Nanostructured Electroactive Materials with large Charge Capacity: Direct field Electrostimulation through connected and non-connected electrodes

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Abstract

Electric field stimulation protocols depend on the electrode material used, but the material's characteristics are often not considered or described sufficiently for optimization. Furthermore, charge capacity is considered only in capacitor-like systems, without considering that intercalation materials offer an internal faradaic charge delivery advantage, with substantially less risk for biological systems. This chapter describes new materials with high charge capacities, appropriate electric field protocols for using them, and examples of neural cultures that can be used to elucidate the biological effects of fields. Mammalian neurons, neuron-astrocyte co-cultures and amphibian spinal neurons are used *in vitro* or as scratch wound models to assess their potential for stimulating tissue repair. Importantly, remote control of dipoles induced in conducting implanted materials is shown to be a new promising approach, and a breakthrough.

Introduction

The electric characteristics of biological systems observed two centuries ago [1] inspired substantial discoveries in physics and are now the basis of modern technologies dependent on electrochemical interactions at the material biological interface. Such matter-biosystems allow sensing, electrical stimulation and design of interfaces for bionic actuation in a large variety of medical applications with increased complexity. In particular, electric eel actuation modes offer new insight. Although empirically observed during the dawn of animal electricity and electromagnetism theories, only recently have they been studied further. [2] Pulsed fields of the same polarity are emitted from stored energy electrocells in the eel's body. These paralyze prey from a distance but also create involuntary movements that betray its location and facilitate its detection. [2] The long range effects can only be carried through ionic gradients and correlated capacitor effects. The actual effect on involuntary muscle contractions correlates with a disturbance of action potentials and transmission of electric signals in the prey and suggests that pulsing allows creation of sustained ionic gradients. The same fundamental behavior is the basis of electrostimulation procedures now used in the medical world. Additional underlying effects are also being discovered. [3-5]

Although the study of interactions between biosystems and external fields or electrodes is a rather empirical world, electrostimulation protocols with the functional objectives of minimizing symptoms (as in Parkinson's disease tremor through deep brain stimulation, DBS) or inducing normal function (as in targeted muscle stimulation, for example bladder sphincter control) also act through body electrolytes,

creating ionic gradients that modify cell behavior and action potentials, thus stopping tremor or inducing unconscious movements. [5]

The process of interacting with living systems through applied fields has clear restrictions. Aqueous based electrolytes that are present in all cases elicit oxygen radical formation if charge is delivered through implanted electrodes. This is derived from water oxidation or dissolved O_2 reduction, so protocols are defined to minimize charge delivery and to achieve net zero charge in short time frames. Generally, little attention is paid however to the actual electrode material even when neurosurgeons inform of specific inflammatory and encapsulation problems.

Electrostimulation protocols for functional therapies in the nervous system rely on capacitive and relatively high impedance electrodes including platinum and its alloys, steel, or titanium nitride. They minimize net charge delivery by using alternating positive and negative pulses (AC fields) with large frequencies. [3] On the other hand, in living systems directional ion transport across tightly sealed epithelia generates electric fields within tissues with a constant or mostly sustained field direction (DC electric field). These endogenous DC fields influence cell behaviors, including control of neuron growth; therefore, the electric field application that aim to repair damaged tissues often mimic this by using DC fields to achieve a sustained ionic gradient. At present, the type of ionic gradient that favors neural growth is not clear but evidence suggests a significant role intracellularly for Ca^{+2} ions [6] during DC field effects on neural cell development. K^+ and Na^+ ions are also known to have growth effects. [7] Empirically however, electrical stimulation is gaining prominence as a therapy for conditions as diverse as non-healing wounds and traumatic injury. [8,9] Generally, these procedures use direct current stimulation, either pulsed or steady, through direct connection to implanted metal electrodes.

Therefore, if an external field is to be applied the DC field direction may be continuous or it may use repetitive pulses of the same polarity to prevent heating effects and capacitor draining effects in the set-up. Pulses of opposite polarity could also be used as long as a big portion of one polarity is sustained. In any case, application of an electric field involves capacitive charging at the electrodes (with very small charge capacity) and a faradaic electronic-ionic transfer between the electrode and the ionic electrolyte medium of variable charge depending on electrode materials and on potentials used. For both the zero net charge AC pulses and the DC continuous and pulsed fields a real charge delivery within the time frame of possible chemical reactions (micro to milliseconds) is occurring. Reported AC field protocols [3] calculated to be net zero charge in the long term still have charge transfer in short time scales.

Such electron-ion transfer at the electrode surfaces involves redox chemical processes and therefore radical formation related to O_2 reduction and H_2O oxidation, eventually leading to tissue damage and enhanced encapsulation and inflammation. Purely capacitive electrodes, like Pt, have as expected, very low charge capacity, basically the formation of a double layer on the surface of the electrode, yielding immediately faradaic reactions from the aqueous biological media at the interface. The possibility of conferring the electrode with added internal faradaic processes therefore is desirable, as is an increase in surface area of an electrode but without increasing its external geometric area.

New 3D structures currently being developed to increase the exposed surface, vascularization and to increase of charge capacity have demonstrated some success in some aspects when the materials are carbon based [10, 11], and also fiber electrodes based on carbon [12], or carbon coated materials with conducting polymers. In neither of those cases are charge capacities reported. (It is worth remarking that charges used for electrodeposition of polymers are not the final charge delivery capacities) [13,14].

Also, a clear alternative to purely capacitive electrodes (e.g. platinum or carbon) is the use of electrode materials that offer an extra charge capacity during electron-ion exchange through an internal redox intercalation process, therefore minimizing the electrochemical transfer to be delivered to the ionic cell media. Indeed, the material should have a large ion-intercalation capacity and permit a large degree of reversibility in the process to have a significant advantage as electrode material. Many of those cases also

allow electrodeposition on previously formed substrates, offering a wide variety of possibilities (like Iridium oxide being deposited on deep brain stimulation electrodes)

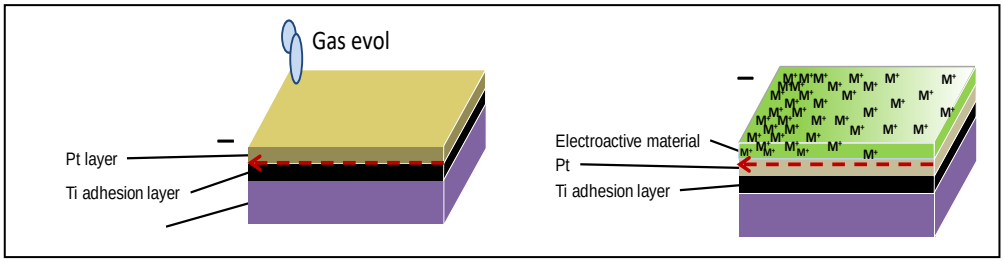


Figure 1. Scheme of a) Capacitive electrodes (H_2 or O_2 gas , and radical formation at the electrode) and b) Intercalation mixed valence electrodes where Na^+ (for example) intercalates (shown as diffusion within the structure). A coating configuration has been assumed but is not needed.

Electrodeposited iridium oxide, named IrOx here, is an amorphous open structure with oxohydroxide character derived from a hydrolysis process of iridium salts combined with anodic deposition. Its chemical mixed valence structure renders a mixed conducting material that allows oxidation state changes along with intercalation and deintercalation processes of H^+, OH^-, Na^+ and other ions, as proven recently [15]. Its charge capacity reaches 5 mC/cm^2 (vs 0.1 mC/cm^2 charge capacity for Pt) [16] when deposited through constant current anodic processes, and 20 to 30 mC/cm^2 when prepared through dynamic anodic potential sweeps.[15] Other materials are also being tested including conducting polymers based on PEDOT (poly(3,4-ethylene dioxythiophene)), with variable number of oxidation states, and nanocarbons like graphene and carbon nanotubes, all with unknown or similar charge capacities to those of IrOx [12].

Through the mixed valence states possible in those materials and the corresponding intercalation processes induced by the applied electric field, an alternative charge delivery process is possible at the interface between conducting material and electrolyte, cancelling most of the radical formation effects. An added advantage usually seen in those materials is a lower impedance and larger tissue tolerance, very possibly related to the same “shock absorber“ effect of the intercalation processes. Along with this alternative mechanism for charge transfer each material offers a peculiar chemistry and microstructure that may result in different prevailing intercalation processes and a variety of possible conductivity changes for the same applied fields.

Still, IrOx or conducting polymers like polypyrrole or polythiophenes with various counterions, fall short of the charge capacities desired for longer period stimulations, mostly because of strong limitations on coating thickness or because of substantial changes in conductivity during preparation. In our scientific approach we could foresee a large scope for significant improvement in conductivity, charge capacity and kinetics of ion exchange through the formation of hybrid materials by use of nanostructuring processes that offer larger conducting interfaces. In particular, carbons are known as additives in many electrochemical devices, and typically improve the conductivity of oxides by segregating on the surface of the particles and allowing percolation when forming composites. [17]

Thus, with the aim of expanding electrode properties to allow longer periods and larger stimulation rates of electric stimulation a variety of new materials and processes to prepare them is being explored. Composites with bioactive molecules or processes that modify the final structure are being tested. Here we will select some examples that have been successful under electric fields. When comparing different material types, a range of micro- and macro-scale surface topographies will be present. Although neurons have been reported to be sensitive to macroscopic topography of insulating materials, the observations described in this work for conducting materials have shown no effect in that respect, and this aspect will not be discussed.

In recent years, new microstructures in coatings of IrOx with improved adhesion [15] and doping [18] , conducting polymers containing biological molecules [19, 20], and new hybrid materials of IrOx–nanocarbons have been developed and tested in neural cell cultures from mammalian and amphibian (*Xenopus laevis*) embryos [21-23]. Their charge capacities are two orders of magnitude larger than Pt, and 10 times larger than those of the individual components and have allowed short term electrostimulation with positive neurite outgrowth, as well as permitting new protocols for electric field application. [24,25]

As mentioned above, electrical stimulation is gaining prominence as a therapy for conditions as diverse as non-healing wounds and nervous system disease or traumatic injury. Generally, these procedures use AC pulses, and few use direct current stimulation, either pulsed or steady, through direct connection to implanted metal electrodes. Such therapies are suggested initially by observations of isolated cells, cell cultures, or as seen below, by models that mimic a nervous system wound using regeneration of an *in vitro* scratch wound model with an external electric field applied. Although modelling a wound *in vitro* through a scratch induced physically was first used for glial proliferating cells, [26] the observations shown below suggest that the study of electric field effects based on scratch wound neuron cultures results a very powerful alternative as repair model [24], if compared with sole cell cultures on electrode materials.

Cultures of dissociated neurons from the embryonic spinal cord of *Xenopus* on insulating materials had been used to demonstrate a variety of reproducible cell responses during electric field stimulation, including axonal migration directed parallel to the field vector and faster migration toward the external cathode. Cells survived well during field exposure (150 mV/mm) because agar salt bridges physically separated the neurons from the electrodes and the potentially harmful electrode products.[25]

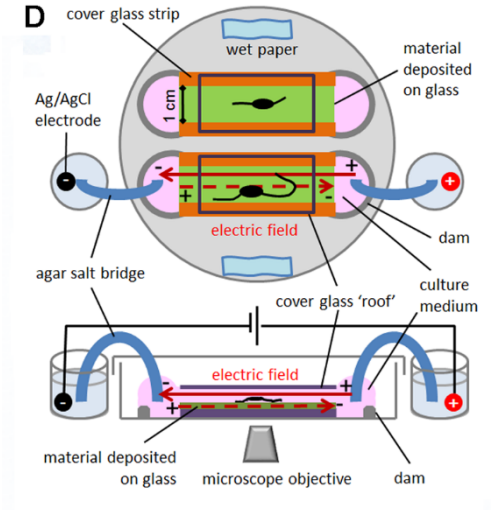


Figure 2. *Xenopus* neuronal studies under an electric field on both insulating and conducting substrates. Materials were not "wired" directly to the power supply. Arrows indicate the imposed external electric field

(solid red arrow) in the culture medium and the dipole (dotted red arrow) of opposite polarity induced within the conducting materials on which the neurons grew. (From reference 25, with permission]

Although the results of those fundamental studies are crucial to understanding the detail of how cells respond to electrical stimulation, for electrostimulation in biological systems use of salt bridges is not possible. In terms of neural repair, the evaluation of the effects of charge delivered relative to the maximum charge capacity of each material, has been performed using DC pulsed fields with constant polarization. The time scale of pulses has been in the order of minutes and relaxation times sufficient to dissipate heat effects, with total charges delivered above and below CSC (charge storage capacity) values. Specifically, DC fields with preprogrammed charge control protocols have been reported for various materials using cultures of rat and mouse cortical neural cells and cultures that modelled wounds [24], and have been shown to induce wound repair *in vitro* in very short periods (less than an hour) using standard connected electrodes, and without using salt bridges to separate immersed electrodes. Repair is particularly significant when wounds are created on the IrOx-graphene hybrid material substrates, even if the same charge is delivered as in other materials with lower capacities [24] To evaluate the possible differences in repair at anode and cathode, both half cells are separated by salt bridges. Surprisingly, as described below, cathode secondary reactions (O_2 reduction) seem to be more significant and decrease neurite outgrowth slightly.

Again, using Xenopus neurons, this time grown on conducting materials, an even more interesting observation has been made recently [25]. The induced dipoles at the immersed conductive material created by the application of an external driving voltage are sufficient to stimulate cells through distant external electrodes. But it is not only the magnitude of the dipole that induces neural growth. Cells behaved differently depending on the specific conducting material on which they were grown. Indeed, the induced field either stimulated faster neurite growth (on IrOx and (Ir-Ti)Ox) or biased the direction of growth toward the cathode of the external field (on PEDOT-PSS).

On the basis of the dipolar effects observed, direct dipolar interaction between conducting materials and neurons, is expected also in the absence of electric fields. Because cells create dynamic fields through action potentials and signal transmission, conducting materials immersed in the cell cultures or implanted could respond to them through dipoles that would also be correspondingly dynamic. Previous reported experiments on various materials may be explained now through that dipolar interpretation [27, 21-24], although dipolar interactions had not been offered as the mechanistic interpretation at the time. Furthermore, behavioral changes have been observed in *in vivo* experiments in rats with electrodes simply implanted, but not connected. [28]

Finite element studies have been made in an attempt to map the field gradients observed by neurons in the presence of external fields which would aid the specific design of field magnitudes and geometries of implants. [29]. Together this suggests that the existence of dipolar interactions between materials and cells may be of use for additional types of electrostimulation devices. Both, direct dipolar interactions with conducting materials and remote control of the induced dipoles on the materials may facilitate alternative designs in new clinical electrostimulation devices, and also offer new interpretations on the neural interfaces established.

Materials with Large Charge Capacity and Nanostructured Hybrid Materials

Oxides-oxohydroxides, coatings and electrochemical methods to achieve good quality coatings

Typical biocompatible oxides are based on TiO_2 and derivatives. [30] However, the lack of conductivity in those cases prevents their use as electrodes (highly reduced Ti oxides have electronic conductivity but are not biocompatible). Among conducting oxides, Iridium is the only case where cell cultures reflect biocompatibility. While the IrOx coatings and precursor solutions are still being studied, new electrodeposition processes have been found to achieve well adhered stable IrOx coatings on Platinum (or

Pt coated glass, carbons or steel) that are stable during cell culture studies. [15] Notably, when such coating is obtained, the charge capacity is also increased five times, even with a roughness, and exposed area, several orders of magnitude lower. Possible differences in iridium oxidation state may arise in our opinion by changing the deposition method, but no detection of such changes has been possible. Clear evidence on the mechanism involved in anodic deposition and redox response is well observed however in the quartz microbalance study coupled with cyclic voltammetry in Figure 3 with and without oxalate additives in a rich K+ media. Deposition from the precursor iridium solutions (Figure 4) is seen in the graph as a mass increase. As potential is increased, a mass loss occurs over the deposition mass, corresponding to deintercalation of K^+ ions and also mass loss corresponding to K^+ intercalation is observed when potential is returning to the original resting potential. The dynamic process yields a conducting hydrated IrOx coating of low impedance which in turn can still be oxidized and reduced further (eg. During electric field application in presence of cells), in which K^+ ions are easily exchanged by H^+ upon immersion in water or during sterilization [15]. Figure 5a and b show the profile (SEM) and macroscopic images of the coatings obtained on Pt coated glass. (Blue as prepared through anodic sweeps deposition, and green upon reduction in sodium phosphate buffer)

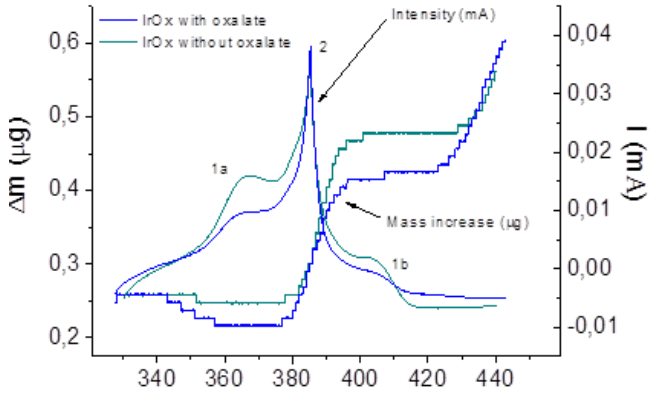


Figure 3. Cyclic voltammetry and quartz microbalance mass change during dynamic electrodeposition of IrOx from aged oxalate and non-oxalate pH 11 solutions, where the same mechanism is evident. Time scale corresponds to an increase in voltage followed by a decrease to the original resting potential .

The precursor iridium solutions that yields IrOx coatings are significant in the development of more complex hybrid materials described below. Despite the claims of some publications [31 and others], the solution does not contain iridium oxide nanoparticles. Although 2 nm nanoparticles are always observed at high intensity electron beams typically used, when lowering voltage and current, small portions of dried solutions evidence clusters of iridium oxo species (dark in Figure 4 a) that evolve within minutes to species with local hollandite type structures K_xIrO_n [32], and eventually to 2 nm metallic Ir particles, with no EDX presence of oxygen or potassium.

a, b

c, d

e, f

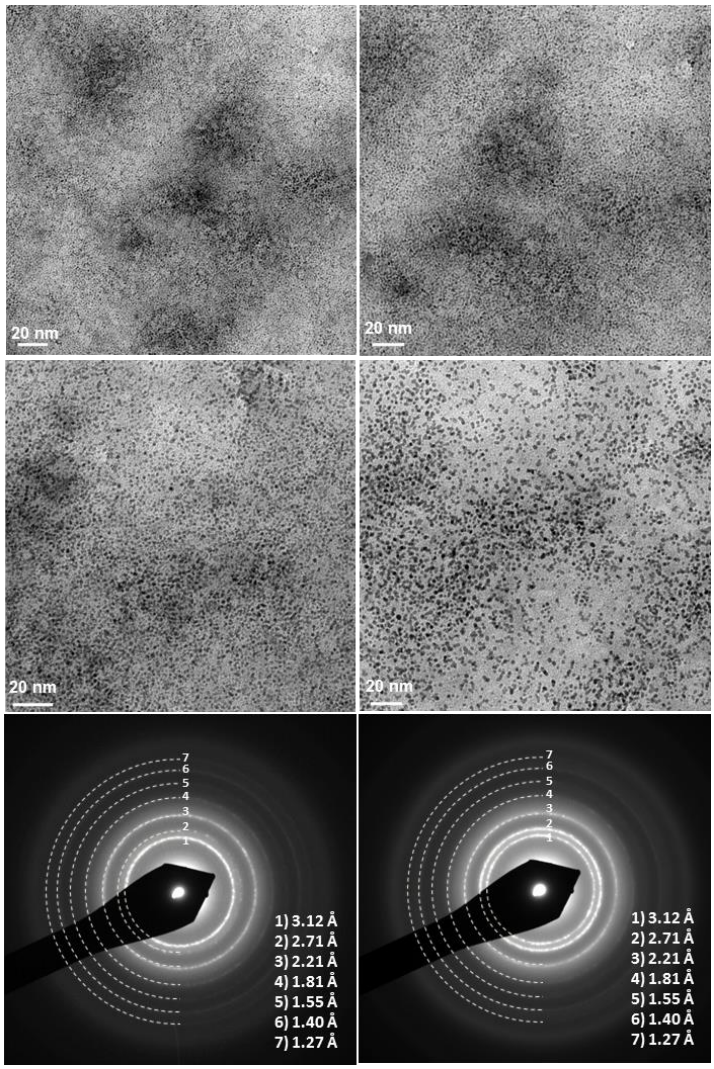


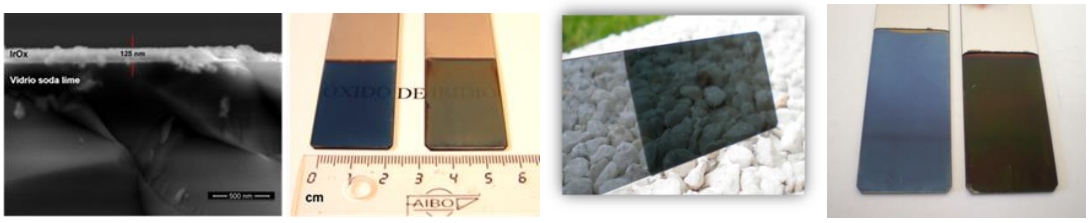
Figure 4. a-d) Sequential time evolution (5 min) in the same zone from oxoiridium species in solution to metallic iridium 2 nm nanoparticles, using a 80 KV electron beam in a 120KV Jeol TEM microscope. e-f), examples of diffraction circles (low resolution involves several particles observed and circles in diffraction) suggesting hollandite quasiamorphous structures, and Ir. Evolution of oxygen and potassium is clear (Acknowledgement J Oro, ICMAB). The final EDX signal (not shown) only contains Ir.

It is that iridium solution the one that would be used for hybrid, IrOx-nanocarbon material, and that has proven to be the driving solution for electrodeposition, as described below. IrOx coatings show the same response than iridium Oxo solutions under electron beams, suggesting that the oxo character is also preserved in the anodic coating, and the possible existence of polyoxometalates in the precursor solutions, (IrOn)x, with variable in oxidation states. In all cases, dynamic deposition yields well adhered and stable coatings during electrochemical cycling and cell culture processes, with an empirical formula $K_{1.7}(CO_3)_{0.2}IrO_{1.1}(OH)_{2.7}\cdot 0.4H_2O$, that after soaking in pure water or neural cells culture media prior to any assay exchanges K^+ for H^+ and yields $IrO_{0.3}(OH)_3.0.4H_2O$. The non-stoichiometry of the phase described here results essential, and if crystallized, IrO_2, without hydration or mixed valence states, does not offer the same electrode behaviour [15].

Conducting polymers with various counterions (X), polypyrrole-X, PEDOT-X bilayers and composites

Thanks to the use of the same dynamic electrodeposition scheme developed on IrOx, other coatings have been prepared with good adhesion and stability upon cell culture studies, something that had not been easy before [19]. In particular, conducting polymers polypyrrole and PEDOT obtained by oxidation of the monomers, pyrrole and EDOT, are the best candidates for bioelectrodes, if the appropriate counterions are

chosen. Given that PEDOT-PSS is rather erratic as substrate of mammalian neuron cell cultures [19], and depending on the coatings used on active materials to induce cell adhesion [19], the authors have approached electrodeposition using alternative counterions, in the understanding that a surfactant like PSS, the commercial and most used one, could generate heterogeneities in the surface. In particular, using amino acids in aqueous solutions with pH above their isoelectric point, highly compatible coatings of conducting polymer coatings have been achieved with the amino acid being a counterion. In that way, electrodeposition is more difficult to achieve but the final polymer is more biocompatible and supports neural cell growth better than other reported polymers. Furthermore, stabilization of polypyrrole versus atmospheric oxidation is achieved by forming bilayers with a common counterion (Polypyrrolelysine deposited on PEDOT-lysine) with better response in mammalian neuron growth than any polymer reported [19]. Although having optimal compatibility, charge capacities for all conducting polymers tested remain similar or inferior to that of dynamically deposited IrOx [15,19].



a) b) c) d)

Figure 5. a) SEM profile images of IrOx coatings using dynamic potential sweeps, b) Macroscopic color changes observed upon reduction for IrOx coatings on Pt coated glass (involving simultaneous Na+ intercalation [19]), c) PEDOT-PSS coating con Pt coated glass, d) PEDOT on polypyrrrole.lysine bilayers on Pt coated glass

The role of Carbons and Nanocarbons in formation of Hybrid Materials

With the aim of achieving materials with larger charge capacities, structural stability and biocompatibility, a number of cases taking IrOx as a basis, have resulted in successful results. Using the same dynamic electrodeposition processes and variable limiting potentials, precursors of iridium oxosolutions, have been mixed with pyrrole, EDOT, or graphite, carbon nanotubes, and various graphene varieties, in combination of two or three elements.

Even though both conducting polymers and IrOx are obtained by anodic depositions, when mixed, electrodeposition does not work. A direct chemical reaction of the monomers and iridium yields a hybrid IrOx-polypyrrole or IrOx-PEDOT hybrid in powder form, where the polymer encapsulates the oxide, evidencing that the polymers forms around the nucleating oxidizing species (IrOx), and such particles prevent electrodeposition [33]. In turn, this evidences that iridium oxide electrodeposition is the key process to develop coatings.

Using a carbon component in an IrOx based material, is an attractive twist given the known behavior of carbon in supercapacitors and batteries and the improvement of the conducting properties of known oxides when admixed with small amount of carbon [17]. In general, it is observed that carbon segregates at the oxide surfaces, offering a percolation road possible for electron conduction. Such resulting microstructure results in better conductivity in the final composite material. By using nanocarbons instead, we expected to find the possibility of finding nanostructured new hybrid materials that may offer significant improvement in properties.

Thus, biocompatible hybrid materials based on IrOx and carbons have been obtained with graphite, graphene oxide, exfoliated graphene and carbon nanotubes (CNT). In all cases, evidence exists [21-23] (see Figure 6) that this time iridium species adhere to carbon in the precursor solution (a and b shown for pristine graphene exfoliated electrochemically from graphite), as opposed to the case of polymers.[33] Figure 6 shows SEM images indicating unambiguously the nanostructures achieved through the interaction and electrodeposition process driven by IrOx (nanocarbons do not deposit in the absence of IrOx). IrOx is sustained by CNT in one of the hybrids in a nanostructure that resembles reinforced concrete. In the case of pristine graphene, graphene oxide, or N-doped graphenes, a millfeuille nanostructured is formed. (Hybrids of IrOx with nitrogen-doped graphene oxide do not allow neurons growth and will not be treated here). [34]

a), b)

c),d)

e),f)

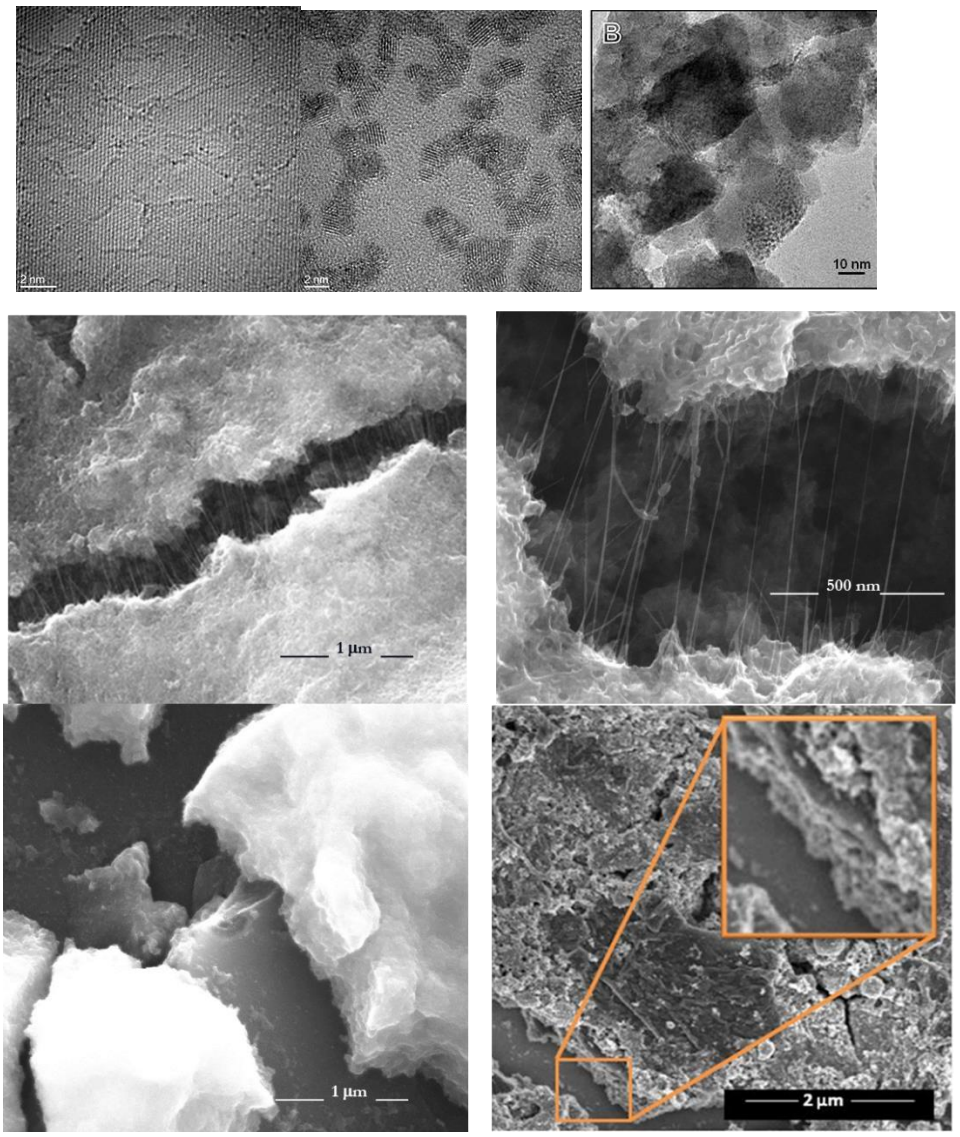


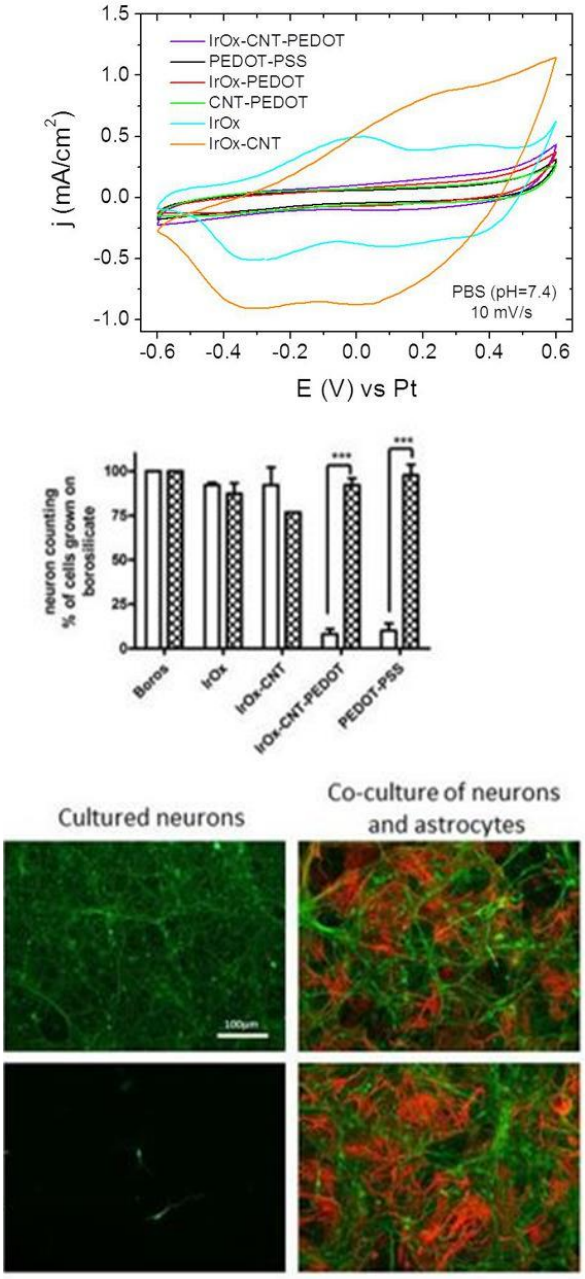
Figure 6. a) High resolution TEM images of pristine graphene obtained by electrochemical exfoliation from pure graphite and of iridium oxo species attached to it b) TEM images showing the adhesion of iridium precursors to the graphene surface. c) SEM images (all 3 microns scale) of nanostructured electrodeposited IrOx-Carbon nanotubes hybrids, and IrOx-CNT-PEDOT hybrids, showing the nanostructure where CNT sustains IrOx as in reinforced concrete, and how PEDOT encapsulates both IrOx and CNT. d) IrOx-pristine Graphene and IrOx- Graphene oxide Hybrids showing the millfeuille nanostructure. Cracks develop only under the vacuum at the SEM microscope , allowing the view of the inner structure

In all cases Cyclic voltammetry for all IrOx-nanocarbons hybrids (see Figure 7a) shows the expected iridium redox processes in all cases but the trihybrid, evidencing that the material has that charge capacity available during stimulation. However, if PEDOT is incorporated, the trihybrid case IrOx-CNT-PEDOT,

only PEDOT processes are available electrochemically suggesting encapsulation of all components by the polymers. Accordingly, IrOx-nanocarbons hybrids, except the trihybrid IrOx-CNT-PEDOT, have charge capacities increased ten times with respect to the dynamic IrOx electrodeposit, reaching 120 mC/cm^2 and the cycling stability after 1000 cycles remains about 70%, implying a 10-fold increase in stimulation time possibilities. IrOx-CNT-PEDOT however has the charge capacity previously observed for PEDOT-PSS again confirming encapsulation. It is significant however that if calculated per carbon atom, the capacity of the IrOx-pristine graphene hybrid doubles that of the one obtained with Graphene oxide, despite the larger roughness and surface exposed in the last case, showing that the effect of conducting pristine graphene is larger. On the other hand, the same enlargement of charge capacities is reached in IrOx-graphite hybrids, but after a few electrochemical cycles, the capacity fades to 10% of the original, through disintegration of the hybrid. Therefore, the formation of nanostructured hybrids offers new possibilities in possible charge delivery only for the combination IrOx-nanocarbons.

As described below, several models of cell growth evaluation have been chosen, but is significant to mention here that the different nanostructures described reflect also in cell cultures. In all IrOx-Nanocarbon hybrids, with the exception of the nitrogen doped graphene, neurons grow well. Again PEDOT cases signify a different behavior. Neurons in cell culture with IrOx-CNT-PEDOT don't grow well, similar to that found for PEDOT-PSS (Figure 7b,c) suggesting that these polymers may not sustain neural growth, and again that PEDOT is encapsulating highly compatible IrOx and nanocarbons. However, if coculture of astrocytes and neurons is performed, both cell types grow and differentiate properly. Furthermore, neural cells grown on top of these materials were more resistant against anti-inflammatory insult. [35] *Xenopus* cells on the other hand find an optimal substrate in PEDOT-PSS based materials with no surface adhesion phases, as shown below, but also on IrOx based materials, with different behaviors in presence of electric fields.

The response of cell cultures represents therefore a significant way of characterizing new materials once a certain cell type is chosen. Thus, PEDOT encapsulation of other components in the trihybrid material is proven by the similarities in voltammetry, capacities and also neurons response to individual PEDOT behavior. Such consideration may be useful in the design of future materials.



a)

b)

c)

IrOx

IrOx -CNT- PEDOT

Figure 7. (a) Comparison of Cyclic Voltammetries and derived charge capacities for all hybrids, and b) and c) neural cell responses. (From ref. 35 with permission from Elsevier)

Neural cell cultures used as models for biocompatibility studies.

Once materials with the desired characteristics have been prepared it is necessary to show that they are not harmful to cells. If they are destined for use in the nervous system, the initial biocompatibility tests typically involve growing cells on the material's surface. These can be clonal cell lines that mimic neural cells (e.g. PC12 cells or neural precursor cells) or primary cultures derived directly from animal tissues (e.g. dissociated sensory neurons from dorsal root ganglia or CNS neurons from the brain or spinal cord). Although mammalian culture systems are highly desirable for modelling the interactions of the neuron-material interface, such *in vitro* models have a significant limitation. Mammalian neurons (especially CNS) require an adhesion layer to promote cell attachment to the substratum; without which mammalian neurons generally do not adhere or extend axons. Adhesion layers, such as polylysine, which extends 1 nm from the

material surface or collagen, which extends 100 nm or more can also influence the outcome. Mammalian neurons growing 100 nm away from the material surface, on collagen, do not 'see' the material chemistry. It is highly probable that this explains variable reports in the literature observed for cells grown on PEDOT-PSS coated in different ways. An attractive alternative *in vitro* model uses embryonic amphibian spinal neurons from *Xenopus laevis,* which do not require an adhesion layer for cell attachment or to initiate robust neurite arbors. Additionally, the neurons grow at room temperature, do not require a CO_2 incubator, axons extend much faster than mammalian neurons, and their cell bodies and growth cones are about twice as large as those of mammalian neurons. Individual cells are also observed easily. Collectively, these conditions make them ideal for time lapse observations of neuron growth and behavior. This is facilitated by using materials prepared as transparent films. Indeed, using time lapse imaging we have demonstrated that PEDOT-PSS and IrOx are favorable substrates for *Xenopus* axon outgrowth, which matched or exceeded that on uncoated glass [25], and have positive outcomes from the above described hybrid materials.

Although amphibian neurons are extremely valuable for testing a novel material's compatibility it is clearly also important to test mammalian cell responses. This is often done using cultures of neurons from the embryonic rodent brain cortex, especially in the context of using the electrodes for nervous system repair but co-cultures of mammalian astrocytes and cortical neurons provide additional value. In all cases, however, atmospheric CO_2 concentration and temperature must be controlled using an incubator and for neurons, the substrate must be coated with an adhesion layer prior to cell plating.

Most of the materials we have described have been tested using the three models. As mentioned, neurons have been found to differentiate among materials more than other type of cells (astrocytes, cocultures astrocytes-neurons, or *Xenopus* spinal cells), although the reason is still unknown.

When the main focus of the study is the use of those materials as electrodes for electrostimulation, direct seeding and growth observation is far from enough. We want to remark here two models that have recently shown new evidence that may modify the study of the electrode materials, its effects on living systems, and the design of new electric field protocols and devices.

In vitro **wound models for studying tissue repair**

A simple theoretical approach when studies are focused on repair, is to induce a wound-like scratch physically on a cell culture and observe the replenishment of the void space over time. A scratch is physically done with controlled thickness in a monolayer of mammalian neurons previously grown on a variety of material surfaces. The rate of wound closure in presence of electric fields is then monitored to model the rate of recovery of the nervous tissue injury and as compared with spontaneous repair.

Figure 8 shows the spontaneous neurite growth into the wounded area at different times after the scratch is made. A clear scar is observed and a maximum neurite growth is found between day 1 and day 3, allowing complete spontaneous healing within about 7 days. By exploiting selective immunohistochemistry techniques that label distinct cell populations it is possible to look at complex behaviors in such cultures. [24]

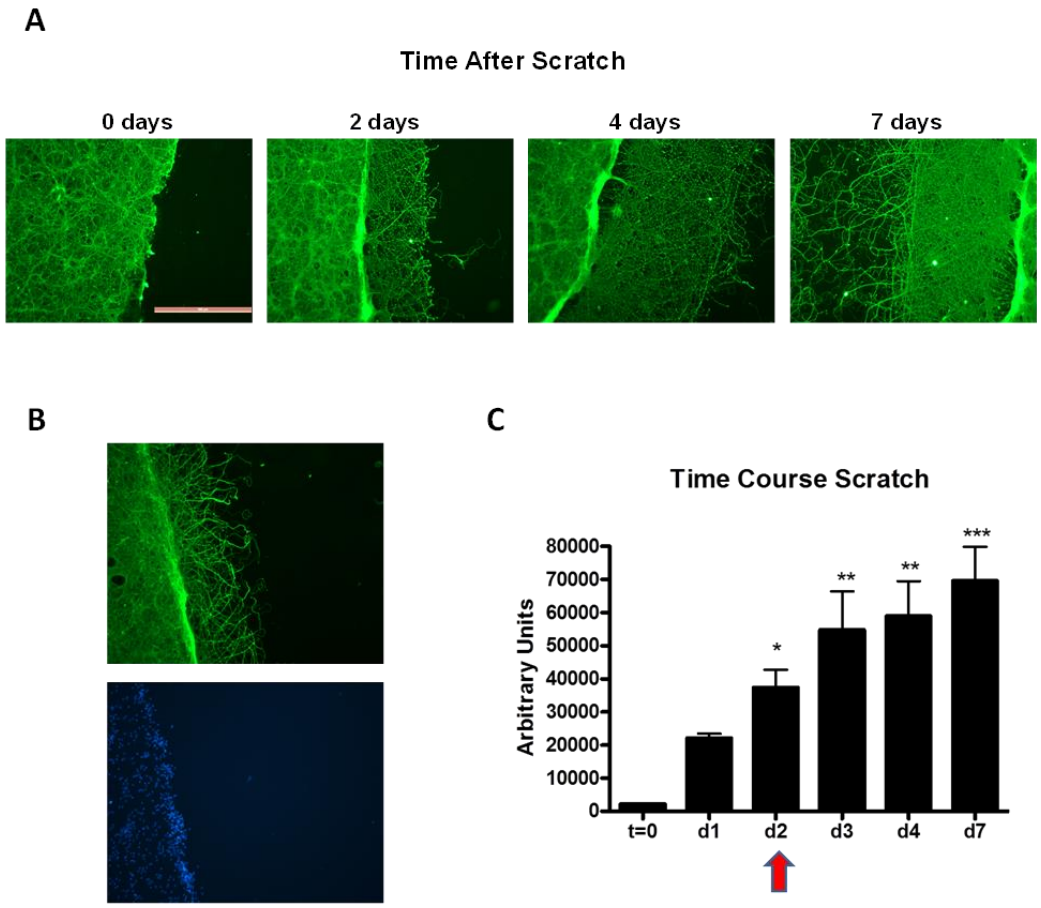


Figure 8. a) Neural cell recovery after scratch wounding of monolayer cultures grown on PEDOT-Polypyrrole-Lysine bilayers (Tau immunostaining), b) Neurite and nuclei images for comparison (benzimide staining for nuclei) and c) Neurite counts using image analyses after different number of days. (Images from ref 24 under permission from Elsevier)

Using this spontaneous repair as reference, the changes in wound recovery upon electrostimulation using a variety of electrode materials shown below, represent the closest model to an in vivo study, and help to determine the specific electric field protocols for which repair is achieved, as a function of material properties.

Establishing safe limits for applying DC or low frequency AC electric fields, for wound repair, depending on materials properties.

When evaluating possible repair, specific responses of neurons to electric fields reported before are significant. A variety of responses have been described for neurons growing on insulating substrates (glass or plastic) and exposed to steady (DC) or oscillating (AC) electric fields *in vitro*. Responses include increased migration rates of neural precursor cells, directed migration (usually to the cathode of a DC field), directional neurite outgrowth (biased toward the cathode the anode or orthogonal to the field vector, depending on cell type) and increased branching [36]. *In vitro* scratch wound cultures akin to those described in Figure 8 have been used to model epithelial wound healing on insulating materials. Indirect electrical stimulation of such cultures has shown that an electric field in the culture medium influences the rate of wound closure for cultured cells including fibroblasts, epithelial cells or keratinocytes [eg.37]. Collectively, these observations suggest that application of an electric field is therefore likely to influence the rate of wound closure in the neuron culture scratch wound model, probably through directional neurite outgrowth.

Typically, those studies cited use conditions in which there are limitations to the electrode materials used. Their charge capacity and the effects of surpassing those values to deliver the charge are detrimental

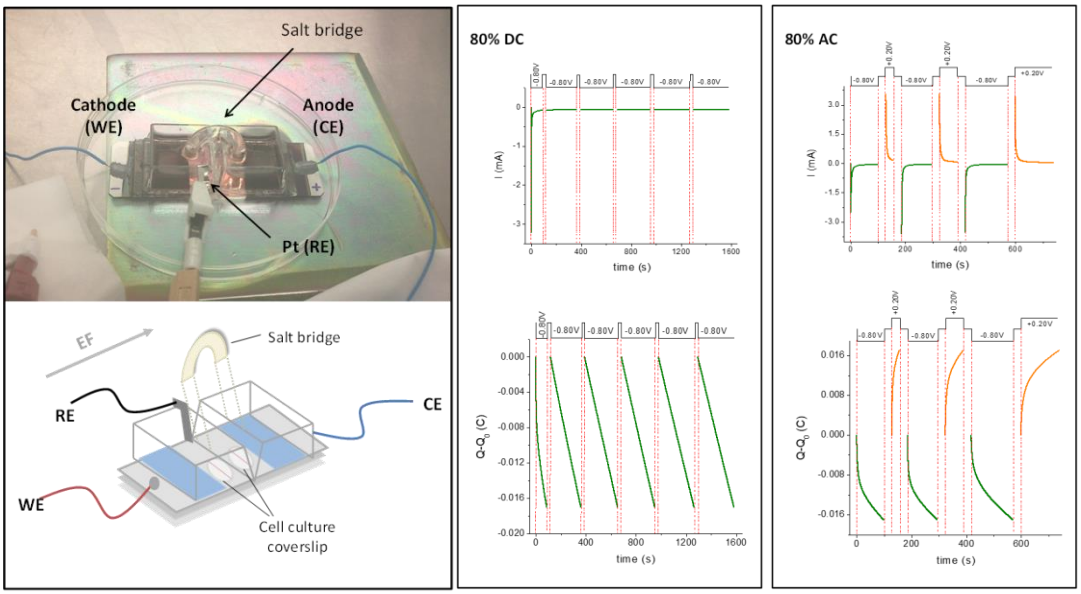
to cells if the electrodes are positioned directly in the culture medium. Therefore, such studies are made by separating cells from electrodes through salt bridges. It is not unusual to find reports where no separation is made, and perhaps is not possible, especially for *in vivo* studies, surpassing the values of charge capacities.

Charge storage capacity studies with various materials shown below define CSC as a decisive point when establishing a DC field. effects. Other parameters, such as heating and geometry are highly related to the material chosen, and therefore the electric field protocol must be defined as a function of those properties. This assessment is clearly seen below.

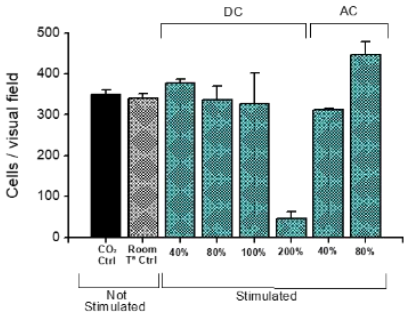
Figure 9a shows an example of pulsed potential electric field protocols with simultaneous current integration, and therefore charge control, that allow the application of constant polarity fields with relaxation times in the order of second to minutes that prevent heating effects.[24] Also included is a protocol of applied potential with alternating pulses (anodic pulses smaller than cathodic pulses) and final charge control, similar to described previously [3] but in larger time scales and larger net delivered charge to induce repair.

Figure 9b and c shows a comparison of cell viability for values of charge delivery below or above the previously measured charge capacity for a particular electrode (bilayer PEDOT-polypyrrole lysine in this case). Independently of the DC or AC mode, cell growth near the electrode drops dramatically once the charge capacity is surpassed. Not only the specific material charge capacity is significant, but also the global capacity from the full electrochemical cell. Such cell may be built symmetrically, with the same material in both sides in the same oxidation states, or modifying the anode to achieve a previously reduced state to magnify the global electrochemically cell capacity. This possibility, which is not present in capacitive materials shows here the full potential since the global capacity may be double even for the same geometry [24].

a)



b)



c)

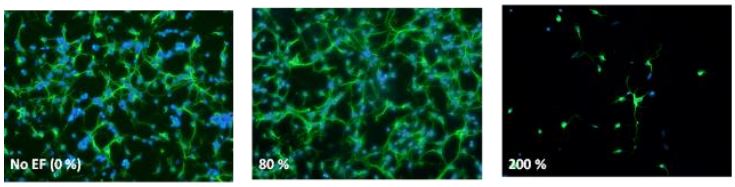
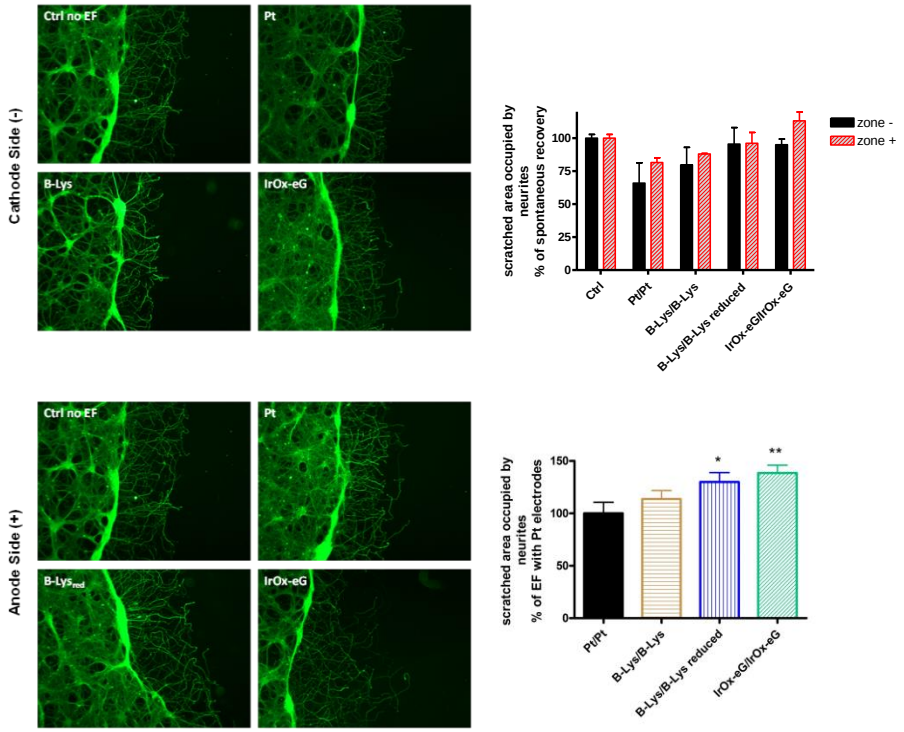


Figure 9. a) Electrochemical cell and EF protocol applied in scratch wound cell cultures and current responses. b) Cell counts after electric field being applied in a cell culture with a symmetrical electrochemical cell and Q delivered below and above electrode CSC limits, 0, 80 and 200% values of CSC of bilayer PEDOT-Polypyrrole-lysine, c) Images of neurons cell cultures after charge delivery below and above charge capacities. [Images from [24] under permission from Elsevier]



a) b)

Figure 10. a) Neurite regrowth during 7 days after scratch on cultures performed on various materials and 5 days after approx. 40 min stimulation with charge 80% of the lowest CSC intercalation material, (PEDOT-polypyrrole lysine bilayer), at anode and cathode sides. b) Quantification of area covered by

neurites and relative comparison with platinum electrode, for various electroactive materials (bilayered polymers and IrOx-pristine graphene hybrid coatings).

When applied to the scratch model, neurite extension into the wound offers additional results. When comparing various electrode materials (see Figure 10), and using the same charge delivered (that of the material with lower capacity) significant results are obtained. The material used as electrode is important, as well as the symmetry of the mixed valence electrodes. With anode and cathode compartments separated by a single salt bridge, differences in each side are also observed.

The result of neurite extension on the scratch wound result clear. For the same charge delivered using various materials, in experiments where up to 80% of the maximum charge capacity is delivered, the materials with largest charge capacity show the best repair. Of course, that charge would only be a small portion of their maximum charge in those cases.

Very significant also is that recovery of the scratch space occurs having used very short electrostimulation time periods. For less than 40 minutes stimulation, cell cultures show that EF induces neurite growth above the spontaneous healing case on the same material (Figure 10b). It is worth comparing also with Pt electrodes because they are so usual in clinical electrostimulation. In that case charge delivered is necessarily above the limits of the capacity of the electrodes, and the scratch model shows that for platinum electrodes growth is inferior to the spontaneous case. Thus, electric fields in net charge mode would not help repair if Pt electrodes are positioned near the lesion, but could with other electrodes.

The largest repair is found for the hybrid IrOx-pristine graphene electrodes, even if the same time and applied charge is used than for others, within the limits of capacity for all. Very significant is also that the behavior is slightly better at the anode, suggesting that O_2 reduction at the cathode may be the main cause of radical formation in the biological media and the origin of most inflammatory responses. For that particular IrOx-graphene material, charge delivery could still be expanded more than five times with respect to bilayer PEDOT- polypyrrole electrodes, before reaching its maximum capacity, working at 100 times more charge than the value of Pt. Thus electrostimulation times could easily be enhanced before finding detrimental effects.

Recently, electrostimulation during months, using AC fields and rehabilitation, yielded actual recovery in spine injury cases, [38,39] with no electrodes or electric field protocols being disclosed. Their results may also benefit from these studies on possible electrode materials.

A promising alternative to direct electric field stimulation: Induced dipolar electrostimulation

It is assumed, and used mostly, that electrodes are made of conducting materials always connected to an external power source. However, unconnected conducting materials immersed in an ionic media will show a dipole between their edges, if a remote external field is applied with external driving electrodes. If the potential is sufficiently large, the dipole may induce electrochemical reactions at any of the poles, negative or positive. This is known in literature as bipolar electrochemistry, [40] and has been rediscovered recently for material preparation, but has never been studied in terms of materials-cells interactions until now.

Several electrode materials described above, have been useful to test the influence of induced fields on the possible growth of neurites, in terms of speed and direction, using them as connected substrates. The *Xenopus* neuron culture system was exploited by us to ensure that cells were in direct contact with the material itself (no intervening adhesion layer). A number of materials, insulators like TiO_2, or pure electronic conducting materials (Au and Pt), or mixed conducting materials (IrOx, PEDOT-PSS or polypyrrole), where compared in absence and presence of the electric fields created by remote external electrodes. Without any electrical stimulation, neurons extended neurites on gold, platinum, PEDOT-polystyrene sulfonate (PEDOT-PSS), IrOx, and the mixed oxide (Ir-Ti)Ox. We showed previously that nano- and macro-scale topographies influence the rate and direction of neurite outgrowth of *Xenopus* neurons on fused quartz substrates [41] so we expected optimal growth to be related to surface texture. However, there was no direct correlation in ranges from nanometers to microns; IrOx and PEDOT-PSS, which are each rougher than glass improved neurite growth rates compared to glass. On the other hand, neurites also grew faster on gold, which is smoother than glass. Additionally, the smoothest (gold) and roughest (PEDOT-PSS) surfaces each encouraged reliable neurite sprouting. Therefore, the results shown below do not correlate with roughness.

After identifying the most suitable materials that supported nerve growth we used time lapse observation to test the ability of those materials to sustain growth safely during 3 h of constant electric field stimulation. Although external fields induce always a dipole between the poles of the immersed conducting material, for magnitudes 100 to 150 mV/mm they induced a large enough voltage between the poles, to generate H_2 (and OH^-) and O_2 (and H^+) at the induced cathode and anode poles. This was visually evident by the gas bubble production and a change in color of the pH indicator in the culture medium at the extremes of the material, (Figure 11). Furthermore, delamination of the gold and platinum coating occurred because of oxidation of the adhesion layer of titanium, which progressed as a wave along the material over time. Indeed, those electric field values were demonstrating the effect of a bipolar effect in the immersed conducting material and suggested that cells will be able to be sensitive to it. Although 100 mV/mm and 150 mV/mm electric fields are safe when used on insulating substrates, only 50 mV/mm was used for growth experiments on conducting materials to prevent undesired effects on the material and the electrolyte surrounding it.

The presence of the imposed external DC electric field in the culture medium induces an electric field indirectly in the conductive substrate material that has a polarity opposite to that in the culture medium (Fig 11). In principle, a different behavior is expected between purely conducting Au or Pt and intercalation materials like IrOx and PEDOT-PSS, because of the expected intercalation/deintercalation of M^+ ions (Na^+, H^+, K^+ etc), and anions like OH^- at the anode. We have used finite element analysis to map the charge gradients that are likely to occur [29] and ionic gradient evaluation is being carried now.

Importantly, at lower external driving fields (50 mV/mm) the material maintained its integrity, no pH changes or gas bubbles were observed and the neurons survived and continued to grow. The results indicate that specific cell growth responses were induced depending on the conductive substrate material in which the field is induced. For example, on PEDOT-PSS neurite growth was directed preferentially toward the external imposed cathode. However, at the same external field condition (50 mV/mm) neurite growth on IrOx or (Ir-Ti)Ox was not directional but the neurites extended faster than on PEDOT-PSS. This suggests that electrostimulation is possible through implanted unconnected electrodes, with defined behaviors for each material type.

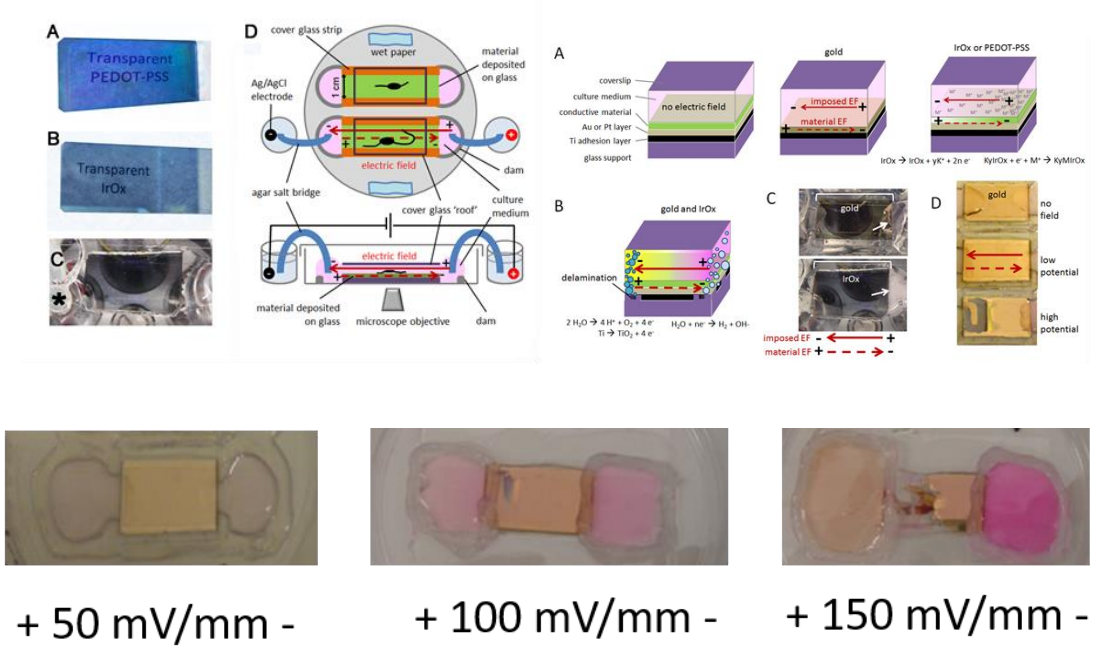


Figure 11. a,b,c) Transparent material coatings used in the study. d) External EF application to material coating immersed in an electrolyte (culture medium) and Setup for inverted microscope observation, and a,b,c,d right: scheme of reactions expected at low and high field. Last row, pH changes observed under external applied fields. pH increase changes the medium colour to pink (H_2 + OH^- formed).

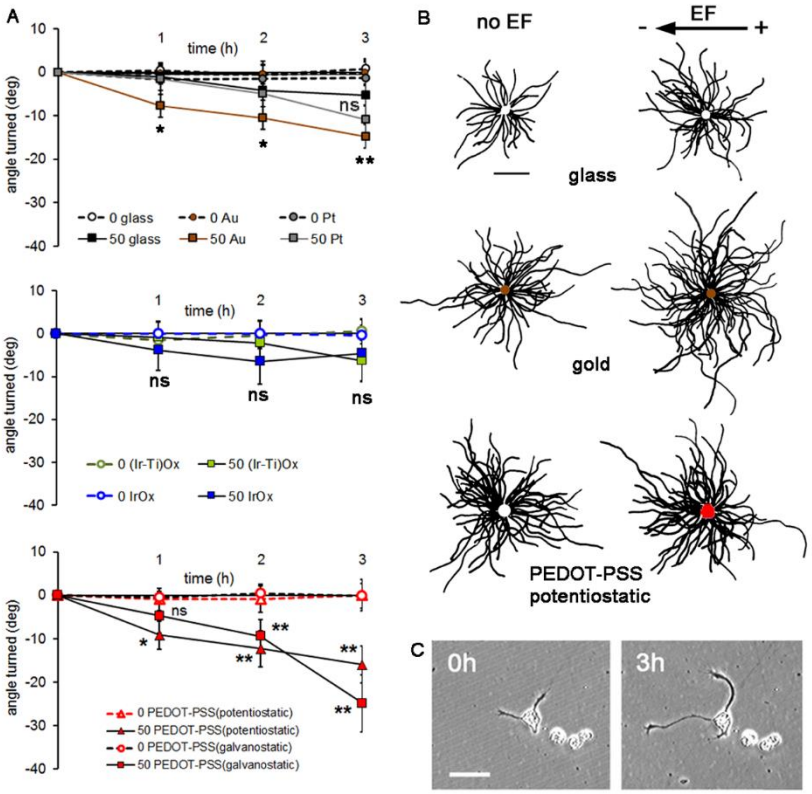


Figure 12. Neuron growth during 50 mV mm^{-1} electric field (EF) stimulation. A) The angle of growth cone migration. Negative values indicate migration toward the cathode; zero indicates randomly directed migration.

Not only do these experiments suggest remote electrostimulation is possible but they also offer a wide perspective of what the cells-material interactions may be. Since neurons develop dynamic action potentials of high local intensity, a conducting material implanted near them could respond in the form of dipoles or multipoles, that would in turn also be dynamic and these fields may influence cell growth and behavior. The two-way road through which interactions can occur opens a vast field of study. Even previous studies with the materials described or others based on nanocarbons etc, may be viewed in a different approach thanks to the observations of bipolar electrodes.

The authors have been aware of recent work [28] where more active behavior of rats was observed upon the implantation of an electrode for Deep Brain Stimulation, prior to the electrode being connected. It is our opinion that such behavior may develop from the direct interaction through neurons and the dipoles induced in the material, and that being dynamic could create a larger overlapping brain wave. If that was true, it would open the possibility of indirect stimulation in the brain through unconnected implants.

Another area ripe for exploration is targeted synthesis of active materials *in situ,* that could act later as a bipolar electrode. A recent report proved that polymerization of conductive polyaniline (PANI) and PEDOT could be accomplished by using aqueous components that polymerize at physiological pH and that genetically target polymer synthesis to neuronal cell membranes [42] The potential to design a therapy that delivers electrical stimulation indirectly to implanted materials that are customized to generate desired electrochemical reactions, or for an implanted conductive material to exploit the natural DC field present at every injury site (no external power source) is an exciting area for future exploration.

Acknowledgements

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