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A neural cell culture study on thin film electrode materials

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Abstract Functional neural stimulation requires good interface between the neural cells and the electrode surfaces. In order to study the effect of electrode materials and surface structure on cell adhesion and biocompatibility, we cultured cortical neurons on thin films of platinum and iridium oxide. We used both flat, as-deposited and laser micro-structured films. The laser micro-structuring consisted of creating regular arrays of micro-bumps or holes with diameters of 4–5 μm and height of about 1.5 μm. The micro-bumps were fabricated onto platinum and iridium film surfaces deposited on borosilicate glass substrates, using mask-projection irradiation with single nano-second pulses from a KrF excimer laser (λ = 248 nm). Amorphous and crystalline (deposited at 250 °C) IrO_2 films were deposited onto the laser micro-structured iridium films by pulsed-DC reactive sputtering to obtain micro-structured IrO_2 films. Cortical neurons isolated from rat embryo brain were cultured onto these film surfaces. Our results indicate that flat and micro-structured film surfaces are biocompatible and non-toxic for neural cell growth. The use of poly-D-lysine as a mediator for cell adhesion onto the thin film surfaces is also discussed.

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Introduction

Neural prosthetic devices employing electrical stimulation of neurons are being used to treat a number of diseases like blindness and Parkinson's disease and have attracted considerable research effort in the past few years [1, 2]. The success of a neural prosthesis relies on the quality of the interface with neural cells. An effective neural interface is one, which provides the needed injection of charge by means of biocompatible electrodes. Biocompatibility is one of the key factors to be considered while designing a neural prosthesis and most generally, it implies non-toxicity, chemical, electrochemical and mechanical stability. The device must be able to perform with an appropriate host response in its intended application. The main factors affecting the quality of an active neural interface are the electrode materials and their surface properties. Platinum is the most commonly used biocompatible electrode material for neural prosthetic applications [3], but recently there has been a growing interest in other materials like platinum–iridium alloys and iridium oxide [4–6]. Neural stimulation applications, which typically employ small-area electrodes, require a high charge capacity per unit area for stimulation. Iridium oxide has an advantage of higher charge capacity through a reversible proton reaction and a corresponding valence change within the porous oxide layers [6]. Studies have also shown that iridium oxide is a biocompatible material and does not form a toxic interface with neurons [7].

The electrode surface topography has a major effect on the cell adherence on the electrode material, and surface modification by micro-fabrication techniques can be used to improve cell adhesion as well as study cell-surface interactions. Scientists have realized that topographical cues may play a crucial role in mediating cell orientation and biocompatibility [8–10]. It is well known that the surface micro-structure of artificial biomedical materials influences cell attachment [11]. The effect of topological modification on different surfaces as glass, quartz [12, 13], metal oxides [14] and silicon [15–17] has been studied and it has been reported that porous silicon enhances growth of neurons [14] and sub-micron and micron scale pillars and wells can improve astrocyte adhesion on silicon [17]. It has also been shown that surface roughness affects neural cell adherence [15].

In order to study the effect of surface modification of electrode materials on neural interfaces, we cultured rat cortical neurons on thin films of biocompatible electrode materials like platinum and iridium oxide. In this paper, we present results of cell survival and viability on both flat as-deposited and poly-D-lysine (PDL) coated films as well as on laser micro-structured films (PDL-coated and non-coated).

Materials and methods

Preparation of thin films

Platinum thin films with thickness 500 nm were deposited on borosilicate glass substrates (Corning Pyrex 7740) at room temperature using a DC Magnetron sputtering technique. A thin adhesion layer of titanium (10 nm) was deposited before sputtering the platinum layer. The sputtering chamber was maintained at a base pressure of $\sim 5 \times 10^{-6}$ Torr. The sputtering pressure and DC power were kept at 5 mTorr and 50 W, respectively. A pulsed-DC system was used for deposition of iridium and iridium oxide thin films on borosilicate glass substrates. The Ir sputtering target had a diameter of 2 inch (purity 99.9%). A thin titanium adhesion layer was deposited in the same sputtering process. Iridium oxide films were reactively sputtered in Ar/O_2 plasma. Prior to deposition the chamber was evacuated to a base pressure of 10^{-7} Torr by a mechanical pump first and then by a cryogenic pump. The sputtering power was fixed at 100 W. The $O_2/(O_2 + Ar)$ mixing ratio (OMR) for the deposition was chosen to be 20% to ensure that the Ir target is not oxidized and that high crystallinity of IrO_2 can be achieved [18]. The sputtering pressure was maintained at 5 mTorr. The substrate temperature for deposition of crystalline IrO_2 thin films was 250 °C and the amorphous films were sputtered at room temperature. The film thickness was measured by profilometry and found to be about 200 nm.

Characterization of the thin films

Raman measurements were performed using a Renishaw in Via Raman Microscope system at room temperature. Spectra were taken with 5,145 Å excitation line of an Ar ion laser. The laser power used for these measurements was about 1 mW. The laser light was focused to a spot size of about 3 μm in diameter onto the sample surface. The Raman spectrum of IrO_2 powder (Alfa Aesar) annealed in air at 750 °C for 48 h was obtained and compared with the reactively sputtered IrO_2 thin films. The crystallinity of the films was examined by XRD in a Rigaku-Rotaflex RU2000 diffractometer using CuK_α radiation. The surface composition of the deposited films was analyzed by X-ray photoelectron spectroscopy (XPS) using monochromatic AlKα radiation in a Perkin Elmer model S500 XPS Spectrometer. Only surface XPS spectra (i.e. without ion etching) were used in this study, because decomposition of IrO_2 was observed during the Ar ion etching. Reference XPS spectrum of iridium film was also recorded.

Laser micro-structuring

A KrF excimer laser (Lambda Physik, model LPX 205i), emitting pulses with a duration of 30 ns and maximum energy of 600 mJ at a wavelength of 248 nm was used to micro-structure the platinum and iridium thin film surfaces. We used the laser fluencies of 0.75 and 1.25 J/cm^2 per pulse for micro-structuring platinum films and for the iridium film the laser fluence was maintained at 1.25 J/cm^2 per pulse. The laser irradiation produced an array of well-defined micro-bumps having diameters of about 2–3 μm. The height of the micro-bumps varied with the variation in laser fluence values. All laser irradiation experiments were carried out in a clean room environment. The excimer laser processed surfaces were studied by optical microscopy, scanning electron microscopy (Hitachi S2400 SEM, equipped with EDAX S2400 EDS) and atomic force microscopy (Autoprobe, Park Scientific Instruments).

Cell culture technique

Rat (Sprague-Dawlley) cortical cells (18-day-old embryo) were prepared in $Ca^{2+}Mg^{2+}$ free Hank's balanced salt solution (HBSS), dissociated with 0.05% trypsin/EDTA (Gibco) and washed in complete medium with 5% fetal bovine serum (FBS). After centrifugation for 7 min at 200*g*, the pellet was gently re-suspended in neurobasal medium supplemented with GlutaMax, 25 μM glutamate

and B27 supplement (Gibco), containing 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were counted using a trypan blue exclusion test and plated at a density of 150×10^3 cells per cm^2 onto 1×1 cm samples (laser micro-structured or flat thin films of platinum or iridium oxide). Glass cover slips (RD German Coverslip 12 mm) coated with poly-D-lysine (PDL) (50 μg/ml) were used as a positive control. Cultures were maintained at 37 °C in a 5% CO_2 incubator for 6 days. Cell survival and viability was assessed by staining with 15 μg/ml fluorescein diacetate (FDA) and 5 μg/ml propidium iodide (PI).

Immunocytochemistry for glia and neurons

Immunofluorescence staining was performed with 4% paraformaldehyde-fixed cells (30 min, room temperature) treated with 0.1% Triton X-100 in phosphate-buffered saline 30 min. Rabbit Anti-Rat NSE (neuron-specific enolase, Polysciences) and Rabbit Anti-Rat GFAP (anti-glial fibrillary acidic protein, Polysciences) were applied for 12 h, followed by Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Molecular Probes) for 12 h. After washing, the cultures were counterstained with 5 μg/ml of propidium iodide and 10 mg/ml Hoechst 33258, for 5 min, washed, embedded in an anti-fade reagent (Invitrogen) and examined with a Nikon Eclipse TE2000-U microscope. Immunostained cells and nuclei were separately counted in five adjacent 0.6 mm^2 fields in each of the samples and cell density was calculated and compared with the control PDL glass slides. For statistical analysis and reproducibility verification each experiment was repeated at least three times.

Results and discussion

Thin film characterization and micro-structuring

Figure 1 shows the Raman spectra of polycrystalline and amorphous IrO_2 thin films reactively sputtered on borosilicate glass substrates. For comparison, the Raman spectrum of IrO_2 powder annealed at 750 °C is also shown. The major Raman modes E_g, B_{2g}, A_{1g} are located at 561, 728, 752 cm^{-1}, respectively [19]. We see all the three modes in the polycrystalline film spectrum. The 728 and 752 cm^{-1} mode is not resolved in the film spectrum and form a wider peak which is dominated by the A_{1g} mode. The spectrum of the amorphous films shows considerably wider peaks due to poor or no crystallinity.

Figure 2 shows the Ir 4f XPS spectra taken from the surface of the polycrystalline (deposited at 250 °C) and the amorphous IrO_2 thin films both reactively sputtered. For comparison the surface XPS spectrum of metallic Ir film is also shown. The binding energies for $Ir4f_{5/2}$ and $Ir4f_{7/2}$ for the metallic films were 63.6 and 60.6 eV, respectively, which are in agreement with the values for pure Ir [20]. The binding energies of the polycrystalline and amorphous IrO_2 thin films are shifted to higher values of 61.6 and 64.6 eV for $Ir4f_{7/2}$ and $Ir4f_{5/2}$, respectively. This 1 eV shift in positive direction illustrates that Ir is in a higher oxidation state, which is in agreement with studies on IrO_2 [21].

Figure 3 shows the AFM images of flat platinum (a) and iridium (c) films and the laser micro-structured platinum (b) and iridium (d) films. As seen from the figure well defined micro-bumps with diameters of about 4–5 μm are formed using a fluence of 1.25 J/cm^2 with one laser pulse. The height of the bumps is in the range of 1–2 μm. The rms roughness for the flat platinum film was found to be 17.3 Å

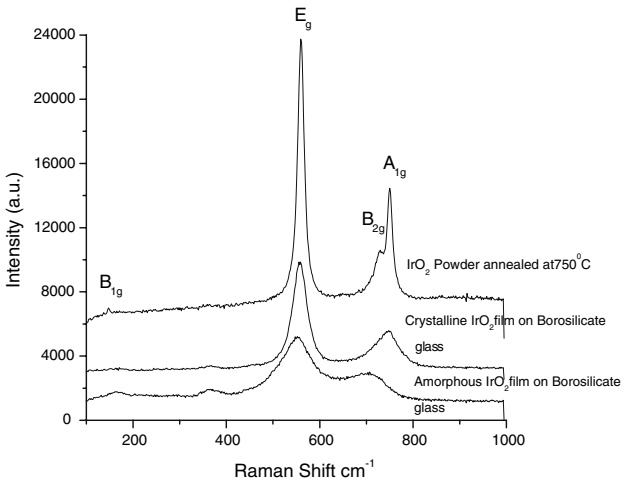

Fig. 1 Raman spectra of as deposited polycrystalline and amorphous IrO_2 thin films compared with the spectrum of the annealed IrO_2 powder

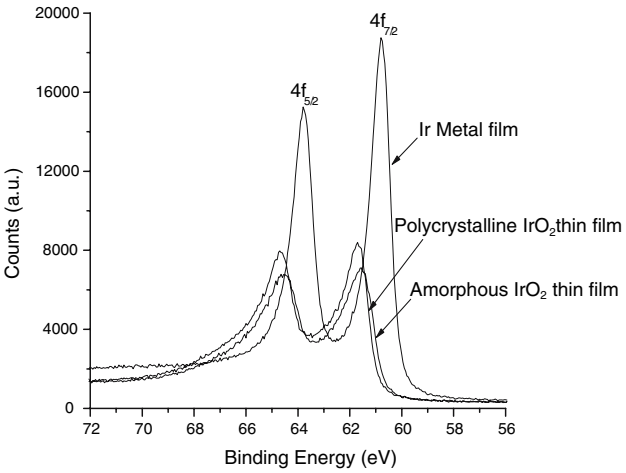

Fig. 2 XPS Spectra of reactively sputtered polycrystalline and amorphous IrO_2 thin films and metallic Ir film

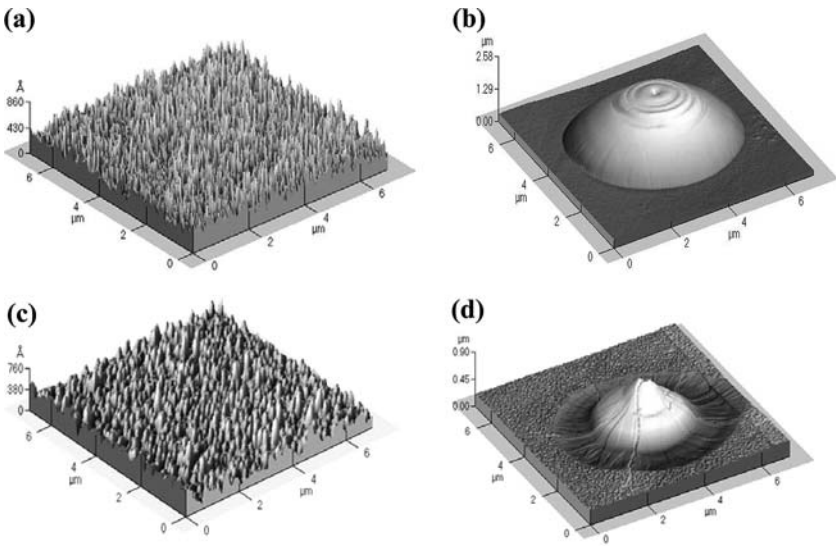

Fig. 3 AFM images showing surface morphologies of flat platinum (**a**), micro-bump on platinum film surface made with a laser fluence 1.25 J/cm^2 (**b**), flat iridium film (**c**), micro-bump on iridium film surface made with a laser fluence 1.25 J/cm^2 (**d**)

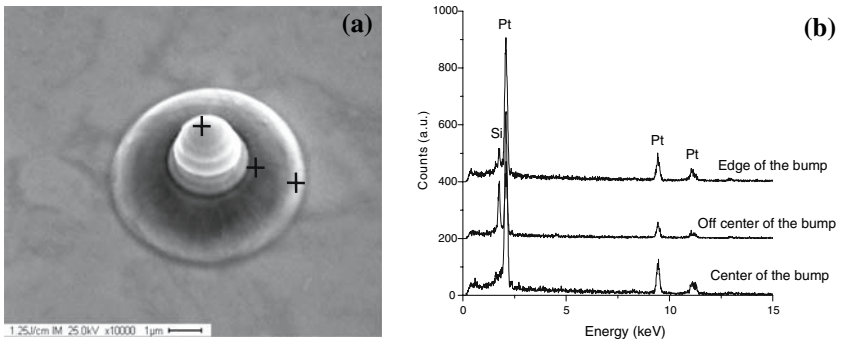

Fig. 4 A SEM image of a micro-bump made on a platinum film surface with laser fluence of 1.25 J/cm^2 (**a**), EDS spectra taken from a micro-bump on a platinum film surface made with laser fluence of 1.25 J/cm^2 (**b**)

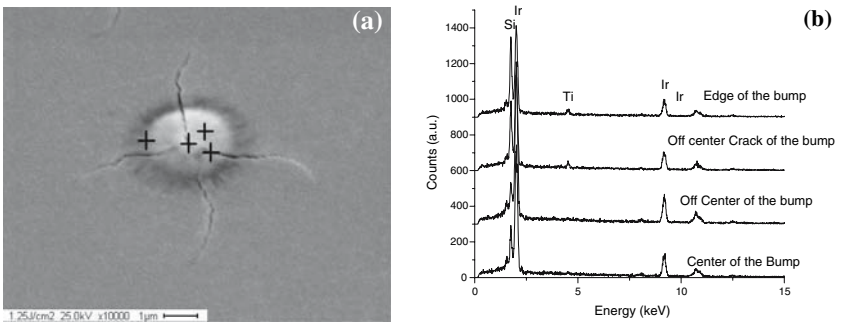

Fig. 5 A SEM image of a micro-bump made on an iridium film surface with laser fluence of 1.25 J/cm^2 (**a**), EDS spectra of a micro-bump on an iridium film surface made with laser fluence of 1.25 J/cm^2 (**b**)

and the rms roughness of the flat amorphous and crystalline IrO_2 films were found to be 103 and 62.0 Å, respectively.

Figure 4a and b show the SEM image and EDS spectra of the micro-bump on platinum thin film made with a laser fluence value of 1.25 J/cm^2, respectively. Figure 5a and b show the SEM image and EDS spectra of the micro-bump on iridium film surface made with a laser value of 1.25 J/cm^2, respectively. The crosses on SEM image indicate the spots from where the EDS spectra were taken. As evident from the EDS spectra of the micro-bump on platinum film surface, we see a higher concentration of platinum at the edge of the bump and the center of the bump. But the off-center area of the bump shows comparatively equal concentration of platinum and silicon. This silicon concentration can be attributed to underlying borosilicate glass that was laser-melted and then contributed to the micro-bump formation. The slight difference in the size and shape of the micro-bumps shown in Figs. 3 and 4 are due to the variations in the size of the openings on the projection mask used to laser fabricate these structures.

In case of the micro-bump on the iridium film surface we see a higher concentration of Si at the edge of the bump

and also on the cracks that are formed on the bump surface. There are also traces of titanium from the underlying adhesion layer, on the edge and the cracks of the bump surface. The concentration of silicon at the off-center spot of the micro-bump on the iridium film surface is considerably lower than that in the off-center spot of the micro-bump on the platinum film surface. This difference in concentration of silicon can be attributed to the fact that the melting point of iridium (2,466 °C) is quite higher in comparison to platinum (1,772 °C), hence the laser-heating is not high enough to cause melting of iridium and fusion with the underlying borosilicate glass substrate. The formation of micro-bumps is a result of rapid local (within the irradiated spots) melting induced by the laser pulse and then rapid self-cooling and solidification. The thermal conductivity of the borosilicate glass substrate (1.14 W/m °C) is very small compared to that of the platinum (73 W/m °C) and iridium (148 W/m °C) thin films. Hence most of the heat energy at the onset of the pulse is distributed laterally in the film. In other words, heat is propagated in a two-dimensional pattern. On cooling, this causes the solidification front to move towards the center of the irradiated spot, eventually causing the center to rise above its original position.

Neural tissue-electrode material interface and cell culture

Among the most important problems that need to be studied in the development of neural implant devices is biocompatibility. The physical and chemical structure of a material can affect the interaction of proteins and cells with that material and hence, the development of appropriate material surfaces that directly contact the neural tissue and the study of neuron-surface interactions in vivo are crucial as well. In a previous work [22] we reported preliminary results on in vitro neuronal growth and survival on flat & micro-structured thin films of platinum and iridium oxide.

The success of neuronal cultures depends strongly on finding an appropriate substrate. This involves careful selection of electrode material and design, using surface modification techniques or control of cell culture conditions. The cell adhesion is mediated by the interactions between receptors on the cell surface and peptide ligands on the material surface. Chemical modification of an electrode surface by coating it with polymers is one such example of surface modification techniques used to promote cell adhesion. In the case of such chemically modified electrodes the coated polymer does not play a role in electrical stimulation/recording, but enhances cell attachment. Therefore we tried to identify a polymer suitable for electrode material coating and in our study we selected poly-D-lysine (PDL) as such a polymer. It is a member of a class of synthetic polymers that can produce chiral secondary structures spontaneously and possesses enough amino groups needed to enhance protein adsorption and neuronal attachment.

The micro-photographs shown in Fig. 6 represent typical neuronal growth onto flat, PDL-coated surfaces. Formation of neurofilaments on the tested surfaces indicated that neural cells could adhere and survive. Our results showed that all tested surfaces coated by PDL are biocompatible and nontoxic for neural cells. Our previous study focused on studying neuronal growth on non

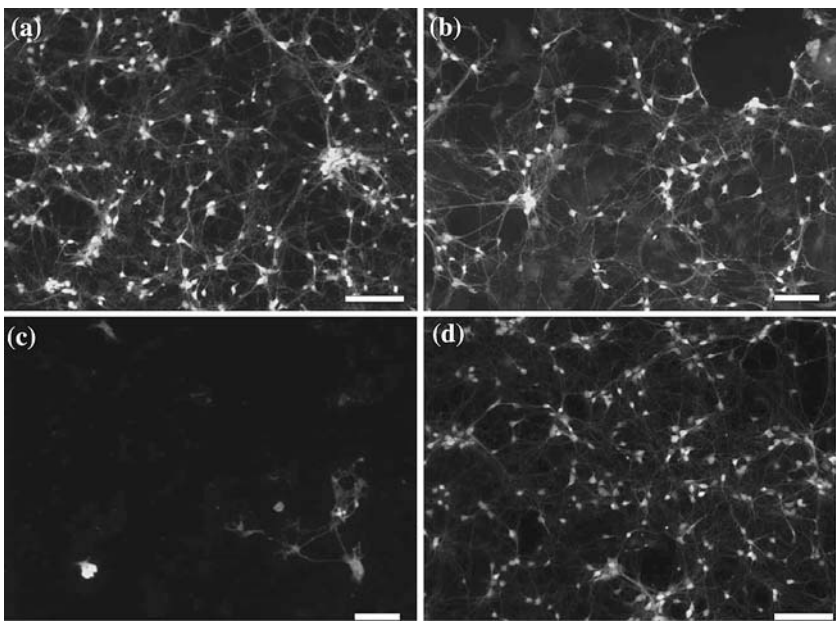

Fig. 6 Immunocytochemistry images on control PDL coated glass cover slip (**a**), PDL coated platinum thin film (**b**), PDL coated amorphous IrO_2 thin film (**c**), PDL coated crystalline IrO_2 thin film (**d**) (magnification 1×10^3, the bars in the images represent a scale of 100 μm)

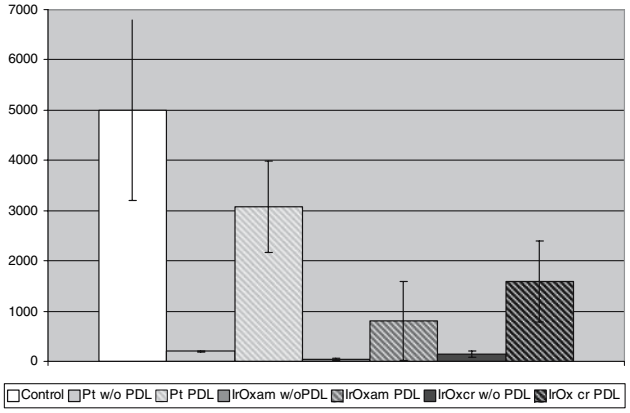

Fig. 7 Number of NSE positive cells per 1 cm^2 plated onto (from left to right) PDL-coated glass (control), flat, PDL-coated thin films of platinum, amorphous IrO_2 and crystalline IrO_2 after 6 days of culture

PDL-coated surfaces, the results indicated poor cell attachment on the tested surfaces [22]. In this study, the PDL-coated surfaces showed better cell attachment and hence we can say that the cell receptors interact with the PDL molecule amino groups, leading to enhancement in cell attachment. Figure 7 shows number of NSE positive cells per 1 cm^2 plated onto PDL-coated glass (control), flat, PDL-coated platinum, amorphous IrO_2 and crystalline IrO_2 film surfaces, after 6 days of culture. Only platinum surface coated by PDL showed a good level of cell attachment and we observed significant cell growth compared to control surfaces as seen in Fig. 7.

We were interested in how the neuronal cells or culture reacts to more complex surface morphology (i.e. micro-structuring) than flat and smooth films. To study this we tested growth of neuronal cells on surfaces with laser micro-structured topography. Figure 8 shows results of neuronal growth and survival on micro-structured thin films of platinum (a, b) & amorphous iridium oxide (c, d). A comparison of Figs. 6 and 8 shows no significant difference in cell growth on PDL-coated, micro-structured thin films as compared to PDL-coated flat surfaces. In Fig. 8d is shown the neuronal growth on arrays of laser-ablated holes covered with an amorphous IrO_2 layer having different spacing (20, 60 and 100 μm). Despite the limited (from cells statistics point of view) array areas, a preference of neuronal growth on the 100 μm spacing array with respect to the others can be noted.

Many different types of micro-electrodes have been developed for use as a neural interface to chronically record single neuron action potentials from ensembles of neurons. Unfortunately, the recordings from these micro-electrode devices are not consistent and often last for only a few weeks. For most micro-electrode types, the loss of these recordings is not due to failure of the electrodes but

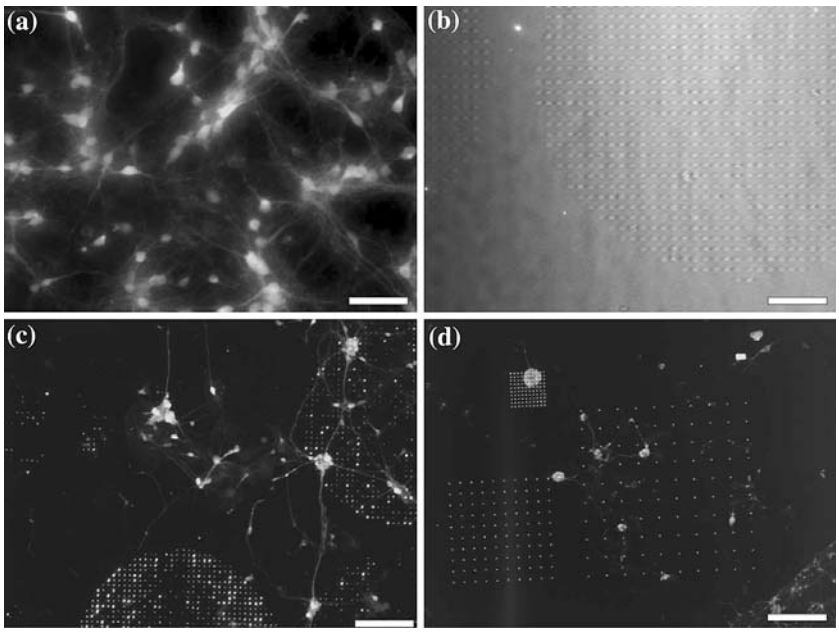

Fig. 8 NSE positive cells per 1 cm^2 plated onto PDL-coated platinum (**a**) (the bar in the image represents scale of 100 μm), cell bodies shown on micro-structured platinum film using reflected light microscopy (**b**) (the bar in the image represents a scale of 50 μm), NSE positive cells per 1 cm^2 plated onto PDL-coated micro-structured amorphous IrO_2 thin film with an array of micro-bumps having small craters at the tip with spacing of 10 μm (**c**) (the bar in the image represents a scale of 100 μm), NSE positive cells per 1 cm^2 plated on PDL coated micro-structured amorphous IrO_2 thin film with arrays of holes with different spacing (**d**) (the bar in the image represents a scale of 300 μm) after 6 days of culture

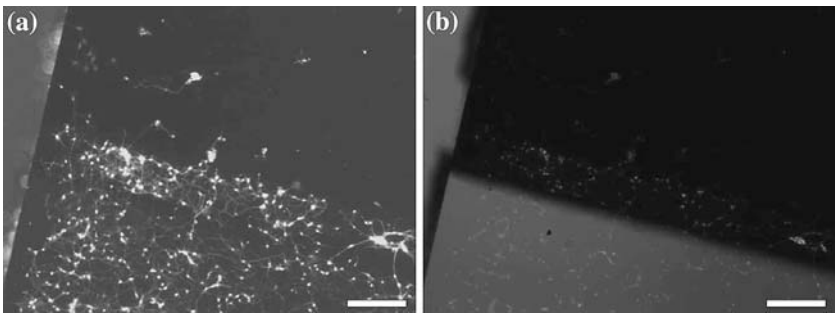

Fig. 9 Immunocytochemistry images showing the neurons at the interface between borosilicate glass substrate and PDL-coated amorphous IrO_2 thin film, in fluorescent light (**a**), and the same with light and fluorescence (**b**)

rather to damage to the surrounding tissue resulting from the formation of nonconductive glial scar. From such a point of view, amorphous IrO_2 is one of the most promising electrode materials for stimulation/recording purposes. Although, IrO_2 is biocompatible and non-toxic for neural cells, we observed poor cell attachment onto IrO_2 surfaces as compared to other tested surfaces. Figure 9a and b shows immunocytochemistry images of neurons growing at the interface of borosilicate glass substrate and the PDL-coated amorphous IrO_2 thin film. As is evident from the figures the density of the neurons is higher on the glass substrate than on the films surface. In order to understand the reason for this higher density of neurons on the glass substrate as compared to the film surface, we characterized PDL-coated cover glass slip and amorphous and crystalline IrO_2 film surfaces with XPS and tried to quantify the PDL coverage by measuring the N_2 content (N1s XPS line) as N_2 in contained in the NH_3 groups forming the PDL molecule. The results from the XPS study showed barely detectable content of nitrogen arising from the amorphous and crystalline IrO_2 films as compared to the glass surface. This could be attributed to the poor wet ability of poly-D-lysine for iridium oxide film surfaces and hence the thickness or amount of PDL on the IrO_2 film surfaces is less compared to platinum and the control glass surfaces. Reduction in the amount of PDL on the IrO_2 films surfaces reduces the peptide ligands that influence the cell attachment on the surface. Hence we see poor cell attachment on these IrO_2 films surfaces as compared to other tested surfaces.

This study was an in-vitro study and hence the results of this study would be very different in case of an in-vivo study, owing to different immune reactions occurring within the body. In the future we plan to study the in-vivo response in more detail.

Conclusions

We were successful in finding conditions for controllable micro-bump formation on platinum and iridium thin films on borosilicate glass substrate by mask-projection irradiation with single nano-second pulses from a KrF excimer laser (λ = 248 nm). Our results indicate that all flat and micro-structured surfaces are biocompatible and non-toxic for neural cell growth. We have some evidence for preferential growth of neurons for an array of laser ablated holes with spacing of 100 μm as compared to other spacing values and we plan to study this in more detail. We used poly-D-lysine as a mediator for cell attachment on the thin film surface and we have observed good attachment of neural cells as compared to non-coated film, results on which were reported earlier [22]. Platinum films were found to be superior to amorphous and crystalline IrO_2 films with respect to neuronal cell attachment properties.

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