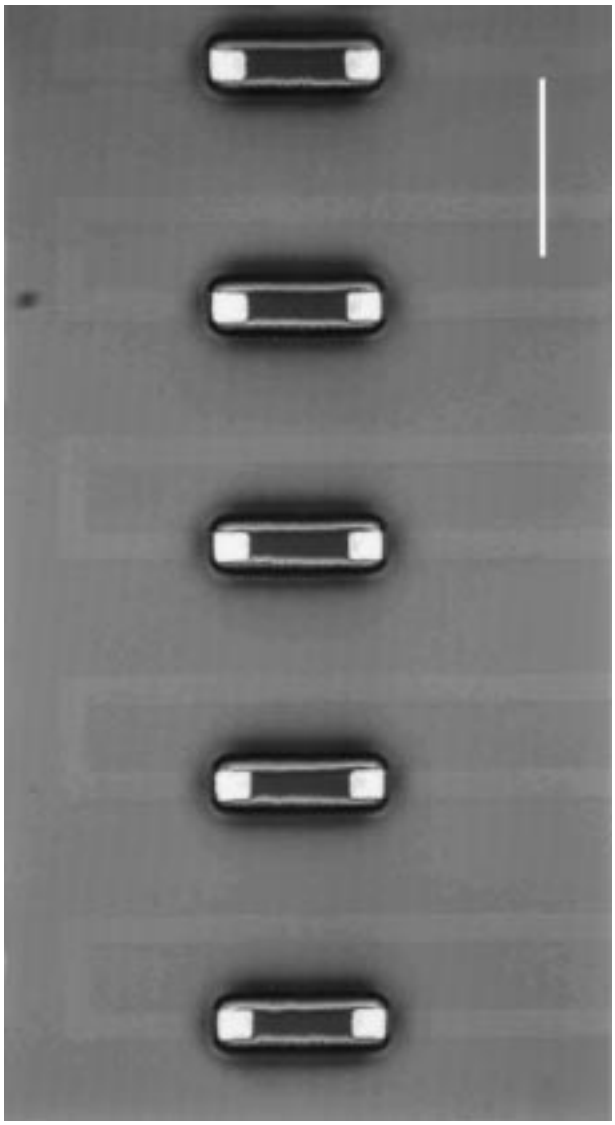

Figure 2 Microgrooves (25 × 100 μm) with gold electrodes at each end on glass suitable for culturing individual neurons (bright field micrograph, scale bar: 100 μm).

esis (SDS–PAGE) and Western blot showed that the protein was nearly 100% pure and only about 5% of the protein had dimerized.

2.3. Chemical functionalization of chips

Plain glass chips (alkali-free glass, DESAG) were sequentially cleaned in acetone/isopropanol/acetone for a total of 15 min and air dried. Silanization was done using a 2% solution of 3-aminopropyltriethoxysilane (APTES, Sigma) in toluene for 1 h at room temperature. The chips were washed with toluene. The heterobifunctional crosslinker *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester (GMBS, Pierce) was dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in 50 mM phosphate buffer, pH 7.0 to a final concentration of 5 mM. To each of the air dried chips 75 μl of this solution was added and after 2 h incubation the chips were washed. The immobilization was completed by adding 75 μl of 50 to 5000 μM Arg-Gly-Asp-Cys (RGDC; Bachem, Switzerland) or 5 to 50 μg/ml Cys-Axonin-1, dissolved in 50 mM phosphate buffer pH 7.4. After a 2 h incubation unbound molecules were washed away with phosphate buffer containing 0.5% Tween-20. The surface density was assessed using iodinated tracers of RGDC and Cys-axonin-1. The RGDC-tracer was prepared by reaction of a Bolton-Hunter reagent (Amersham) with the free amino-group, whereas the Iodogen-method (Pierce) was used for protein labeling. Biofunctionalized chips were stored in phosphate buffer at 4 °C.

Gold surfaces were etched for 3 min in an oxygen plasma and treated with 50 mM mercaptoethane sulfonate (MES, Fluka) in 50% ethanol in H_2O. The peptide or protein solution was then applied for direct immobilization through their Cys-residue. The MES-treatment prevents denaturation of the recombinant axonin-1 and also efficiently reduces non specific binding of other proteins [10].

Lanes and grids of RGDC on glass were created using photolithographic methods. The pattern (5, 10, 15 or 25 μm line width) was first produced in a positive photoresist (Shipley, Germany). The peptide was then immobilized as described above, using iso-octane as solvent for the silanization. The resist was finally removed with an ultrasonic acetone treatment for 2 min leaving lines of covalently immobilized RGDC.

2.4. Primary cell cultures of chicken DRG neurons on chips

Dorsal root ganglia (DRG) were dissected from 10-day old chicken embryos as described by Sonderegger *et al.* [11]. Ganglia were dissociated by trypsinization and trituration and cultured in serum-free defined medium [12].

Peptide- and protein-coated chips were sterilized for 30 min in 70% ethanol and washed with phosphate-buffered saline (PBS) prior to seeding of the cells. Dissociated neurons were plated at low density (5000 cells cm^{-2}) in order to obtain isolated neurons. After a 20 h incubation (37 °C, 10% CO_2) cells were fixed and washed twice with PBS. An inverted light microscope equipped with phase contrast optics was used for culture examination. Isolated cells were selected and the length of the longest neurite measured and plotted as described by Chang *et al.* [13].

3. Results and discussion

3.1. Surface densities of immobilized peptide and protein

Radiolabeled tracers are unique tools to quantitatively determine surface coverage. The structural difference of the iodinated RGDC-tracer and the native RGDC, however, allows only an estimation of surface densities. Using an RGDC-concentration of 5 mM, a coverage in the range of 100 pmol cm^{-2} was determined which is comparable to surface densities found by other groups [14, 15].

When Cys-axonin-1 was immobilized using a concentration of 50 μg/ml, surface densities of 400 ng cm^{-2} on glass and 600 ng cm^{-2} on gold were achieved. This

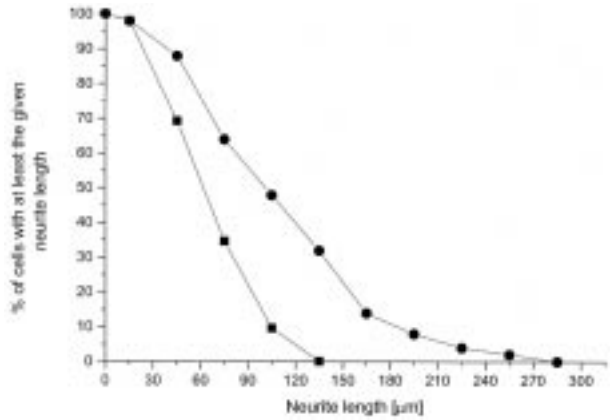

Figure 3 Cumulative histogram of neurite lengths from separated neurons cultured on RGDC (•) and Cys-axonin-1 (■) derivatized surfaces.

coverage corresponds to a dense protein monolayer. A linear decrease of the surface density was observed when Cys-axonin-1 was applied in a ten times lower concentration. Thus, the density of the adhesion protein can be adjusted to a predefined value.

3.2. Neurite outgrowth on functionalized surfaces

Dissociated DRG neurons of chicken embryos were first cultured on surfaces derivatized with various concentrations of RGDC or Cys-axonin-1. The outgrowth of neurites on surfaces treated with 50 μM RGDC was comparable to the outgrowth observed on chips treated with 500 and 5000 μM RGDC. After 20 h in culture, 80% of the neurons showed processes longer than one cell diameter.

In contrast, only 40% of neurons showed neurite outgrowth when cultured on glass or gold surfaces functionalized with Cys-axonin-1 at surface densities of 400 ng cm^{-2} or 600 ng cm^{-2}, respectively. When the protein surface density was reduced to 40 ng cm^{-2}, no outgrowth of neurites was observed indicating that a relatively high surface density of molecules is required. This is in agreement with the model of Kunz *et al.* [16], postulating a heterotetramer of two axonin-1/Ng-CAM dimers at cell contacts. These contacts are essential for the induction of neurite outgrowth. It is supposed that neurite outgrowth in natural systems is also controlled by high local densities of axonin-1 molecules in the cell membrane.

The measured neurite lengths were put in relation to the cumulative fraction of the investigated neurons (Fig. 3). The cumulative histogram of neurite lengths shows clearly that RGDC induces neurite outgrowth more efficiently than Cys-axonin-1. The mean value of the neurite length is 124 ± 59 μm on RGDC, whereas it is 80 ± 29 μm on Cys-axonin-1 ($n = 50$). Therefore, by an overall derivatization of the microgroove ground (glass and gold) with RGDC cell adhesion will be maintained and neurite outgrowth promoted, whereas the Cys-axonin-1 on gold electrodes is expected to improve

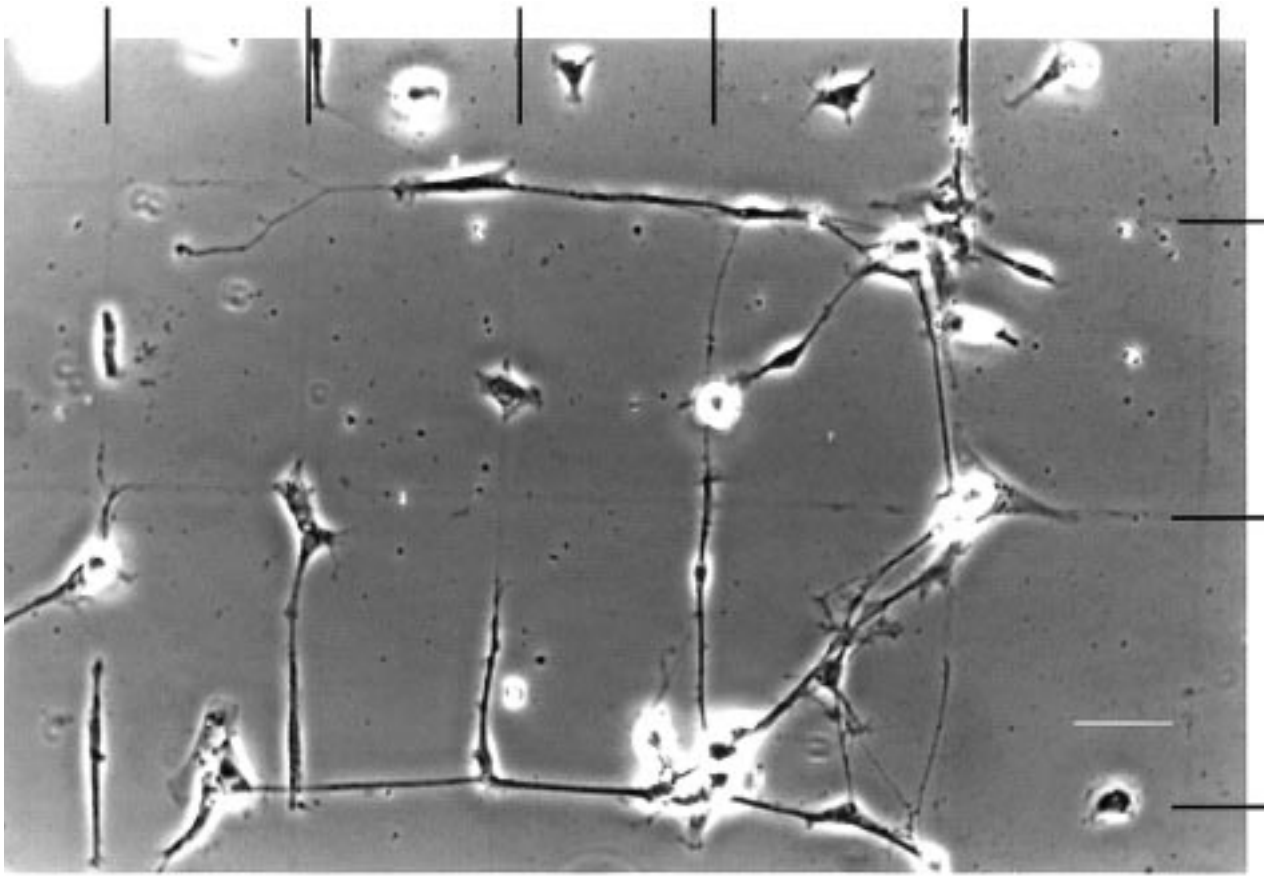

Figure 4 Phase contrast micrograph of a neuron culture on RGDC-patterns (5 μm line width) on glass. Black bars indicate the position of peptide lines (scale bar: 50 μm).

the cell–electrode contact. This may reduce the impedance and result in enhanced electrical signals [6].

Adhesion and neurite outgrowth of dissociated neurons on patterns of RGDC were investigated. The covalently immobilized peptide is still functional, despite the exposition to acetone required to remove the resist in the photolithographic process. Surprisingly, we observed that the outgrowth along narrow lines of 5 to 15 μm (Fig. 4) was more frequent than along 25 μm lines. Analogous experiments with Cys-axonin-1 failed because the immobilized protein was denatured and lost its functionality. Alternative methods for a localized immobilization of proteins are currently under investigation. In summary, RGDC can selectively be immobilized in the microgrooves allowing combination of chemical and topological structuring of the surface.

4. Conclusions

The presented results open new possibilities to produce designed surfaces for research and new applications. The combination of topological and molecular structuring is very promising for the investigation of molecular functions under defined conditions on one hand, and for the creation of dedicated surfaces for cell biology experiments on the other. For neural sciences the addressing of individual cells in a network with microelectrodes will provide a new dimension allowing investigations of neural processes on a very fundamental level.

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