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Full Length Article

Biosafety assessment of conducting nanostructured materials by using co-cultures of neurons and astrocytes

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ABSTRACT

Neural electrode implants are made mostly of noble materials. We have synthesized a nanostructured material combining the good electrochemical properties of iridium oxide (IrOx) and carbon-nanotubes (CNT) and the properties of poly(3,4-ethylenedioxythiophene) (PEDOT). IrOx-CNT-PEDOT charge storage capacity was lower than that of IrOx and IrOx-CNT, but higher than that of other PEDOT-containing hybrids and Pt. Cyclic voltammetry, SEM, XPS and micro-Raman spectroscopy suggest that PEDOT encapsulates IrOx and CNT. In our search for a cell culture platform that could optimize modelling the *in vivo* environment, we determined cell viability, neuron and astrocyte functionality and the response of astrocytes to an inflammatory insult by using primary cultures of neurons, of astrocytes and co-cultures of both. The materials tested (based on IrOx, CNT and PEDOT, as well as Pt as a reference) allowed adhesion and proliferation of astrocytes and full compatibility for neurons grown in co-cultures. Functionality assays show that uptake of glutamate in neuron-astrocyte co-culture was significantly higher than the sum of the uptake in astrocytes and neurons. In co-cultures on IrOx, IrOx-CNT and IrOx-CNT-PEDOT, glutamate was released by a depolarizing stimulus and induced a significant increase in intracellular calcium, supporting the expression of functional NMDA/glutamate receptors. LPS-induced inflammatory response in astrocytes showed a decreased response in NOS2 and COX2 mRNA expression for IrOx-CNT-PEDOT. Results indicate that neuron-astrocyte co-cultures are a reliable model for assessing the biocompatibility and safety of nanostructured materials, evidencing also that hybrid IrOx-CNT-PEDOT nanocomposite materials may offer larger resistance to inflammatory insults.

1. Introduction

Among the various biomaterials implanted in biological systems, electroactive materials are the least known and are still under development. Implants of neural electrodes in soft tissues are mostly composed of inert materials like Pt, Au, C, Pt-Ir or TiN; however, side effects consisting of astroglial encapsulation, persistent inflammation, radical formation and loss of neurons have been described after their use (Marin and Fernandez, 2010), resulting in decreased function and degradation of the electrode. Other materials such as iridium oxide (IrOx) and conducting polymers are thought to decrease electrical impedance and improve electrode behaviour. In particular, dynamically electrodeposited IrOx shows optimal biocompatibility, high charge storage capacity (CSC) desired for extended direct electric fields to be applied, and high stability (Cruz et al., 2012). Furthermore, carbon nanomaterials and their combination with IrOx leads to fully compatible hybrids for mammalian neurons (Baldrighi et al., 2016; Carretero et al., 2014, 2015; Pérez et al., 2015) with enhanced properties (low impedance and increased charge capacity) and large potentiality as electrodes for neuroregeneration (Lichtenstein et al., 2017). On the other hand, conducting polymers have attracted attention due to their versatility, usual low cost and expected flexible structure; a goal for future soft tissue electrodes (Balint et al., 2014). However, the neural biocompatibility of polypyrrole (PPY) and poly(3,4-

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ethylenedioxythiophene) (PEDOT) polymers depends largely on cell type, on the counterion used and on the polymer substrate (Balint et al., 2014; Boehler et al., 2017; Moral-Vico et al., 2013; Vaitkuviene et al., 2013). The physico-chemical characteristics of the nanostructured materials are relevant since they may determine the nature of their interactions with neural cells and tissue, and can eventually modify cell morphology, activity or neural differentiation (Polak and Shefi, 2015).

When assessing *in vitro* the biosafety of materials, the biocompatibility test itself thus raises the question of which culture model could be the most adequate for material evaluation, and which material parameters could be acting either to favour or to deter cell adhesion, growth and differentiation. Mammalian and non-mammalian cells, primary neurons, neuron-like differentiated PC12, or neuroblastoma-derived cell lines like SH-SY5Y were used, many times with contradictory results (Gilmour et al., 2016; Gordon et al., 2013). *In vitro*, the adhesion, growth and differentiation of neural cells are highly dependent on the substrate. They need a support on which to adhere in order to be viable. Primary mammalian neurons do not survive on uncoated glass or plastic, whilst astrocytes do (Solà et al., 2011). Furthermore, astrocytes are known to support neuron adhesion, development and guidance *in vivo* and are also an optimal support for the adhesion and growth of neurons *in vitro* (East et al., 2010; Fallon, 1985).

Over the past few years, we have developed nanostructured hybrid materials based on IrOx, nanocarbons and conducting polymers, some of them with largely enhanced charge capacity as electrodes (Carretero et al., 2014, 2015; Moral-Vico et al., 2013, 2014; Pérez et al., 2015). In order to assess their biocompatibility, we studied, as an initial approach, the viability of primary neuronal cell cultures grown on them, and significant features have been observed. While IrOx and binary hybrid materials such as IrOx-Carbon nanotubes (IrOx-CNT), IrOx-graphene or IrOx-graphene oxide allow reproducible growth of neurons (Carretero et al., 2014, 2015; Cruz et al., 2012; Pérez et al., 2015), conducting polymers like polypyrrole or PEDOT show variable behavior in terms of adhesion and growth of primary neuronal cell cultures (Collazos-Castro et al., 2013; Guimard et al., 2007; Moral-Vico et al., 2013, 2014).

These three types of conducting nanostructured materials are at the forefront of new electrodes for the neural system; together with iridium oxides and nanocarbons, polymers constitute the main axis of future neural electrodes and implants because of their flexible mechanic properties, and further research on their behaviour is necessary. Their combination in hybrid materials could offer an improved electrode/substrate for neural biocompatibility. Given that nanocarbons as carbon nanotubes (CNT) and graphene, either alone or as IrOx hybrids, yield materials with optimal neuronal biocompatibility and great enhancement of electrochemical properties (Carretero et al., 2014, 2015; Lichtenstein et al., 2017; Lopez-Dolado et al., 2016; Pampaloni et al., 2017; Pérez et al., 2015), the formation of triple hybrids of IrOx, nanocarbons and conducting polymers is desirable in terms of flexibility. In addition, they could also evidence the role of conducting polymers in biocompatibility terms, depending on the exposed surface, and could shed light on the role of different material components in cell behavior.

Since a large degree of variability has been observed for the neuronal viability with some materials, especially on conducting polymers, it is worth expanding on *in vitro* biocompatibility studies of materials using astrocytes and neuron-astrocyte co-cultures beyond the usual neuronal studies. This study aims to compare primary neurons with astrocyte cultures and with co-cultures of both as this combination, from our point of view, is the *in vitro* assay that most closely resembles the *in vivo* situation.

In the present work we have tested the biocompatibility of materials by using primary cortical astrocytes and co-cultures of cortical astrocytes and neurons and compare it with that of the cortical neuron cultures previously reported. Furthermore, we take into account the properties of the materials as electrodes and develop a new tri-hybrid material that combines three different types of chemistry (inorganic metal oxides, flexible polymers, and nanocarbon-oxide hybrids) to discern possible substrate effects on neural cells. Biocompatibility is also studied in terms of glutamate uptake and neurotransmitter release as a measure of neural function and the influence of the material on the inflammatory astrocytic response. In order to do so, a variety of simpler materials are used in comparison with the tri-hybrid concerning their specific physicochemical properties and their role as cell substrate for the various culture types. A summary of substrate materials includes IrOx, PEDOT-PSS [poly(3,4-ethylenedioxythiophene) polystyrene sulfonate], IrOx-CNT, the new synthesized tri-hybrid IrOx-CNT-PEDOT and Pt (most commonly used neural electrode in clinical practice). The study implies a search of a method to decide if a material is adequate or not in the neural environment. If the material is adequate in terms of biocompatibility and good electrochemical behavior, it can be used as both stimulating and recording electrodes. And depending on the properties, as AC or DC electrostimulator, and thus, an electrochemical characterization is also given.

2. Materials and methods

2.1. Preparation and characterization of nanostructured materials

All materials were prepared as coatings on Pt conducting substrates using electrodeposition processes that improve adhesion and thickness control, as previously described (Carretero et al., 2014; Cruz et al., 2012; Moral-Vico et al., 2013) and in Supplementary Material. A new hybrid phase IrOx-CNT-PEDOT was prepared and its electrochemical properties and neural biocompatibility was compared with previously reported nanostructured materials. In all cases, the materials are macroscopically homogeneous and well adhered to the Pt substrate and the final coating had a thickness to assure transparency to facilitate cell culture studies.

Physicochemical and electrochemical characterizations of the coated materials were done as described previously for some of them (Carretero et al., 2014, 2015; Cruz et al., 2012; Moral-Vico et al., 2013; Pérez et al., 2015).

- Scanning electron microscopy (SEM) was carried out on QUANTA FEI 200 FEG-ESEM at 10–30 kV in high vacuum conditions. The sample was placed horizontally in the SEM holder, the electron beam perpendicular to it, in a standard way for standard images. The thickness of the coatings was measured by placing the transversal fractured substrate in parallel with the electron beam.
- Confocal Microscopy for roughness evaluation was performed using a Leica DCM 3D instrument with a 100x lens and ambient conditions of temperature and pressure. The data acquisition was repeated twice for each of the samples and the area captured was 128 × 95 μm^2. Subsequently the data was processed with the MountainsMap Premium Software. Roughness for the coatings was evaluated as Sq, the standard deviation of heights as seen from confocal microscopy
- X-ray photoelectron spectroscopy (XPS) and Raman spectra procedures are described in Supplementary material.
- The electrochemical behavior of the coatings was studied by cyclic voltammetry (CV) using 10 mV/s scan rate, with the same potentiostat and electrochemical cell system used in the synthesis as described in Supplementary Material, using sodium phosphate buffer (PBS, 0.10 M Na_2HPO_4, and 0.10 M KH_2PO_4 at pH 7.4) as electrolyte and with parallel 1 cm^2 electrodes separated 1 cm from each other. On the basis of the corresponding CV, charge storage capacity was determined, to evaluate the extent in which the material may be used as electrode in DC mode if applying electric fields.