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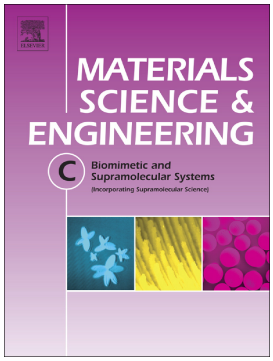

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TiO_2 surfaces support neuron growth during electric field stimulation

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Abstract

TiO_2 is proposed here for the first time as a substrate for neural prostheses that involve electrical stimulation. Several characteristics make TiO_2 an attractive material: Its electrochemical behaviour as an insulator prevents surface changes during stimulation. Hydration creates -OH groups at the surface, which aid cell adhesion by interaction with inorganic ions and macromolecules in cell membranes. Its ability to neutralize reactive oxygen and nitrogen species that trigger inflammatory processes confers biocompatibility properties in dark conditions. Here, physicochemical characterization of TiO_2 samples and their surfaces was carried out by X-ray diffraction, X-ray photoelectronic emission spectroscopy, scanning electron microscopy, atomic force microscopy and by contact angle measurements. Its properties were related to the growth parameters and morphology of amphibian spinal neurons cultured on TiO_2 samples. Neurons adhered to and extended neurites directly on TiO_2 surfaces without pre-coating with adhesive molecules, indicating that the material permits intimate neuron-surface interactions. On TiO_2 surfaces the distal tips of each extending neurite and the neurite shafts themselves showed more complex filopodial morphology compared with control cultures on glass. Importantly, the ability of TiO_2 to support neuron growth during electric field exposure was also tested. The extent of growth and the degree of neurite orientation relative to the electric field on TiO_2 approximated that on glass control substrates. Collectively, the data suggest that TiO_2 materials support neuron growth and that they have potential utility for neural prosthetic applications incorporating electric field stimulation, especially where intimate contact of neurons with the material is beneficial.

Introduction

Biomaterials are used increasingly in the treatment of nervous system disorders and new biomedical strategies are being developed to promote repair and regeneration of neuronal tissue. [1] In particular, electrical stimulation is emerging in neural prostheses and novel treatments for spinal cord lesions and for peripheral sensory feedback in limb prostheses. [2, 3] Intracellular and extracellular chemical, physical and electrical gradients control activities such as cell proliferation, cell migration, electrical signalling in the nervous system and axon outgrowth. Consequently, these gradients play important roles in processes including, tumour cell metastasis, spinal cord repair, epithelial wound repair, tissue regeneration and establishment of left-right body asymmetry. [4]

Wounded tissues have an endogenous electric field (EF) that contributes to wound healing. For example, the unwounded mammalian corneal epithelium maintains an electrical potential across itself (inside positive); a consequence of directional ion transport. Upon wounding this potential collapses to zero at the wound centre but is maintained distally, resulting in a lateral voltage gradient (EF) within the sub-epithelial tissues, with the cathode at the wound centre. In this system the resulting EF controls cell migration and the axis of cell division during the re-epithelisation phase of wound closure. [5, 6] The ability to control wound closure electrically has prompted development of electric therapies to aid wound healing but the optimal stimulation intensity, duration, wave shape and even the electrode materials may not yet be optimal. [4, 7]

Embryonically, the central nervous system develops from a sheet of neuroepithelium that maintains a voltage gradient akin to adult skin or cornea. Therefore, the nervous system develops within a direct current electric field (EF) and damaged nervous tissue has a persistent endogenous EF associated with it. [8] Electrical stimulation has been used to attempt to aid spinal cord repair in adult mammals, including humans. In the context of nervous system repair the stimulation can be steady DC, oscillating biphasic DC or high frequency current pulses. [2-

4, 9-11] Steady and slowly oscillating DC stimulation is intended to encourage regrowth of neurons whereas rapidly oscillating EFs generally aim to stimulate function of residual circuits. [12]

One problem with indwelling electrical stimulators is that even state of the art (typically metallic, platinum or a platinum alloy) electrodes produce undesirable electrochemical side effects, such as heating and pH changes that induce tissue necrosis upon long term use, especially at high stimulation intensities.[2] Another consideration is that the inherent properties of the materials play important roles, dictating whether cells interact with neural prostheses and how they behave upon stimulation. For example, the material surface charge has a strong influence on the extent of neural adhesion and the direction of neurite growth, with different surface charges even promoting growth directed in opposite directions during electric stimulation. [13] This property may be useful for guiding nerve growth during regeneration but it can only be exploited by better understanding how neuron growth is controlled by new types of materials.

The present study tested the hypothesis that TiO_2 would provide a safe substrate for neuron growth, even during DC electrical stimulation, therefore proving its suitability for potential use in nervous system regeneration therapy. TiO_2 has many attractive properties and it is in wide use because of its high chemical stability and low production cost compared to other materials. [14] Due to its behaviour as an insulator TiO_2 presents favourable electrochemical properties under electrical stimulation, avoiding possible unwanted and harmful surface or surface-media interface changes, such a pH changes. [15] Moreover, it has been reported that hydroxide (OH) formation occurs on the surface of TiO_2 when it is hydrated and at pH 7. Under those conditions the OH groups in acidic sites are easily deprotonated and become more reactive. The creation of this surface charge at the interface is responsible for the interactions with inorganic ions and macromolecules present in the cell membrane. [15] These reactions, which occur under physiologically relevant conditions, are conducive to cell interaction and cell adhesion to the

surface substrate. Furthermore, TiO_2 possesses anti-inflammatory properties due to its ability to inhibit or neutralize reactive oxygen and nitrogen species (ROS and RNS) in dark conditions and the biocompatibility of TiO_2 is attributed to this property. [16] These species trigger the inflammatory mechanism cascade caused by a foreign substance such as an implant or prosthesis. Consequently, TiO_2 prevents the formation of a protein layer and the recruitment of fibroblasts and glial cells, which would otherwise encapsulate and reject the implant as the inflammatory response escalates. [17, 18] The mechanism of ROS and RNS inhibition has been studied but still not elucidated. It has been proposed to be mediated by redox reactions but this is unlikely in physiological media because the redox conditions necessary for the proposed phase changes do not exist. [16, 19, 20]

Collectively, these electrochemical and surface properties support the suitability of TiO_2 as a candidate substrate for cell growth. Indeed, neurons grow on TiO_2 surfaces. [21] Our study extends this concept by examining nerve growth on TiO_2 surfaces during electrical stimulation. This is the first report to consider directional nerve growth on TiO_2 during electrical stimulation.

Materials and Methods

Substrate Preparation

The TiO_2 ceramic disks were prepared from TiO_2 nanopowders synthesized by the precipitation route, as described elsewhere. [22] Ti (IV) isopropoxide (Aldrich 95%) was dissolved in absolute ethanol at 70°C for 24h under constant stirring. Afterwards, hydrolysis takes place in the presence of humidity. The prepared precipitated nanopowders were sieved through 100 µm mesh and then calcined at 600°C for 12h to eliminate organic residuals. Their particle size was finally reduced by using an attrition mill for 5h in isopropanol and sieved again through 100 µm mesh. This procedure assures homogeneity of the particle size distribution. The prepared titania powders were isostatically pressed and sintered in air at 1400°C for 4 hours. Dense TiO_2 disks (20 mm diameter) were obtained from this compact by microcutting, and underwent thermal treatment in air at 1400°C for 4 hours to eliminate sharp edges from the surface caused by

microcutting. Sharp edges would mean that the surface morphology would play an important role in the direction of cell growth apart from electrical stimulation. Thermal treatment was chosen based on the results obtained in a previous cell culture study, where different commercial TiO_2 powders were processed by different thermal treatments. [22]

Cover glass (22 X 22 mm, no 1.5 thickness) was used as a control growth surface. Both TiO_2 and glass surfaces were not treated with any adhesion layer (e.g., no poly-L-lysine or extracellular matrix) prior to cell plating.

Characterization of TiO_2 substrates

The phase composition of TiO_2 samples was studied by X-Ray Diffraction (XRD) in an X-Ray difractometer X'Pert PRO from Panalytical with a monochromer for λ kα_1. Surface composition of the TiO_2 substrates was characterised by X- ray photoelectronic emission spectroscopy (XPS) with a VG Escalab 200R Spectrometer. Surface morphology was observed by Scanning Electron Microscopy (SEM) with a HITACHI TM-1000 microscope, working at 10 kv. With this equipment, metallization of the sample surface is not required. Deep studies of the TiO_2 microstructures were carried out with a FE-SEM Hitachi S-4700. Also Atomic Force Microscopy (AFM) was used for the morphological and topographical study of the TiO_2 surfaces. A Cervantes from NANOTEC Electrónica equipment, with WSxM 5.0 Develop 4.2 image software, was used. Contact angle (θ) of TiO_2 substrates with a watery media was determined by the Sessile water drop method by using a Tensiometer Easy DROP standard from KRÜSS, and a digital camera. This method determines the surface energy of the solid sample by placing a drop of liquid with known parameters of surface energy on the material. The shape of the drop, and specifically the contact angle is measured. For this latter calculation the Young-Laplace method was used. Finally, the relative density of the TiO_2 samples was calculated by the Archimedes method.

Cell Cultures

Primary cultures of neurons were prepared from stage 20 *Xenopus laevis* neural tubes according to the method of with minor alterations. [23, 24] All neural tubes for one experiment (one per chamber) were pooled in the Ca^{2+}–Mg^{2+}-free Steinberg's solution (disaggregating medium). Disaggregating medium was then replaced with an appropriate volume of culture medium (200 µl per chamber) and the resulting cell suspension was triturated gently. Culture medium consisted of (v/v) 20% Leibowitz L15 medium, 2% penicillin (5000 IU/ml)/streptomycin (5000 µg/ml), 1% fetal bovine serum (all from ICN Biomedicals) made up in Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.44 mM $Ca(NO_3)_2$, 1.3 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.9).

For cultures seeded onto cover glass or TiO_2 materials the cell suspension was plated directly onto the cover glass (22 mm x 22mm) or onto the TiO_2 disk, which rested in a 35 mm dish. Another coverslip was placed gently on top of the drop of medium to prevent evaporation. After about an hour to permit cell adhesion the top cover glass was floated off by adding additional culture medium and discarded. The coverglass or the TiO_2 disk with adherent cells was inverted (cell side down) over a drop of medium in the central trough in the centre of the EF chamber (Fig 1) and secured to the glass support spacing strips using a non-curing silicone compound (Dow Corning MS4). The EF was initiated 1 to 2 h after plating. Alternatively, about 6 to 9 h after plating the cells on both substrates were fixed for cytoskeleton labelling with rhodamine phalloidin without EF stimulation.

Electric Field Application

The procedure for application of EFs to cells *in vitro* has been described previously [25, 26] The base of the EF chamber was a 100 mm-diameter x 20 mm tissue culture dish to which two 12 x 50-mm strips of No. 1 cover glass were secured parallel to each other, 1 cm apart with silicone sealant (Dow Corning 3140RTV). Chambers were allowed to cure for at least 24 h before use.

Cells plated onto cover glass or TiO_2 disks were allowed to adhere for about 1 hour before being inverted over the central trough (cells were on the roof of the EF channel), which contained a drop of culture medium. Electrical contact with the culture chambers (Fig 1) was made using two ~20-cm-long U-shaped tubes filled with Steinberg's solution gelled with 2% agar. One end of each bridge rested in a Steinberg's-filled beaker which, in turn, contained a Ag/AgCl electrode connected to a DC constant current electrophoresis power supply. The other end of each bridge terminated in a pool of culture medium continuous with that in the central trough containing the cells. The EF applied was 150 mV/mm for 3 hours. The EF across the coverglass or disk was measured directly using a multimeter. Regardless of whether exposed to an EF or not, the cells were kept in the dark (protected from light) from the time of plating.

Data Collection and Analysis

EF responses of neurons on glass control

Data for neurons on cover glass were collected as photographs on an Axiovert 25 Zeiss inverted phase-contrast microscope. The cells in each chamber whose neurite paths could be identified unambiguously were photographed hourly for 3 hours. The length and angle turned by the growing tip (the growth cone) of each neurite was measured each hour as described in (Figure 1A). [23] For angle measurements; -90 deg indicates that the growth cone faces the cathode directly, +90 deg means it faces the anode directly and 0 deg means It is oriented perpendicular to the horizontal EF. The angle of turning relative to the EF vector and the percentage of neurites that turned more than 15 deg during the 3 hours in each direction were determined. In Figure 1A, the example shows a growth cone that turned 126 degrees toward the cathode (indicated by a minus sign) during 3 hours.

Fluorescence imaging of fixed cells

The opacity of the TiO_2 samples prevented use of time lapse phase contrast microscopy, so cells cultured on TiO_2 disks were fixed at the end of the 3 hour experiment and labelled with rhodamine phalloidin to assess morphology with and without EF stimulation. [23] Neurons on

cover glass or TiO_2 were fixed with 4% Formaldehyde in Phosphate Buffered Saline (Sigma) with 0.1% Tween-20 (PBST) and f-actin was stained with 1:50 rhodamine phalloidin (Molecular Probes). Vectashield was added before imaging to prevent photobleaching.

Data were collected from photographs. All cells in each chamber whose neurite paths could be identified unambiguously were photographed for analysis. Photographs were taken on a Zeiss inverted epifluorescence microscope (Axio Cam MRm with Axiovision software). For analysis, the photographs of neural cells cultured on coverglass and TiO_2 substrates under EF conditions were compared with the corresponding photographs from cell cultured without EF stimulation. The directional responses were expressed qualitatively by superimposing the neuronal cell bodies and tracing the neurite paths at the end of experiment. In Fig 1B the angle of the growth cone faced is compared to the angle of the initial neurite segment as it emerged from the soma. Percentages of growth cones facing towards the cathode after 3 hours were calculated.

Results

Characterization of TiO_2 substrates

The chemical composition and topography of the samples were examined because they are key parameters for neuronal cell adhesion and differentiation. X-ray diffraction of the surfaces showed that prepared TiO_2 rutile was a single phase (JCPDS 21-1276) with no signal of crystalline secondary phases (Figure 2). X-ray photoelectronic emission spectroscopy XPS O1s spectra showed evidence of OH groups linked to the TiO_2 structure (not physisorbed). The Ti2p spectrum confirmed that titanium on the sample surface was in the Ti^{4+} state and therefore appeared as TiO_2.

The prepared titania disks were rigid and opaque, 20 mm in diameter, 2 mm thick and cream-coloured (Fig 3A). The 98% relative density established using the Archimedes method indicated that the substrate was not porous. SEM micrographs from the surface (Figure 3B and C) confirmed the low porosity and revealed a heterogeneous grain size ranging from 10 μm to 80

μm. FE-SEM revealed a complex microstructure of terraces on the surface of individual grains (Figure 3F and G). The height of these terraces had a distribution range from 180 nm to 362 nm (Figure 3G). Furthermore, AFM micrographs showed a surface roughness in the range of nm (around 100 nm), which was also observed in the grain surfaces (Figure 3D). The contact angle measured for the TiO_2 disks was 34 degrees (Figure 3E), which indicated a highly hydrophilic surface, and consequently, good contact with the culture media.

The substratum affected neuronal morphology

Neurite growth was assessed on TiO_2 by labelling neuronal morphology in cells fixed after 9 to12 h in culture. Rhodamine phalloidin, a fluorescent marker for filamentous-actin, labelled the whole cell including the neurite and fine details of growth cone structure. Without any EF stimulation the labelling revealed morphological differences on TiO_2 substrates compared to glass controls (Fig 4). Wider lamellipodia and numerous filopodia were observed for growth cones on the TiO_2 substrate. Differences were quantified by measuring the neurite lengths and calculating the number of branches per neurite, the number of filopodia per growth cone and the density of filopodia on each neurite (Fig 5). Although neurites were shorter on TiO_2 (54 ± 3 μm) than on glass (73 ± 4 μm) their morphology was more complex on TiO_2. Growth cones on TiO_2 had wider lamellipodia with more filopodia per growth cone (5.6 ± 0.2) compared with cells cultured on glass (4.6 ± 0.2). On TiO_2 they had more growth cone filopodia (5.6 ± 0.3) and more filopodia per neurite (0.10 ± 0.01 per μm) compared with cells on glass (4.6 ± 0.2 and 0.06 ± 0.01 per μm, respectively). Therefore, there were more points of cell-substratum interaction for neurons developing on TiO_2 relative to glass. Overall, (without EF stimulation) TiO_2 supported neuron growth at least as well as glass.

TiO_2 was also a favourable substrate during EF stimulation. There were fewer growth cone filopodia after EF treatment on TiO_2 but the mean neurite length, the density of filopodia along the neurite and the number of branches were the same for EF stimulated and unstimulated cultures. Whereas on glass EF stimulation decreased the mean neurite length and extent of

branching compared to unstimulated cells, on TiO_2 the EF increased the number of growth cone filopodia. Therefore, in general, TiO_2 supported neuron growth during EF stimulation better than glass control. For example, on TiO_2 there was more EF-induced branching than on glass ($P < 0.0001$) and the EF induced a small but significant increase in neurite length compared to EF treated cells on glass ($P < 0.05$).

EF stimulation biased growth toward the cathode on glass control

Initial experiments using tissue culture glass control substrates confirmed that neurons responded with directional growth in an EF (Fig 6). Neurites on glass growing without EF stimulation extended at 22 ± 2 μm/h and they grew in random directions, meandering on average by only 1 ± 6 deg (n = 41). However, during 3 h in an EF at 150 mV/mm they grew faster (25 ± 1; n=102) and their growth direction was biased, turning -17 ± 4 deg toward the cathode (indicated by the negative sign; p=0.008 compared to no EF). Cathodal turning was at the expense of anodal turning; 33% of growth cones would be expected in each cathode, anode or perpendicular-facing category for random growth. Prior to EF application, 38%, 33% and 29% of neurites faced towards the cathode, perpendicular and anode respectively. After EF stimulation 44% of growth cones faced towards the cathode but only 17 % faced toward the anode ($p < 0.002$). 39% are faced towards the perpendicular direction (Figure 6A). During stimulation time 39% of neurites turned towards the cathode but only 20% turned towards the anode (Figure 1B).

Rutile surfaces supported cathodal growth during EF stimulation

Dynamic observation of growth was possible for neurons on transparent substrates (glass control) but the TiO_2 rutile disks were opaque, preventing direct time lapse observation. As *Xenopus* growth cones advance they leave a neurite 'trail' behind them, so the path the growth cone has taken is preserved faithfully in the shape of the neurite (e.g. Fig 1B). This was exploited to evaluate directional neurite growth on TiO_2 substrates. Responses on rutile were determined for fixed, labelled cells with and without EF stimulation and were compared to

control neurons grown on glass. Composite drawings made by superimposing the neuronal cell bodies and tracing the neurite path at 3 hours were compared (Figure 7A). Growth was uniform when no EF was present, 49% and 53% facing the 'cathode' side on glass and TiO_2, respectively. On the other hand most growth cones faced the cathode with EF application on both substrates, 61% and 60% respectively on glass and TiO_2 (Figure 7B). Therefore rutile was as good at supporting cathodal reorientation as glass.

Discussion

The Influence of Substratum on Neuronal Morphology

Cells behave differently on different substrates due to the material's particular surface characteristics, with the cell-material interface dictating subsequent cellular responses. For neurons the microstructure, composition and surface charge each contribute to the morphology and number of cells that adhere to materials. [13, 27, 28]

The phase composition of the materials produced in this study is based on a single phase of TiO_2 rutile, which was confirmed by XRD. The existence OH groups on the surface have been demonstrated by XPS (Figure 2). When a TiO_2 surface layer is in contact with an aqueous (biological) medium complex processes occur at the oxide-liquid interface; hydration of the oxide causes hydroxide formation at the surface and acid-base reactions at the OH groups induce charges at the oxide-liquid interface. At pH 7, which is physiologically relevant, OH groups linked to acidic regions lose some protons, creating a superficial charge. This charge is responsible for the electrostatic surface interaction with inorganic ions and macromolecules such as the proteins, lipids and polysaccharides present in the cell membrane, thereby promoting cell attachment. Although our data are restricted to neurons cultured without pre-coating the surface with any attachment factors it is likely that these rutile surfaces would promote attachment of a variety of other cell types also, either by direct electrostatic cellular adhesion or by electrostatic adsorption of growth promoting molecules to which cells could then adhere.

The chemical and microstructural properties of TiO_2 surfaces are responsible for the surface energy of the material in contact with aqueous media. A low contact angle indicates a low surface energy and therefore better wetting of the surface by the liquid medium, hence adhesion of cells to the surface will be easier. The TiO_2 substrata used here had a low contact angle value of 34 deg (Figure 3G) indicating that it is conducive to interaction of the material surface with the medium. In addition to neurons we also observed non-neuronal cells (myoblasts and fibroblasts) growing on the materials in our primary cultures, suggesting that the material is also conducive to growth of a wider range of cell types.

Electrostatic attraction of cells to the material is crucial for differentiation but surface topography also plays an important role in cell attachment, morphology and subsequent cell function. For example, nanostructured topography improves cell-substrate interactions, probably via membrane molecules involved in cell attachment at the nanometer scale. The surface of the TiO_2 material studied here has nanostructures in the form of repeating terraces superimposed onto a complex micrometre scale granular surface pattern (Figure 3). Surface roughness can affect growth of cells, including neurons. Indeed, the *Xenopus* neurons used in this study are sensitive to parallel nanogrooves as shallow as 14 nm with widths as narrow as 1 um. [27]

The site of neurite initiation on the cell body, the direction of growth cone filopodia orientation and the rate and direction of subsequent neurite outgrowth were all determined by the subtle substratum topography. Although the mean neurite length on TiO_2 was shorter, neurons had more filopodia, both along the neurite shafts and on their growth cones compared to controls on glass (Figure 5). This confirms that TiO_2 is a good substrate to support neurite growth and that it is biocompatible for the neurons. The morphology with numerous filopodia is indicative of a good cell-substratum interaction and suggests that the topographical nanostructures on the surface may contribute to filopodia production as was shown previously for these neurons on arrays of nanogrooves. [27] The reduced overall neurite length on the textured TiO_2 surfaces may be due to neurites pausing to contemplate sending out branches (evident as residual lateral

filopodia) in response to topographical cues. The inability to perform time lapse observation of cells on the opaque surfaces prevented testing this idea.

Neuronal growth on TiO_2 during EF stimulation

For cells on TiO_2 some parameters of cell morphology did not change upon EF stimulation; for example, the mean neurite length, the number of branches per cell and the filopodia density along the neurite were similar with or without EF stimulation (Fig 5). Therefore, the conditions that control the cell-material interaction did not change under EF stimulation, permitting cell growth and survival. This has positive implications for the use of TiO_2 in neural prostheses because the material–cell interaction was not influenced negatively by stimulation.

A physiological EF as low as 10 mV mm^{-1} (~0.5 mV across a growth cone) causes growth cones to turn, usually towards the cathode but the growth substrate plays an important role in the response. Here, 150 mV/mm was used, to approximate the endogenous EFs that exist naturally in the *Xenopus* neural tube and to permit comparison with previous studies. [29] The synergistic relationship of the net surface charge and the external EF determines the rate and direction of growth cone movement. [13] It was shown here that 44% of *Xenopus* growth cones cultured on glass substrates grew toward the cathode during EF application (Figure 7). When an electric field is applied, the percentage of growth cones facing the cathode increased at the expense of those facing the anode on glass and TiO_2 (Figure 7). These results are highly promising for situations where promotion of oriented growth is desired using electrically active biomaterials implanted in the nervous system (e.g. nerve regeneration).

Conclusions

In this work TiO_2 was shown to be a good substrate for neurons growth, even during exposure to an electric field. *Xenopus* neurons growth on the custom-made TiO_2 disks was more complex than when on glass, with neurites and growth cones having more numerous filopodia. Additionally, a DC EF orientated neural cell growth towards the cathode on both glass and TiO_2

surfaces. This behaviour has not been reported previously for neurons growing on TiO_2 surfaces. The observation that the response on TiO_2 was similar to that on glass indicates that directional cues are read by growth cones on these ceramic surfaces in the same way as on inert, insulating surfaces, such as glass or plastic. Additionally, cathodal EF-induced orientation was not affected by the rough surface topography or the chemical composition of TiO2. Collectively, the data show that TiO_2 is an attractive candidate material for electrical stimulation of cell growth *in vivo*.

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List of Figures:

Fig. 1 EF application and quantification. A) EF chamber set up and analysis for cells on transparent materials (tissue culture plastic or glass coverslips). The EF is shown by the arrow; the parallel channel is the no EF control. B) EF application on opaque TiO_2 disks. Cells were grown on TiO_2 materials, which were then inverted cell side down over the central channel prior to EF application. Analysis was from actin labelling of fixed cells. The neurite emerged from the cathode-facing side of the cell (blue) but the growth cone turned to face the anode (yellow). In all schematics the lids of the culture dishes are omitted for clarity. Scale bars are 50 µm.

Fig. 2. Chemical analysis of the prepared material. A) XPS scan of the TiO_2 substrates. B) XRD of sintered samples show a single phase TiO_2 rutile composition.

Fig. 3 Physical features of TiO_2 substrates. A) TiO_2 disk. Scale is 1 cm. B and C) Scanning electron (SEM) micrographs. D) AFM micrograph of a 2µm x 2µm area. E) Contact angle by the Sissel drop method of water on the TiO_2 surface. F and G) Higher magnification SEM showing grain surface terraces.

Fig. 4 Fluorescence micrographs of *Xenopus* neurons on glass (left column) and TiO_2 substrates (right column). Scale bar 50µm.

Fig 5. Neuron morphology on glass or TiO_2 surfaces with (black) or without (white) EF stimulation. A) Mean neurite length. B) Mean number of filopodia on each growth cone. C) Mean number of neurite branch points per cell. D) Mean density of filopodia along neurite shafts (number per µm). Number of neurons on glass no EF = 8; glass + EF = 18; TiO_2 no EF = 52; TiO_2 + EF = 48. Number of growth cones on glass no EF = 14; glass + EF = 23; TiO_2 no EF = 144; TiO_2 + EF = 99. Error bars are sem. P values are 2 tailed from a Student's t-test, assuming unequal variance.

Fig. 6 Direction of neurite growth in a 150 mV/mm EF for 3 hours. A) Percentages of neurites facing the cathode, anode or perpendicular before and after applying EF. B) Percentages of neurites that turned towards the cathode, anode or did not turn more than 15 deg. Neurites have turned to face the cathode and away from the anode. N = 84 growth cones.

Fig. 7 Composite drawings made by superimposing the neuronal cell bodies and then tracing the neurite paths for cultures on TiO_2 substrates. Drawn from fluorescence images of cells fixed after the 3 hour experiment. A) No EF control (top) and B) EF (150mV/mm) (bottom).

Figure 1

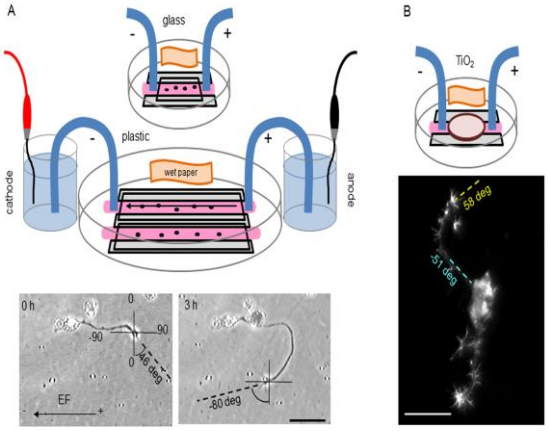


Figure 2

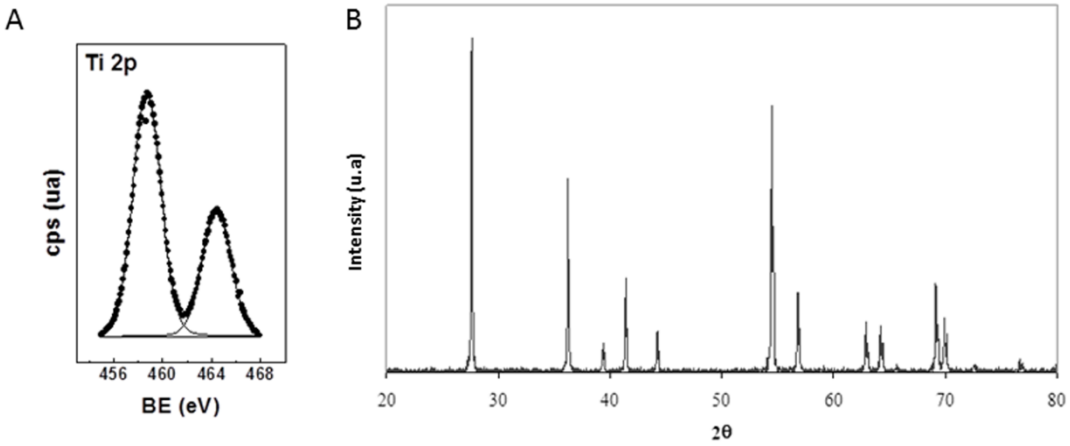
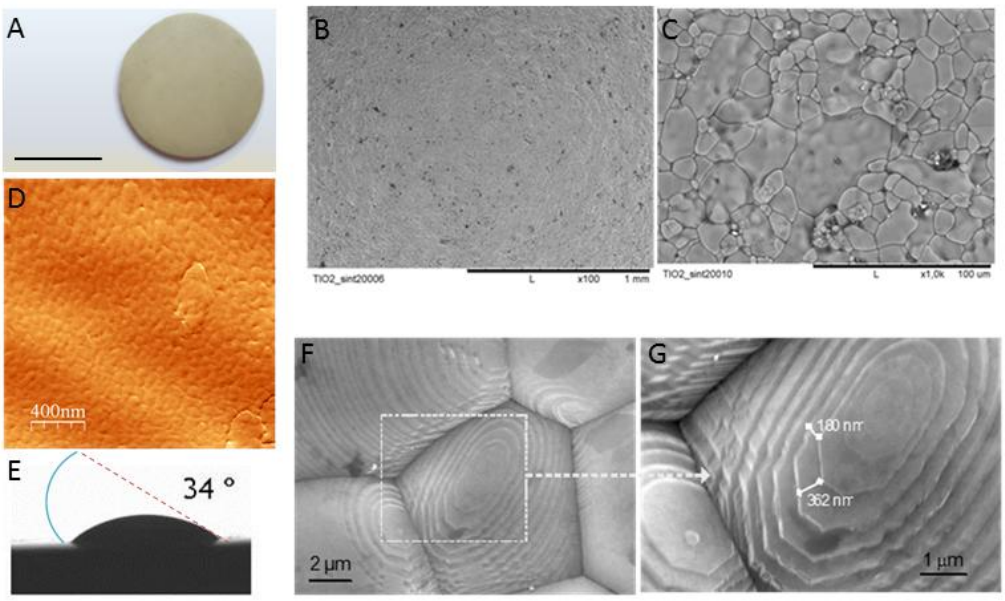


Figure 3



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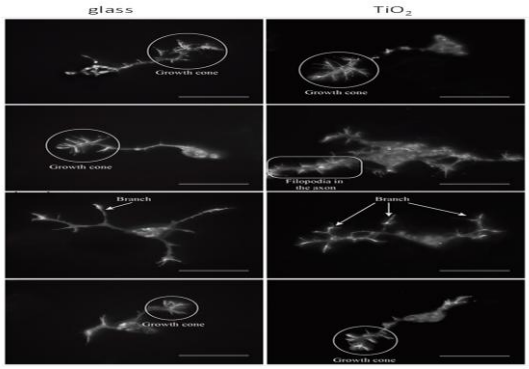
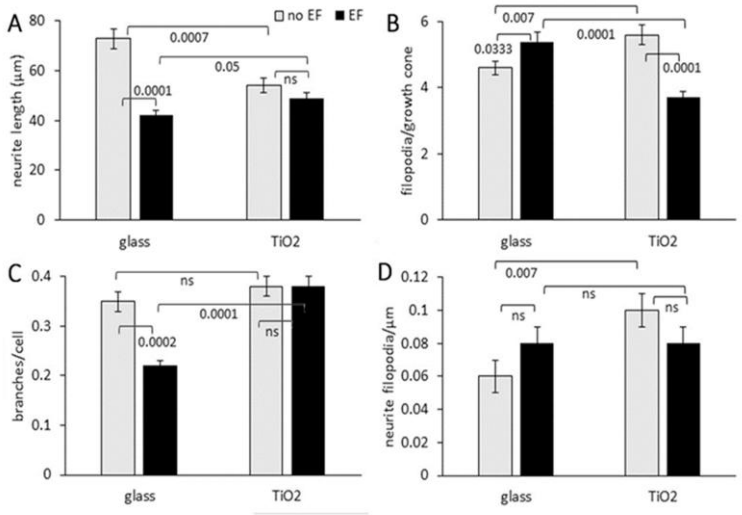


Figure 5



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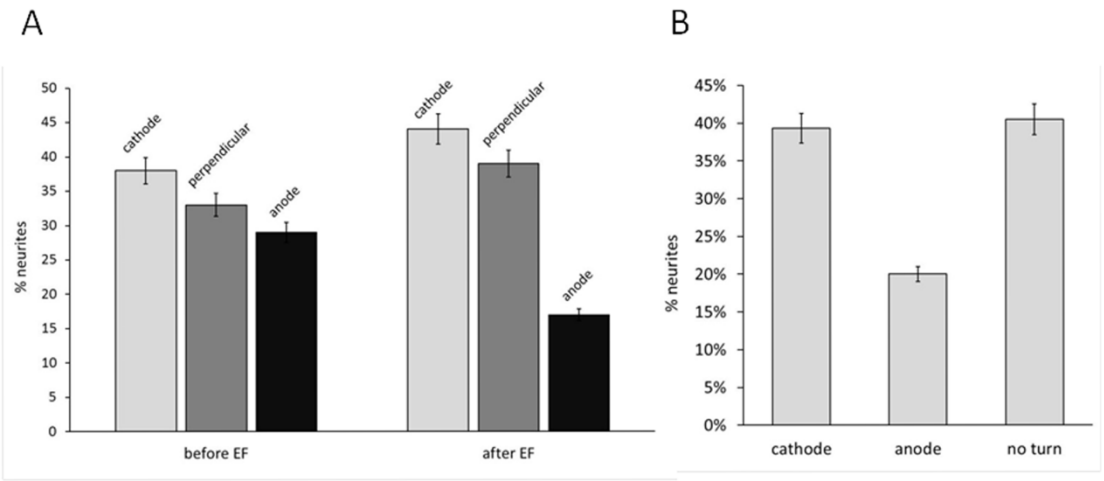

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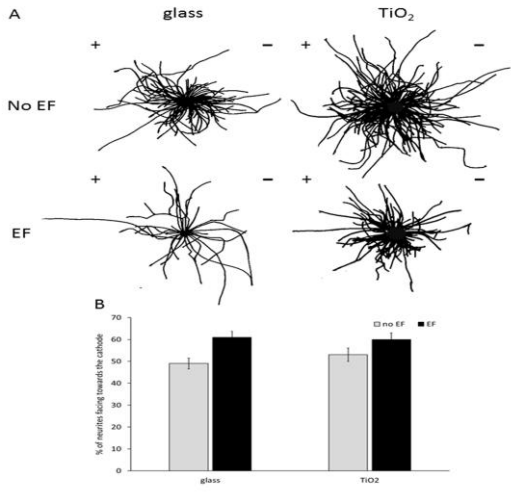

Highlights

- *Xenopus* neurons growth on the TiO_2 surfaces showed neurites and growth cones having more numerous filopodia than those growths on glass.
- Orientated neural cell growth towards the cathode was observed on both, glass and TiO_2 surfaces, with similar responses.
- TiO_2 is an attractive candidate material for electrical stimulation of cell growth *in vivo*.