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Neuronal cell growth on iridium oxide

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ABSTRACT

Iridium oxide is an attractive material for the development of novel multi electrode array (MEA) systems that provide electrodes for stimulation as well as recording single neurons. In this study the biocompatibility of pure iridium and different iridium oxides that differ characteristically in their surface roughness was investigated using two different biological test systems, insect and vertebrate neurons. Iridium oxide surfaces were coated with Concanavalin A and poly-(D)-lysine. In detailed investigations (R_a value determination, contact angle measurement, marker enzyme assay) the surface characteristics of non-modified and coated iridium oxide films were analysed, demonstrating that the materials can be successfully coated. Furthermore, we show that locust neurons grow well on all substrates tested, while chicken neurons need coated surfaces for proper adhesion. Increasing the roughness of iridium oxide films, which in principle could improve cell adhesion, did not improve the neurocompatibility. These results show that in future applications iridium oxide films can be used with surface morphologies previously shown to be optimal for stimulation purposes (cauliflower-like surface structure).

1. Introduction

A better understanding of the function of neuronal networks, the basis for learning, cognition, and information storage in the brain, is of great interest to neuroscientists. The investigation of neuronal networks formed in cell culture provides the opportunity to gain insights into basic principles of the development, structure, and function of neuronal assemblies such as outgrowth of neurites, synapse formation, and synaptic plasticity outside the much more complex situation *in vivo*. To obtain deeper insights into the function of such neuronal networks in culture it is essential to have tools which allow investigations over long time periods (days, weeks), preferably with non-invasive electrophysiological techniques. Planar multi electrode arrays (MEAs) provide such tools for long-term recording of neuronal activities as well as for the stimulation of single neurons or subpopulations. Various MEAs have been developed for *in vitro* experiments [1–8]. To get an even more detailed understanding of information processing in neuronal networks, an optimal resolution of signalling processes is required, ideally with low cell densities that allow for the stimulation and recording of single cells. Single cell stimulations require small-area electrodes with a high charge injection capacity per unit area. The size of the electrodes and the amount of the stimulus current that must be produced are directly determined by the efficiency of electrical charge transfer from the electrode to the cells. The most common stimulation electrode materials are platinum and gold [1–3,6], but in the last years the interest shifted more and more towards iridium oxide [9–15]. Previous studies show that iridium oxide is an excellent candidate for the use as a charge injection material for stimulation electrodes [16–20], because the value of its charge delivery capacity is about a factor of 20 higher and the safe water window is larger compared to the values of platinum [13,19]. Therefore, the size of the electrode can be lowered for the same amount of current that must be applied for activation of one cell. The high charge delivery capacity occurs through a reversible proton reaction and associated valence transition within the oxide film [21].

An optimal interaction between neurons and electrodes on a MEA with iridium oxide electrodes requires biocompatibility, more precisely neurocompatibility, of iridium oxide. In this context, biocompatibility does not only mean that cells survive on the material [17,12,18], but rather that an optimal growth of neuronal cells on the substrate occurs, which is a prerequisite for network formation. Cell adhesion plays a key role [22,23]. It was shown that surface texture, including topography [24], wettability as well as

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chemical coating with bioactive molecules, may influence adhesion and therefore cell growth.

In this study, two different biological test systems were used – thoracic neurons from adult locusts [25,26] and auditory brainstem neurons from embryonic chicken [27]. The robustness and undemanding nature of locust neurons in primary culture turn them into an excellent biological test system for the testing of electrodes for single cell stimulation on MEA systems. First, locust neurons may routinely be obtained in a large size range (20–120 μm), and, second, insect neurons grow and develop with low densities in cell culture. Thus the insect neurons provide an excellent test system for our long-term goal, the further reduction of the size of the electrodes so that they finally become suitable for recording and stimulation of much smaller vertebrate neurons. It would be futile, however, if it turned out that the electrode material should turn out to be incompatible to vertebrate neurons. For this reason we also included tests with chicken neurons.

2. Materials and methods

2.1. Substrate

All sputter experiments were performed on a Nordiko NS 2550 top–down magnetron tool. Prior to sputtering, the chamber was evacuated to at least $4 * 10^{-6}$ mbar by means of a cryogenic pump. During sputtering, the system pump was throttled to a pumping speed of 144 L/s. The Ar flow to the plasma chamber was held constant at 100 sccm. Before iridium oxide film deposition a Ti adhesion layer of 25 nm thickness was deposited on the 100 mm in diameter oxidized Si substrates. For the iridium oxide film deposition the sputtering forward power was fixed at 180 W and the sputtering pressure was maintained at 15 mTorr by means of a throttle valve. To gain different iridium oxide morphologies the gas flow $Q(O_2)$ determining the O_2 partial pressure for the deposition was varied between 1.7 sccm and 90 sccm. Films with typical surface morphologies were referred to IrO#1 (1.7 sccm), IrO#2 (10.4 sccm), IrO#3 (45 sccm) and IrO#4 (90 sccm), respectively. The pure iridium was deposited with the same parameters, just without any oxygen supply. Film thicknesses were measured by profilometry and were found to be in the range of 100 nm.

The different substrates for the investigations, available as square 1 cm^2 pieces, were cleaned in a 1:1 mixture of acetone and ethanol, followed by distilled water in an ultrasonic bath for 3 min each to eliminate potentially harmful substances deriving from the fabrication process. In every experimental series iridium and the different iridium oxide surfaces were used in an uncoated as well as a coated condition. In experiments with insect cells, Concanavalin A (Con A, Sigma) was chosen as coating substance, whereas in the experimental series with chicken cells, poly-(D)-lysine (PDL, Sigma) was used. Con A was diluted (0.2 mg/ml) in phosphate buffered saline (PBS, Sigma), whereas PDL was dissolved in distilled water (0.1 mg/ml). The coating solutions were applied for at least 2 h at room temperature to the desiccated substrates. Afterwards the substrates were rinsed three times with PBS and water, respectively. As a negative control, cells were cultured on uncoated and coated (see above) glass coverslips (Ø 12 mm, Marienfeld), which were sterilised by an ethanol flame.

Scanning electron microscopy (SEM) images were made with a Zeiss Gemini 620 electron microscope at 3 kV.

Atomic force microscopy (AFM) images were made with an Explorer atomic force microscope from Veeco and give information about surface structure and differences in roughness of uncoated and coated iridium oxide surfaces.

2.2. Contact angle measurements

The wettability of the uncoated and coated base materials was assessed by static contact angle measurements using the sessile drop method. The measurements were performed using a contact angle meter (OCA 20, DataPhysics) with appropriate software (SCA 20, DataPhysics). A drop of Milli-Q water was placed on the solid surface of the base material. A droplet of water (5 μl, ultrapure grade) was formed through a capillary and put on the different test surfaces. The contact angles were measured from the left and the right side of the drop and values were averaged. Measurements were repeated three times for each base material on three different batches.

2.3. Marker enzyme assay

To investigate the quality of the Con A and PDL-coating and to determine the protein density on substrates a marker enzyme assay was performed using horseradish peroxidase (HRP) [28]. Con A conjugated with HRP was commercially obtained (Sigma), while the linking process of PDL and HRP was done with an EZ Link Plus Activated Peroxidase Kit (Pierce). The experiments were carried out by coating all test substrates with HRP-conjugated Con A (0.2 mg/ml) or HRP-conjugated PDL (0.1 mg/ml). Coated substrates were inserted into 3 ml photometer cuvettes containing 3 ml o-phenylenediamine dihydrochloride (Sigma–Aldrich; 0.8 mg/ml in 50 mM citric acid–NaOH, pH 5.5) and 30 μl of 3% hydrogen peroxide (v/v) in bidistilled water. HRP catalyses the conversion of o-phenylenediamine by hydrogen peroxide to 2,3-diaminophenazine. The solution in the photometer cuvettes was continuously stirred and inserted into a Spectrophotometer (PCP 6121, Eppendorf) every 30 s to measure E_{389}. During the absorption measurement the substrate was outside of the solution. The more coating substance on the iridium oxide surfaces the more enzyme is available and the faster is the chemical reaction towards 2,3-diaminophenazine. Therefore, high absorption values indicate a high turnover of substrate and this again reflects a high enzyme concentration. Assuming that most of HRP is coupled to Con A or PDL, respectively, a high enzyme concentration demonstrates a high coating protein concentration. Due to the brief experimental runs, the amount of oxidation product resulting from uncatalyzed reaction is negligible. Overall, six measuring points were taken per substrate which resulted in a total measuring time of three minutes. Experiments were repeated three times. The slope of a linear approximation of the measuring points in each case gives a degree of absorbance. The steeper the slope the more absorbance could be measured.

For calibration of the total amount of adsorbed protein, the enzyme activities of known amounts of labeled proteins were measured [28].

In order to determine whether or not the HRP-labeling changes the adsorbance of Con A or PDL, coating with pure HRP was performed according to the protocol as described above. Additionally a displacement assay was applied. For the assay coating was performed as described above, but known amounts of unlabeled Con A or PDL were added to the HRP-labeled protein.

2.4. Animals

Male and female adults of *Locusta migratoria* were used within one week after their final moult. Locusts were obtained from a crowded colony maintained at the Institute of Biology II. The insects were kept under a constant light/dark cycle of 12/12 h and were fed with wheat seedlings and wheat bran.

For chicken embryonic neuron cultures, fertilized eggs of White Leghorn chickens were obtained from a local poultry farm and incubated until embryonic day 6.5 in a forced draft incubator. All experimental procedures in this study conformed to German laws and were approved by the local authorities (Animal Research Committee, RWTH University Hospital Aachen, Germany).

2.5. Cell culture

Locust cells were prepared in modified Leibovitz L-15 cell culture medium (Sigma–Aldrich). The following substances were added: 200 mg/l glucose, 80 mg/l fructose, 35 mg/l L-proline and 6 mg/l imidazole as 10x stock solution in Leibovitz L-15. Furthermore, modified Leibovitz L-15 medium contains 1% (v/v) L-glutamine solution (200 mM, Invitrogen) and a mixture of penicillin (10.000 units/ml) and streptomycin (10 mg/ml) (Sigma–Aldrich) and Amphotericin B (250 μg/ml, Sigma–Aldrich) solution. The osmolarity was enhanced to 390 mOsmol/kg by addition of 10 mM glucose, 10 mM fructose and 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) [26]. The meso- and metathoracic ganglia were dissected for the isolation of neurons. After removal of the ganglionic sheath, cells were dissociated enzymatically by treatment with dispase (Invitrogen) solution (2 mg/ml in Hank's balanced salt solution, Sigma–Aldrich) for about 30 min and subsequent trituration of ganglia. After centrifugation for 3 min at 1200 rpm (Sigma Laboratory Centrifuges 3–15) a defined amount of supernatant was discarded and the cells were resuspended. The amount of residual medium determined the density of cells. For the experiments, cell suspensions that derived from 4 animals were pooled. A defined volume of 70 μl cell suspension was plated on each pretreated substrate. Cells were allowed to adhere to the surface for about one hour. Afterwards, 3 ml cell culture medium, as specified above, was added. The cultures were maintained at 30 °C in an incubator. Since the cell density is non-homogeneous, plating a defined number of cells was not possible.

Chicken embryonic brainstem cultures were prepared as previously described [27]. Briefly, embryos were decapitated and tissue preparation was performed in ice-cold Hank's balanced salt solution (Gibco/Invitrogen) under a preparation microscope. Tissue chunks were treated with trypsin/EDTA (Gibco/Invitrogen) for 14 min and gently triturated. Dissociated cells were briefly resuspended in Dulbecco's modified Eagle's medium/F12 (1:1; Gibco/Invitrogen) containing 10% fetal calf serum (Gibco/Invitrogen) to stop trypsin activity, and rinsed once (Hank's balanced salt solution) before being resuspended in Dulbecco's modified Eagle's medium/F12 (1:1) containing 2% of the serum supplement B27 (Gibco/Invitrogen). All media contained 20 units/ml penicillin/streptomycin. Cells were seeded on base materials (see above) in a controlled density of 400 cells/mm^2 and cultivated in a humidified incubator at 37 °C with a 5% CO_2 atmosphere for 3 days.

2.6. Analyses and immunocytochemistry

The total number of cells in the insect cell cultures was determined at the first and the third day *in vitro*. All cells on the surface of the substrate were counted to