

● PERSPECTIVE

The impact of graphene on neural regenerative medicine

There is increasing interest in studying carbon-based nanomaterials (CBNs) for use in regenerative medicine. Some carbon crystalline structures, such as graphene, carbon nanotubes/nanofibers, boron nitride nanosheets/nanotubes and fullerenes, as well as disordered structures, such as diamond-like carbon, glass-like carbon, and amorphous carbon, are now being considered as promising scaffolds (Ferreira et al., 2015; Kabiri et al., 2015), and therefore, studies of their biocompatibilities have begun to be reported in recent years. There are recent examples of the successful use of graphene-based substrates as interfaces for neuronal growth while retaining neuronal signaling properties without alteration (Fabbro et al., 2015) or as a bioscaffold for neuronal regeneration after spinal cord injury (Palejwala et al., 2016); nevertheless, the potential of CBNs as a neural interfacing material for neural repair and regeneration remains poorly understood.

Graphene is nothing but a single layer of graphite. It is a single, monatomic layer of carbon atoms neatly arranged in the form of hexagons. Graphite is constituted by multiple layers of graphene that hold together due to Van der Waals bonds. This means that graphene is not naturally available; rather, it is a part of graphite. There are several techniques that can be used to obtain graphene, e.g., the scotch tape method, which is also known as mechanical exfoliation, or chemical vapor deposition (CVD). Thus, getting good-quality graphene is very difficult, no matter the technique used (Romero et al., 2016). Recent research specifically suggests that graphene holds great potential in the biomedical field because of its extraordinary physical and chemical properties: controllable surface morphology, flexibility (controlled thickness), hydrophilic nature, electrical conductivity and, in some cases, transparency (depending on the thickness), chemical stability and the ability to absorb proteins and substances of low molecular weight (Kumar and Chatterjee, 2016). These properties may contribute to the transfer of chemical stimuli and biological signals to cells and thus the facilitation of cell maturation and differentiation.

It has been known for more than a decade that some graphene composites can promote and stabilize cell attachment, can induce the formation of functional neuronal networks (Thalhammer et al., 2010), and can be used as tunable and tailorable substrates to study nerve cell biology *in vitro* and *in vivo* (Edgington et al., 2013). Nevertheless, not all structural compositions of graphene, in the form of a substrate or in the form of a suspension, possess the same biological response or biocompatibility. In many occasions, the applicability of graphene has been hampered by evidence of nanotoxic effects on different cell types (Chen et al., 2012), which might be due to impurities in the materials during their preparation and surface functionalization. In particular, there is an interesting discussion about the interaction of cells with pristine graphene, graphene oxide and hybrid graphene particles in the form of coating and composites (Kumar and Chatterjee, 2016). Since there are few reviews detailing the cytotoxicity and biocompatibility of graphene and its derivatives, rigorous surveys are needed to evaluate the use of these materials as supporting substrates for tissue regeneration and the associated cell responses (Kumar and Chatterjee, 2016). Although graphene and some derivatives show excellent biological outcomes *in vitro*, in particular for neural tissue engineering applications, when considered for *in vivo* use, their performance is not well understood because of potential concerns of toxicity over the long term, since most experiments published to date have been performed over the short term (Bianco et al.,

2013). Nevertheless, graphene has been successfully tested in rats for the ingrowth of regenerating axons after spinal cord injury, and no area of pseudocysts around the scaffold suggestive of cytotoxicity was found (Palejwala et al., 2016). Nevertheless, prior to clinical use, these materials will have to be subjected to more comprehensive studies in animals and under different experimental conditions.

Recently, a novel graphene-based material has been synthesized and presented as a particular nanometer-thin nanocrystalline glass-like carbon film (NGLC) (Romero et al., 2016), composed of curved graphene microflakes connected by an amorphous carbon matrix. Its exceptional mechanical, electronic, and thermal properties, together with the possibility to be chemically tuned to become biomimetic, attracted the attention of our group to test the biocompatibility of this material. For this purpose, we utilized the progenitor cell line SN4741 (Son et al., 1999), derived from *substantia nigra* dopaminergic cells obtained from transgenic mouse embryos, to try to accurately assess the biocompatibility and cell behavior response to this nanomaterial (Rodriguez-Losada et al., 2017). NGLC treatment induces nerve cell differentiation and decreases cell proliferation (Sviderskaya et al., 2002). There are very extensive reports in the literature that indicate that processes of quiescence are related to differentiation mechanisms, but only this quiescence process has been associated with NGLC and SN4741 cells (Rodriguez-Losada et al., 2017). We show in our study (Rodriguez-Losada et al., 2017) how the immortalized SN4741 cells die because they initiate a maturation and aging process, as evidenced by the expression of SP30, which implies a decrease in the number of immature dividing cells. The use of MTT is not a perfect method for determining cytotoxicity when it is used with this type of substrate in cells with a variable growth speed in function of its maturation as we show in our work. Moreover, the proliferative capacity of an adult neuron is practically nonexistent, so when the differentiation process ends, the adult neuron continues with the aging process (activation of the senescence process) until death (Ding et al., 2001). For this reason, the fact that cells growing in NGLC flakes or on top of NGLC surfaces showed a decrease in the number of cells in the carbon-containing systems, compared to the control, is an interesting result that supports the fact that our material favors the processes of differentiation (Rodriguez-Losada et al., 2017). When cells of embryonic/transgenic origin are used, we should consider that we are working with immortalized and undifferentiated cells and that their division capacity is therefore undetermined. For this reason, they do not have a terminal growth pattern. They are not mature cells, nor can they be considered adult cells. Additionally, the effect of NGLC transforms the cells to differentiated-matured cells, which in fact represents an interesting phenomenon. If cell morphology is assessed, these cells will show a pure fibroblastic-like structure in most cases. Instead, our NGLC allows not only growth and adhesion of cells but also differentiation and cell contacts (Rodriguez-Losada et al., 2017). This capability is very desirable for the communication and survival of adult cells. In fact, NGLC induced neuronal differentiation or maturation into dopaminergic cells without using any cell growth factors such as GDNF or BDNF or any Laminin matrix, allowing spontaneous cell growth. Thus, this result itself is relevant for neuronal regeneration, thereby making our work relevant. The capacity of NGLC to promote networking-like figures, rearranging the cells in such a way that their architecture changes but fewer nuclei can be counted, makes us think that it does not really have to be like that, since the cellular disposition seems to change from a monolayer to cell grouping. Nevertheless, other nanostructured materials like boron nitride nanotubes functionalized with specific moieties have shown a differentiation process in mesenchymal stem cells (MSCs) when assessed at both the gene and phenotype levels (Ferreira et al., 2015). However, the main difference of



this work with respect to the NGLC is that MSC culture needs a complex culture medium with a large number of differentiators and maintenance factors, and these cells can direct their differentiation process toward different lineages (Claros et al., 2010; Ferreira et al., 2015). Meanwhile, the culture medium for SN4741 cells contains only essential medium and fetal serum without any differentiating factor or inducer. Consequently, the scalable, inert NGLC material (Romero et al., 2014) is demonstrably the inducer of the morphological, genetic and protein changes of the cells. Therefore, we think that the use of nanotubes, even if they are functionalized, always poses a risk to cellular health because they are incorporated into the inner part of the cells and cannot be degraded. Cultures in the presence of or in contact with the CBN produce the same effect without being invasive. Our work attempts to clarify the true phenomena that mediate NGLC-induced cell differentiation, although we believe that this process is a set of features influencing cellular plasticity as a whole: chemical factors such as the degree of oxidation, the groups exposed to the surface and the degree of wettability, as well as physical aspects such as conductivity, surface roughness, porosity or Young's modulus. Some of these aspects must be methodically studied in relation to the cellular differentiation capacity of this type of material before drawing conclusions that allow us to choose the most suitable materials for use in biomedicine.

We have tried in this paper to highlight the technical and methodological issues arising in the research of graphene-based materials, such as NGLC, for biocompatibility, focused on its use in biomedicine and showing the potential problems of cellular assays. Graphene derivatives, such as NGLC, affect the balance between proliferation and differentiation by reducing the proliferative capacity and by increasing the differentiation process. Thus, this balance is not only good but desirable and contributes to a change of focus in the search for better scaffolds. We understand that a substrate that allows inappropriate proliferation of immature or mature (non-terminal) cells is likely to be considered dangerous because it may potentiate malignant cells involved in oncogenic processes. At the same time, the use of cell types other than SN4741 cells should be contemplated, including primary cultures of different cell types and sources. Obviously, electrophysiological characterization is necessary in order to conclude the existence of a neural network formation. These ideas invite us to reconsider the model of the ideal scaffold to be used in regeneration, with a greater emphasis on the concepts of functionality and cellular differentiation rather than just on obtaining substrates that allow for cellular growth and colonization.

Nevertheless, since concerns for the toxicity of certain types of graphene persist, a clear objective for future research must be to clarify the degree of toxicity of each graphene-based material *in vivo* before presenting any clinical trials. Some of the recent reviews on the biosafety of graphene-based materials (Bianco et al., 2013) conclude that for all types of graphene nanoparticles, it is important to investigate and critically evaluate the potential short- and long-term health risks and toxicity hazards after acute, sub-acute and chronic exposures using *in vitro* and *in vivo* (small and large animal) models. For clinical translation of any graphene-based biomedical application that requires its systemic administration, formulations with high purity, dispersibility in aqueous media, and controlled physiochemical properties are highly desirable.

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