Stimulus responsive graphene scaffolds for tissue engineering

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Abstract

Tissue engineering (TE) is an emerging area that aims to repair damaged tissues and organs by combining different scaffold materials with living cells. Recently, scientists started to engineer a new generation of nanocomposite scaffolds able to mimic biochemical and biophysical mechanisms to modulate the cellular responses promoting the restoration of tissue structure or function. Due to its unique electrical, topographical and chemical properties, graphene is a material that holds a great potential for TE, being already considered as one of the best candidates for accelerating and guiding stem cell differentiations. Although this is a promising field there are still some challenges to overcome, such as the efficient control of the differentiation of the stem cells, especially in graphene-based microenvironments. Hence, this chapter will review the existing research related to the ability of graphene and its derivatives (graphene oxide and reduced graphene oxide) to induce stem cell differentiation into diverse lineages when under the influence of electrical, mechanical, optical and topographic stimulations.

***Index Terms*— Differentiation, Graphene, Scaffold, Stem Cells, Stimulation, Tissue Engineering**

1. INTRODUCTION

Recent developments of medicine and technology have led to an outburst of research on regenerative medicine allowing the emergence of new and enhanced biomaterials with the potential to fully heal damaged tissues and organs. Primary studies were focused on skin equivalents for treating burns but with the increasing needs of medicine, different tissue types started to be engineering such as bone, blood vessels, liver, muscle and nerve conduits [1]. Today, research is focused more significantly in stem cell technologies. The capacity of these cells to self-renew and generate differentiated progenies gives them the ability to treat numerous diseases and injuries all over the body [2]. For improved results in this field it is essential to use substrates that enable cell attachment, proliferation and differentiation [3,4]. Indeed, materials that are able to initiate, stimulate and sustain the series of complex events that lead to cell differentiation produce more favourable results when implanted in the human body [2,5]. The design of scaffolds is therefore vital for cells to proliferate, differentiate and eventually generate the desired tissue [6]. According to the literature, the choice of the material and consequently scaffold's mechanical properties has a direct impact in tissue formation by inducing cell differentiation into the desired phenotype through mechanotransduction [6,7]. For that reason, it is important to use materials that possess intrinsic characteristics for sustaining cell growth and inducing differentiation which may possibly lead to important progresses in stem cell research.

Graphene is a single-atom thick sheet of hexagonally bonded carbon atoms arranged in a two-dimensional (2D) honeycomb structure with unique electronic, physical, chemical and mechanical properties, including high electrical conductivity, elasticity and high molecule absorption [8,9]. Presently considered the thinnest, strongest and stiffest material [10], graphene is leading to numerous promising advances in fundamental science, especially in nanobiology and nanomedicine [11]. Moreover this material has a great potential to improve the performance of a broad range of biomedical devices since it is highly biocompatible, has low toxicity [12,13] and exhibits low inflammatory responses [14]. Furthermore graphene and its derivative,

graphene oxide (GO) have the capability of being easily functionalized [15], which has made them popular in several fields of biomedical engineering including anti-bacterial [16], viral [17] and parasitical applications [18] as well as bioassays [19], biosensors [20], cancer cell targeting, imaging and therapy [21-25] and stem cell-based tissue engineering (TE) [25-27]. As a result, graphene-based materials are now receiving increasing attention in the field of TE [26]. Many researchers have already proposed graphene as one of the best candidates for accelerating and guiding stem cell differentiations into specific lineages [28]. In fact, its exceptional properties led graphene to be considered as a powerful platform that not only allows stem cell attachment and growth but also enhances the differentiation of stem cells into specialized cells [29]. It has already been demonstrated that graphene is an effective substrate to promote the adherence of human osteoblasts and mesenchymal stromal cells [30], to stimulate osteogenic [31], myogenic [32] and neuronal [33] differentiation of human mesenchymal stem cells (hMSCs) and to induce the differentiation of neural stem cells (NSCs) in three-dimensional (3D) porous structures [34].

However, despite of the significant developments in applications using graphene-based materials, the stem cell-based TE using graphene is still a field of science that is not entirely mastered, in particular the efficient control of the differentiation of stem cells into specific cell-types of interest, especially in graphene-based microenvironments [35]. Additionally, because it is possible to use stimuli to control stem cells' physiological activities, such as its viability, division, migration and differentiation [36-40], graphene has recently been investigated as a template and/or electrode for inducing stem cell differentiation under various stimulation types.

Therefore, in the following, the capacity of graphene-based materials to induce stem cell differentiation when under the influence of various stimulations (including electrical, mechanical and chemical) will be revised.

2. Graphene Substrates As Platforms For Directing Cellular Differentiation: From 2D To 3D

Scaffolds play a very important role in the success of TE, since they are able to not only provide cell support and attachment, by acting as a biomimetic structure, but also to modify cellular responses over time and to supply growth factors and drugs [41]. For the fabrication of an ideal scaffold several parameters should be taken in account, such as the mechanical strength so that it can support the predesigned tissue structure, porosity, absence of toxicity, ability to transport oxygen, provision of attachment sites for cells, provision for nutrients and biodegradability [42]. Although the use of 3D scaffolds is the most common stem cell culture method in TE [43], the use of 2D constructs can also be very advantageous, since, for example, the smart layering of 2D sheets can facilitate the creation of a more organized structure allowing a more appropriated cell placement.

The fabrication of 2D graphene templates is usually accomplished by two main methods [29]. Firstly, by the chemical vapour deposition (CVD) method [44], in which thin graphene sheets are produced. For allowing the enhancement of graphene's excellent features, including its electrical conductivity and mechanical properties, the fabrication of 2D graphene scaffolds by this method led researchers to start to see graphene as a potential material for TE applications. Alternatively, 2D graphene constructs can be fabricated through the chemical exfoliation of graphite [45], that allows the production of GO and reduced graphene oxide (rGO) sheets. In this case, it is essential to perform the exfoliation appropriately once it was already demonstrated in previous works [28] that the surface chemistry of graphene determines the surface adhesion properties, which can directly lead to the increasing or decreasing of cells' proliferation and differentiation. In fact the lack of standardization of graphene based materials remains a significant problem for the reproducibility of the results, ion since the several methods reported for the production of graphene sheets can have, as final products, materials with different number of layers and/or chemical groups, affecting the behaviour of cultured stem cells in a different manner [46]. Many researchers have already

demonstrated that the use of graphene as a 2D scaffold has a great potential to enhance the proliferation and differentiation of a vast number of cells [32,34,47-49]. However, for a successful *in vitro* implementation of stem cell differentiation systems, the use of 2D scaffolds may not be always sufficient, since effective differentiation requires appropriated features such as a suitable topography with cell growth channels as well as the natural synergistic effects of cell to cell and cell to extracellular matrix (ECM) interactions that can only be assured by 3D systems [43]. In addition, their high heterogeneity, limited scalability and low reproducibility are making 2D scaffolds a more less attractive system when compared with 3D ones since they are not entirely compatible with the development of in vitro models that accurately simulate the native stem cell niche [34].

Concerning the importance of the topographic cues for cells behaviour reported above, the effects of graphene-based polymer composites in the form of 2D and 3D scaffolds on the biological response of osteoblasts were investigated by Kumar *et al.* [50]. As shown schematically in Figure 1, first, the authors fabricated the scaffolds by incorporating 1% (wt/wt) of GO and rGO in polycaprolactone (PCL) nanocomposites, being that the 2D substrates were circular discs (5 mm diameter and 30.5 mm height) prepared by compression moulding, whereas the 3D macroporous scaffolds were prepared by the salt leaching method. Then the cells were cultured on both 2D and 3D substrates and biological studies were performed in order to observe cells' behaviour. They demonstrated that there is in fact a difference in the cellular responses obtained by the two different substrates. They found that the cells tend to organize into aggregates in 3D scaffolds in contrast to 2D ones where cells tend to spread and become randomly distributed.

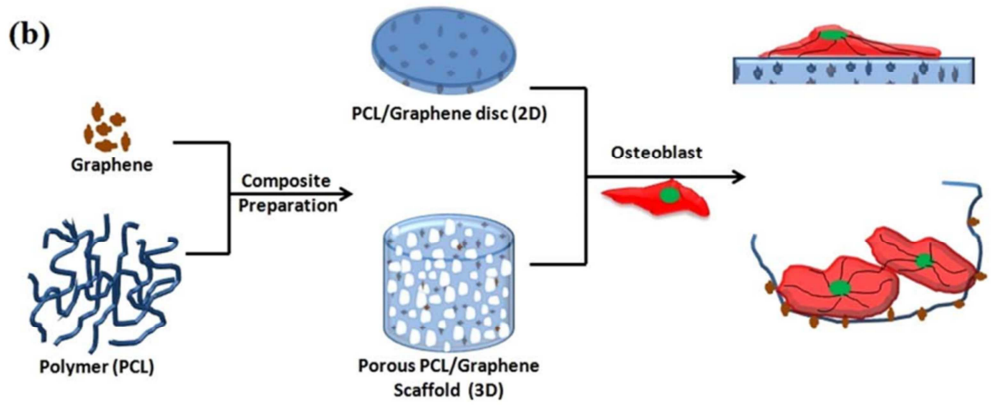

FIGURE 1- (b) Preparation of planar 2D and porous 3D PCL/Graphene composite and schematic representation of *in vitro* osteoblast response on 2D and 3D scaffolds. Reproduced with permission [50]. Copyright 2015 WILEY PERIODICALS, INC.

As a result, the use of 3D scaffolds in TE is becoming a more popular method for stem cell culture, since it not only allows a substantial improvement of stem cell viability and function but also because it is a method that has higher efficiency, consistency and predictability [43]. Although graphene has proved to have a great potential in this field it is still a very recent material, meaning that there is a limited number of studies related with 3D graphene structures. However, its exceptional properties are exciting more and more the scientific community to develop further methods in order to provide 3D graphene scaffolds with several topographies.

Primary attempts for the fabrication of 3D graphene structures were reported by Chen *et al*. [51] in which they performed the direct synthesis of 3D foam-like graphene macrostructures by using the CVD method, allowing the production of thin films named by them as graphene foams (GFs) (Figure 2).

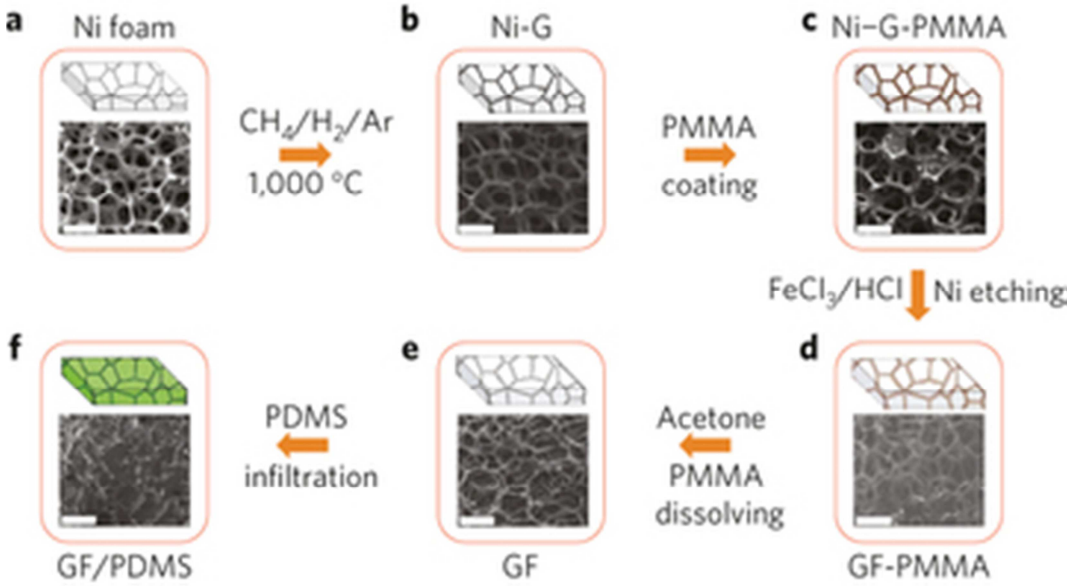

FIGURE 2- Synthesis of a GF and integration with polydimethylsiloxane (PDMS). (**a,b)** CVD growth of graphene films (Ni–G) using a nickel foam as a 3D scaffold template. **(c)** An as-grown graphene film after coating a thin poly(methyl methacrylate (PMMA) supporting layer (Ni–G-PMMA). **(d)** A GF coated with PMMA (GF-PMMA) after etching the nickel foam with hot HCl (or $FeCl_3$/HCl) solution. **(e)** A free-standing GF after dissolving the PMMA layer with acetone. **(f)** A GF/PDMS composite after infiltration of PDMS into a GF. All the scale bars are 500 µm. Adapted by permission from Macmillan Publishers Ltd: Nature Materials [51], copyright 2011.

This was an important step in graphene's research since it excited the development of more studies using 3D graphene scaffolds in the field of TE, in particular, the investigation of the ability of this graphene structures to regulate cell behaviour and induce stem cell differentiation. Consequently, Crowder *et al.* [31] investigated the use of 3D GFs as cell culture substrates for the evaluation of its capacity to promote osteogenic differentiation of hMSCs. The GFs were produced by growing graphene on 3D Ni scaffolds, in which Ni was posteriorly removed by $FeCl_3$ etching. Their results indicated that 3D GFs are capable of supporting the attachment and viability of hMSCs and also of inducing the spontaneous osteogenic differentiation of hMSCs without the need of extrinsic biochemical manipulation, demonstrating once more the potential of graphene in biomedical applications.

Later on, the capacity of 3D GF to act as a biocompatible and conductive scaffold for NSCs was evaluated. In this study Li *et al.* [52] reported the first use of a 3D graphene porous foam, as a novel scaffold for inducing the proliferation and differentiation of NSCs *in vitro*. The synthesis of the 3D GFs was made by the CVD method using a Ni foam as a template, in which the Ni foams were removed afterwards by appropriated chemical solvents. The results presented in this study indicate that these graphene structures are able to effectively support NSC growth as well as induce more cells' proliferation when compared with the traditional CVD-grown 2D templates, since it was verified an upregulation of Ki67 protein expression (known as a cellular marker for proliferation). Additionally, it was also demonstrated by phenotypic analysis that 3D GFs tend to enhance the NSCs differentiation towards astrocytes and neurons in particular.

Another study concerning the use of 3D GFs to promote the repair of neural injuries was presented by Serrano *et al.* [53]. In this paper, it was reported a new method for the fabrication of the 3D porous structures, in which scaffolds were produced by a biocompatible freezer-casting procedure. The possible utility in neural tissue regeneration was assessed using embryonic neural progenitor cells which allowed the study of the cellular adhesion, morphology and viability as well as the neuronal/glial differentiation. The results proved that highly viable and interconnected neural networks were formed on these scaffolds having presented both neurons and glial cells and synaptic connections.

Later on, Shin *et al.* [54] reported the fabrication of GO-impregnated biomimetic matrices composed of poly(lactic-co-glycolic acid) (PLGA) and collagen (GO-PLGA-Col) by performing electrospinning for the enhancement of the myoblast differentiation. After analysing the physicochemical and mechanical properties of the hybrid scaffold as well as its biocompatibility and biofunctionality, they found out that GO dispersed and blended adequately with collagen in the hybrid matrices. Moreover, it was demonstrated that the hydrophilicity of the PLGA matrices significantly increased due to the blending of GO and Col which led the GO-PLGA-Col hybrid matrices to become a more favourable microenvironment for the attachment and proliferation of the C2C12 skeletal myoblasts as demonstrated in Figure 3. Furthermore, the results

observed in Figure 4 demonstrate that these hybrid 3D matrices were able to stimulate the myogenic differentiation of myoblasts, being this differentiation further enhanced under the culture conditions of the differentiation media, which was explained by the synergistic effect of GO and Col. Recently, Girão *et al.* [55] showed the feasibility to prepare GO-Col scaffolds, taking advantage of their chemical electrostatic interactions and resulting in porous structures suitable for cellular microenvironments.

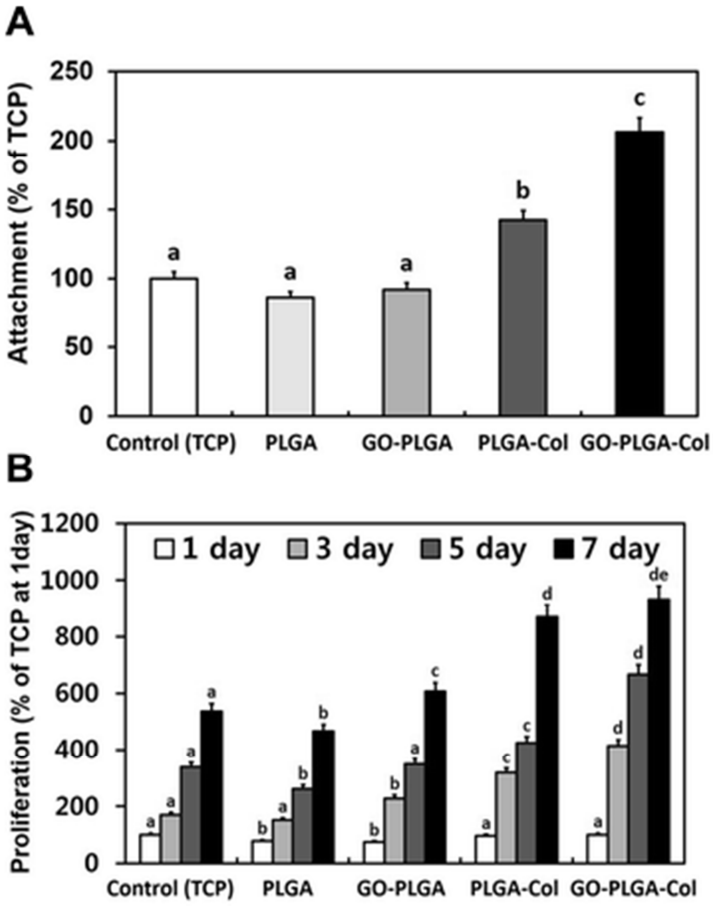

FIGURE 3- Initial attachment and proliferation of C2C12 skeletal myoblasts. **(A)** Initial attachment of C2C12 skeletal myoblasts on tissue culture plastic (TCP), PLGA matrices, GO-PLGA matrices, PLGA-Col matrices, and GO-PLGA-Col matrices were measured using a CCK-8 assay at 6 hours after seeding. **(B)** Proliferation of C2C12 skeletal myoblasts was measured using CCK-8 assay on 1, 3, 5, and 7 days after incubation. The different letters in **(A)** denote the significant differences between the control and experimental groups, $p < 0.05$. The different letters in **(B)** denote the significant differences between the control and experimental groups at the same time

point, $p < 0.05$. If two groups have the same single letter (a, b, c, etc.), there is no significant difference between them. If a group is marked with a dual letter (eg, de), it has a significant difference from the control and other groups marked with ‘a’, ‘b’, or ‘c’, but does not from another group marked with ‘d’. Reproduced with permission from [54]. Copyright 2015 BioMed Central.

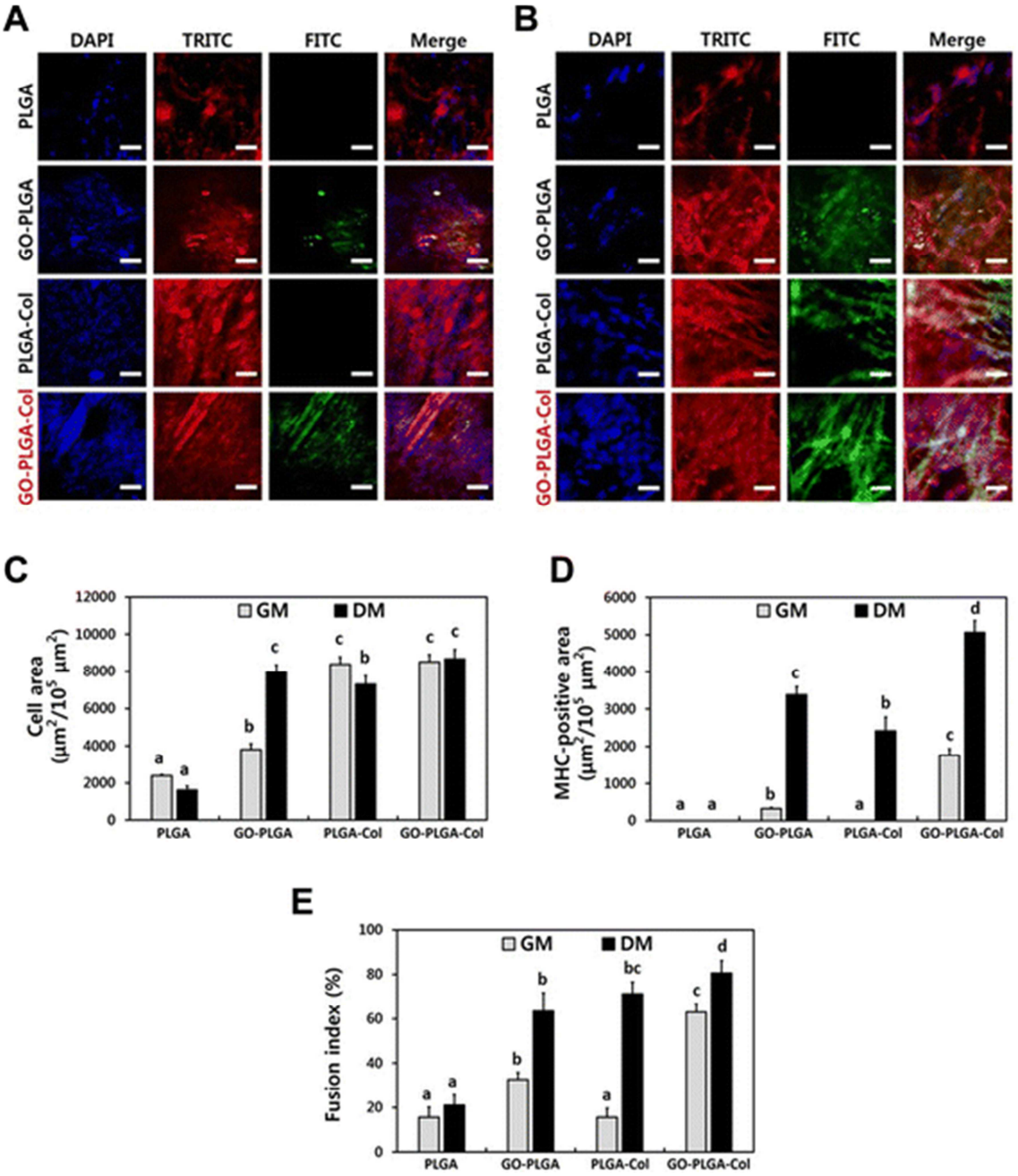

FIGURE 4- Myogenic differentiation analysis with immunofluorescence staining. Two-photon excitation fluorescence images of C2C12 skeletal myoblasts in **(A)** growth media (GM) and **(B)** differentiation media (DM). The cells were cultured in

GM for 2 days and then cultured in GM or DM for additional 5 days. The cell nuclei were counterstained with DAPI (blue), the F-actins were stained with TRITC-labelled phalloidin (red) and the myosin heavy chains (MHCs) were stained with FITC-labelled anti-MHC antibody (green). The scale bars are 50 μm. Quantification of **(C)** the cell area, **(D)** MHC-positive area, and **(E)** fusion index. The fusion index was calculated as a percentage of the nuclei number in multinucleate myotubes with more than two nuclei to the total number of nuclei. Quantitative analysis was performed using ImageJ Software. The different letters in **(C)** and **(D)** denote the significant differences between each experimental group, $p < 0.05$. The different letters in **(E)** denote the significant differences between each experimental group, $p < 0.05$. If two groups have the same single letter (a, b, c, etc.), there is no significant difference between them. If a group is marked with a dual letter (eg, bc), it has a significant difference from the control and other groups marked with 'a', but does not from another group marked with 'b'. Reproduced with permission from [54]. Copyright 2015 BioMed Central.

Afterwards, in order to produce a more efficient scaffold Nieto *et al.* [56] synthetized a graphene foam/polylactic acid–poly-ε-caprolactone copolymer hybrid (GF-PLC) scaffold by a dip-coating method that enables retention of the porous 3D structure. For biocompatibility tests, hMSCs were cultured on the scaffolds for a period of 28 days. Hybrid scaffolds revealed to be a good substrate for supporting chondrogenesis. Therefore, this study demonstrated that 3D GFs combined with other materials are also advantageous for TE applications, in particular for this case, it is suited for musculoskeletal applications, such as the growth of new cartilage in order to replace damaged one.

3. GRAPHENE-BASED STIMULUS RESPONSIVE SCAFFOLDS

Previous works have already demonstrated that by stimulating stem cells it is possible to control several physiological activities of the cells, including its differentiation [36-40]. In addition, graphene has already proved to be a good structure for inducing stem cells' differentiation when several stimuli are applied [57-59]. As a result, in the last few years, the possibility of controlling stem cell fates by applying different stimulations to graphene scaffolds has attracted an increasing attention of many researchers in the field of stem cell-based therapies.

3.1. Electrical Stimulation

Electrical stimulation plays an important role in the guidance of stem cell differentiation [60,61] and because of its particularity of being a non-invasive method it is now being used as a promising therapy, in particular for neurological diseases [62,63]. In addition it is considered one of the most advantageous stimulations once it is a more controllable, quantifiable and reliable technique that allow the precise delivery of the stimulus through the use of electrodes [64].

Previous works [37,65] have already demonstrated that the use of electrical stimulation indeed influences cells' behaviour being already established that it effectively controls various stem cell's physiological activities such as cell viability, division, migration and more importantly cells' differentiation. Due to its favourable electrical conductivity and high transmittance [10] graphene is considered a promising material for modifying the behaviour of electrically sensitive cells. In fact, since neural cells and their functions are based on electrical activities, the unique electrical properties of graphene suggest that it could be a successful platform for neuronal stimulation and treatment [66]. Moreover, graphene not only has adequate chemical characteristics for the effective attachment of neural tissues [67], but also is susceptible to have its electronic properties tailored in order to match the charge transport that is necessary for electrical cellular interfacing [68,69].

The first work that reported the use of graphene combined with electrical stimulation was presented by Heo *et al.* [70]. The authors proposed the fabrication of an enhanced electrical stimulator supported by a substrate that combines the superior properties of graphene with the non-cytotoxic properties of the polymer polyethylene terephthalate (PET) [71] in order to minimize cellular damage after electrical stimulation. Thus, non-contact electrical field stimulation, produced by charge-balanced biphasic stimuli, was applied through the flexible, transparent and non-cytotoxic graphene-PET film electrodes in order to stimulate neural cells that were cultured in a dish and to further examine its effects on cell-to-cell coupling. Their results showed a superior effectiveness in the shape of cell-to-cell interaction of human neuroblastoma cells when weak electric field stimulation of 4.5 mV/mm with pulse duration of 10 seconds during 32 minutes was applied. Additionally, for this stimulation they also observed a significant increase in the strength of the existing cell-to-cell couplings as well as an increment in the number of cells which enabled the formation of new cell-to-cell couplings (Figure 5). The observed altered cellular interactions were explained based on possible changes on the regulation of the endogenous cytoskeletal proteins fibronectin, actin and vinculin. Hence, this work allowed, for the first time, to demonstrate the benefits of using graphene in electrical stimulation therapies, opening tremendous opportunities for graphene-based materials in the field of TE.

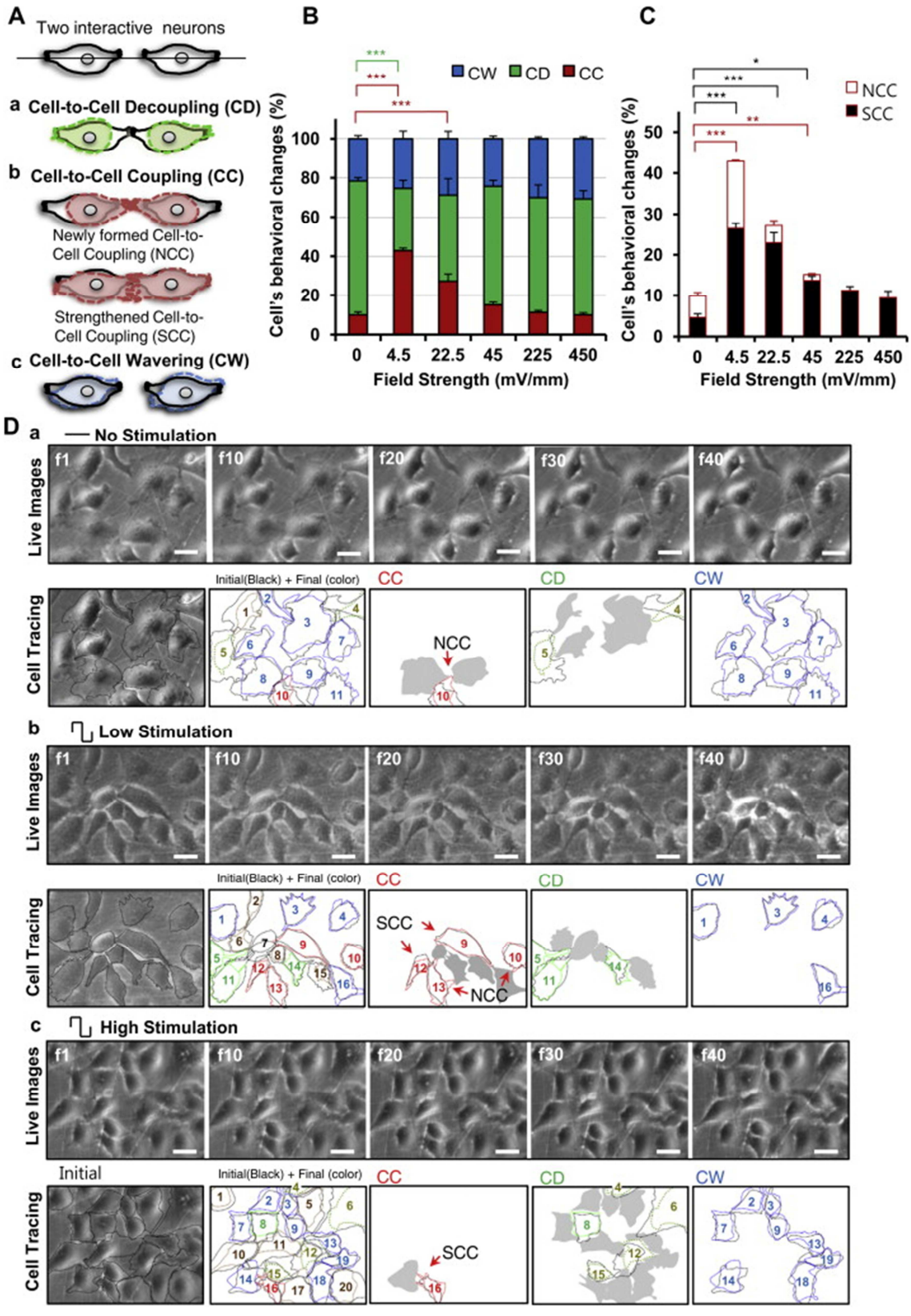

FIGURE 5- Cell-to-cell interactive reactions to electric field stimulation. A. Schematic illustrations of cell-to-cell interactive reactions between two separated cells under electric field stimulation. **(a)** Cell-to-cell decoupling (CD). Cells belonging to the CD group separated from each other after stimulation. **(b)** Cell-to-cell coupling (CC).

The CC group was further classified into two groups: The newly formed cell-to-cell coupling (NCC) group and the strengthened cell-to-cell coupling (SCC) group. The NCC represents a group of cells that respond to electric field stimulation by forming new contacts between cells. The SCC represents a group of cells strenthening existing contacts between cells after electric field stimulation. **(c)** Cell-to-cell wavering (CW). Cells belonging to the CW group exhibit a wavering behavior following electric field stimulation. **B.** A bar graph categorizing behavioral reactions to electric field strengths. The percentage of cells categorized as CC (red) is the highest at 4.5 mV/mm and the second highest is at 22.5 mV/mm. On the contrary, the percentage of cells categorized as CD (green) is the lowest at 4.5 mV/mm and the second lowest is at 22.5 mV/mm. There was no effect of electric field on the CW (blue) group. **C.** The categorization of CC cells. When we further categorized CC into two groups, there was a clear effect of electric field on NCC. In particular, at 4.5 mV/mm, the lowest electric field stimulation, the percentage of cells belonging to NCC was the highest among other electric fields. Under the weak electric fields, cells exhibited a trend of increased percentages of SCC. **D.** A typical example of a cellular reaction to the electric field. **(a)** Live images and tracing of cellular changes with no electric field stimulation. “f1” indicates the 1st image and “f40” indicates the 40th image taken from the optical microscope during stimulation experiment. The initial shape of the cell is represented as a black tracing line and the final shape of the cell is represented with color coding (CC-red, CD-green, and CW-blue). With no stimulation, the majority of cells were categorized into the CW group. Few NCC and SCC were observed with no stimulation. **(b)** Live images and tracing of cellular changes with 4.5 mV/mm. At 4.5 mV/mm, the largest percentages of cells were categorized as CC and we observed clear NCC and SCC. **(c)** Live images and tracing of cellular changes at 450 mV/mm. At 450 mV/mm, the majority of cells reacted to the electric field and was categorized into CD and CW groups. The scale bar represents 30 μm. Values from the bar graphs are mean (n = 6 for 22.5 mV/mm, 45 mV/mm, and 225 mV/mm, and n = 10 for 0 mV/mm, 4.5 mV/mm and 450 mV/mm). Significantly different $p < 0.05$, 0.01 and 0.001 are represented by *, ** and *** symbols. Reproduced from Heo *et al.* [70], Copyright 2011, with permission from Elsevier.

Meantime, in the study of Park *et al.* [34], already mentioned above, a transparent graphene electrode was used for the first time for the electrical stimulation of already differentiated cells of hNSCs (neurons and glia cells) in order to confirm their neuronal activity. Therefore, a series of voltage pulses typically 1 ≈ 10 of 500 mV monophasic/cathodic voltage pulses with 1 ≈ 100 ms

duration in a second was applied to the differentiated cells from hNSCs by using graphene electrodes. The fluorescence intensity of the cells which indicated calcium levels inside the cell was continuously monitored with a fluorescence microscope. By observing the results before and after electrical stimuli, they found out that cells exhibited increased calcium levels, since its fluorescence intensity increased significantly after stimulation. In addition, by plotting the fluorescence intensity versus the stimulation time period they observed that electrical stimulation caused an increase of fluorescence intensity between 60 to 70%. Thus, their findings clearly suggested that the differentiated cells from hNSCs on graphene films were operating as a neuron, and more importantly that graphene films can indeed be used as neural-stimulation electrodes.

The response of NSCs cultured on graphene substrates to electrical stimulation has been also studied by Tang *et al.* [72]. Herein, a series of 10-100 ms monophasic cathodic pulses with intervals of 5 s was applied in the cultured graphene substrates. After several tests, they established a stimulation threshold current of 0.5-1 μA. Furthermore, their findings confirmed not only that graphene is able to improve neural performance and electrical signalling in the network but also that it can work as a 2D material for conducting electrical current to neurons, confirming, hence, its potential to act as an effective neural interface for TE applications.

The effects of electrical stimulation in 3D graphene porous structures were also investigated in the work presented by Li *et al.* [52] that first proved that these scaffolds were able to support NSCs growth and induce cells' proliferation, as mentioned in the previous section. Here, 3D-GFs were used as a cell stimulation electrode by being subjected to a series of monophasic cathodic pulse produced by a function generator, in which it was demonstrated that the stimulation threshold was 20-30 μA. To evaluate the influence of electrical stimulation, the differentiated NSCs cultured on the 3D structures were stained with Fluo-4 AM dye before stimulation in order to monitor the change of intracellular calcium ion concentrations, once it is known by previous works that submitting a neuron under voltage pulse stimuli may favour the opening of calcium ion channels, consequently increasing calcium ion concentrations in the cell, which results in a superior fluorescence intensity of the dye present in the cells [73]. The results presented in this

study show an increase of 50-60% in fluorescence intensity after electrical stimulation, clearly demonstrating that 3D-GFs also have the capacity to operate as an effective conductive platform for electrically stimulate cells, which validates that 3D graphene structures exhibit a great potential for advanced strategies in several areas of regenerative medicine.

Despite of graphene effectiveness in acting as a viable material for regulating cells' behaviour it may not always produce the most desirable results when submitted to electrical stimulation. For that reason, researchers started to study and fabricating enhanced graphene-based structures combined with other materials in order to act as a platform for applications based on electrical stimulation. Concerning this, Zhang *et al.* [74] studied the performance of a new amphiphilic rGO template with an enhanced charge injection capacity in the electrical stimulation of neural cells to assess its capacity to act as a viable interface for the passage of electrical current. To obtain the rGO sheets and further fabricate this template, GO sheets were simultaneous submitted to covalent functionalization and thermal reduction treatments. The covalent functionalization was performed by applying methoxy poly(ethylene glycol) (mPEG) chains on the surface of the rGO sheets, allowing the enhancement of this material as it not only allows the material to have a high dispersibility in various solvents, which enables several post-treatment processes, but also increases its charging capacitance. By performing calcium imaging tests in the PC12 neural cells cultured on the amphiphilic mPEG-rGO films and hydrazine-rGO sheets, the authors found out that the electrical stimulation, executed by applying a series of 1-100 ms monophasic anodic with durations of 10 s and potentials lower than 0.6 V, significantly increased the percentage of cells with higher action potentials cultured on the mPEG-rGO films due to their higher charge injection. These findings not only demonstrated that this new amphiphilic mPEG-rGO material is capable of being used for neural prostheses applications in a safer and efficacious manner but also that graphene can be combined with several other materials in order to effectively modify electrically sensitive cells.

In another work Berit *et al.* [75] investigated the suitability of graphene to work as a material for electrical stimulation purposes when combined with other materials, by analysing the electrochemical properties of the fabricated graphene-based electrodes. As it was already recognized that graphene had

the ability to improve the electrical interface between neuronal cells and electrodes, the authors proposed the use of this material as a biocompatible coating for commonly used electrode materials, more specifically, for gold and silica (SiO_2). After performing electrochemical and Raman characterization on both gold and SiO_2 electrodes coated with graphene, they found out that graphene on SiO_2 substrate is a more promising material combination for the fabrication of superior stimulation electrodes.

More recently, the ability of graphene-based materials to serve as viable platforms for the regeneration of optical nerves by using electrical stimulation was studied by Yan *et al.* [76]. Optical nerves are usually damaged due to atrophy, apoptosis or death of retinal ganglion cells (RGCs) and could cause serious problems, such as the permanent visual field loss and irreversible ocular diseases. The use of electrical stimulation to heal injured neurons *in vitro* and to improve the survival rates and axon growth of central nervous system *in vivo* has already produced reasonable results. Despite this, the use of electrical stimulation based therapies still remains a challenge, mostly due to electrode's characteristics, which have to include high safe charge injection limit (Qinj, i.e. electrochemical capacitance), high performance for long periods of stimulation and high biocompatibility. Graphene has a low Qinj, which forces researchers to combine graphene with other materials to achieve success. Hence, in this work, the authors proposed a new graphene-based electrode for the regeneration of optic nerve via electrical stimulation. The fabrication of the electrode was performed by co-electrospinning biocompatible PLGA with the polypyrrole functionalized graphene (PPy-G)/ethanol dispersion. To synthetize the PPy-G, the polymerization-enhanced ball milling method was utilized (Figure 6 (a)), which enables the efficient introduction of electron-acceptor nitrogen increasing the material's capacitance and also allowing it to remain a conductive platform for charge transportation. Moreover, randomly oriented PPy-G/PLGA nanofibers and aligned PPy-G/PLGA nanofibers with different percentages of (w/w) PPy-G were fabricated under the same electrospinning conditions, as shown in Figure 6.

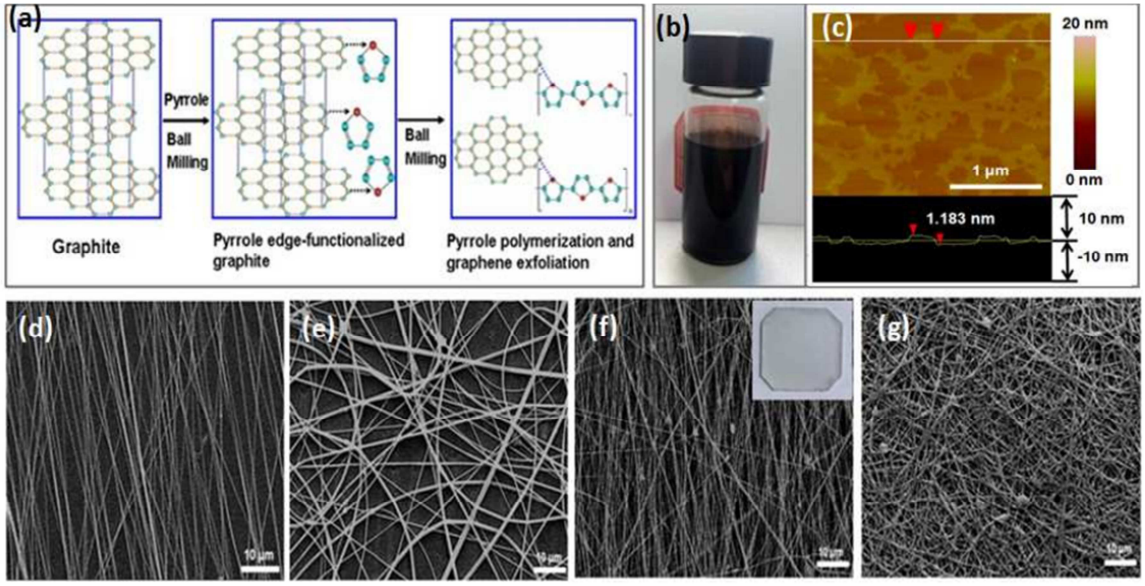

FIGURE 6- (a) Schematic synthesis of the PPy-G via the polymer polymerization enhanced ball milling method. **(b)** Digital photo of the PPy-G well dispersed in ethanol. **(c)** AFM micrograph of the PPy-G. **(d)** SEM micrograph of the aligned nanofibers from 1% (w/w) PPy-G/PLGA dispersion compared to **(e)** that of the random nanofibers prepared at the identical conditions. **(f)** SEM micrograph of the aligned nanofibers from 6% (w/w) PPy-G/PLGA dispersion compared to **(g)** that of the random nanofibers synthesized at the identical conditions. Inset **(f)** is the digital photo of the nanofibrous scaffold used for electrical stimulation. Reproduced with permission from Yan *et al.* [76]. Copyright 2016 American Chemical Society.

In order to investigate the biocompatibility of the proposed substrate, first, its cytotoxicity was evaluated by using the CCK-8 assay. As observed in Figure 7 (e), the RGC cell viability was higher than 80% in all the substrates, demonstrating that the as-synthesized PPy-G/PLGA nanofibers are highly biocompatible substrates. Moreover, the authors demonstrated that the PPy/G-PLGA nanofibers containing 6% (w/w) PPy-G are the best substrate structure for cell attachment and growth since it showed a cell viability of approximately 100% (similar to the control substrate). Then electrical stimulation was applied to the PPy-G/PLGA based aligned nanofibers, with an optimized step potential of ± 700 mV/cm. For effects of comparison, PPy/PLGA aligned and random nanofibers were also submitted to the same electrical stimulation as well as random PPy-G/PLGA nanofibers. The results showed that electrical stimulation led to a clear enhancement in the viability, neurite outgrowth and antiaging

ability of RGCs. The influence of aligned or random nanofibers was also evaluated by analyzing scanning electron microscopy (SEM) images of RGCs that were seeded on these both types of substrates (Figure 7 (a), (a'), (b), (b'), (c) and (c')).

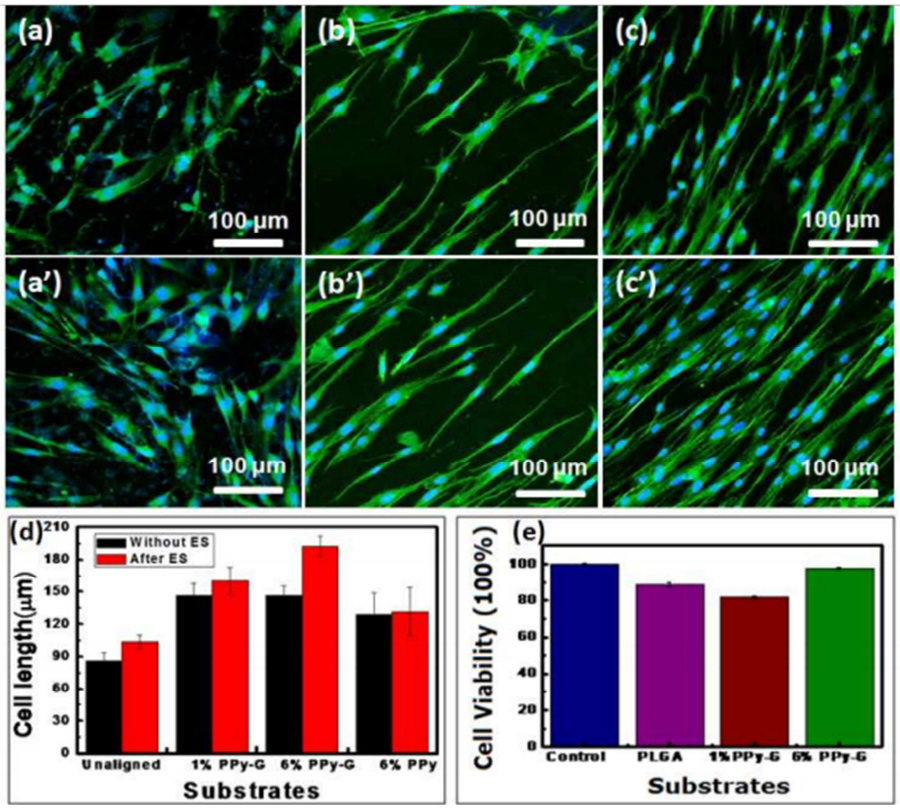

FIGURE 7- Confocal microscopy images of RGC cells seeded on (a) the random PPy-G/PLGA nanofibers without ES and **(a')** after ES; **(b)** the aligned PPy-G/PLGA nanofibers with 1% (w/w) PPy-G without ES and **(b')** after ES; **(c)** the aligned PPy/G-PLGA nanofibers containing 6% (w/w) PPy-G without ES and **(c')** after ES. **(d)** The average cell length of RGCs without and after ES. **(e)** Cell viability of RGCs cultured on the different substrates. ES conditions: Step potential was pulsed between -700 and 700 mV/cm. ES was performed 1 h everyday and lasted for 3 days. Reproduced with permission from Yan *et al.* [76]. Copyright 2016 American Chemical Society.

They found out that although electrical stimulation has promoted cell growth and density in both types of nanofibers, the aligned nanofibers were capable of allowing a longer cell length (137% improvement in cell length after

electrical stimulation), as shown in Figure 7 (d), which not only demonstrates that electrical stimulation enhances the regeneration of RGCs but also that aligned fibrous substrates are more favourable to do this.

The regeneration of optic nerve via electrical stimulation on graphene substrates was confirmed also by the study of Wenjing *et al.* [77], which proposed another graphene-based electrode for the enhancement of RGCs behaviour. The authors produced highly capacitive nitrogen-containing graphene (CG) by incorporating nitrogen-containing conducting polymers with graphene. To do this they used the polymerization enhanced edge-functionalized ball milling techique which consists in polymerizing the monomer with the heat that is produced by the milling friction force. Afterwards, the aligned CG nanofibrous scaffolds were submitted to electrical stimulation by using a modified electrospinning approach. They found out that electrical stimulation significantly enhanced RGCs viability as well as its antiaging ability. Moreover, a 173% improvement in cell length was registered after stimuli. Thus, their findings suggest that regeneration of optic nerve via electrical stimulation is indeed possible, but only by using proper nanoelectrodes.

At this point, graphene had already proved that it could be a potential material for induncig stem cell differentiation via electrical stimulation, however, despite the studies mentioned above, there was still few investigations that could confirm this possibility. Thus, to address this challenge and evaluate, for the first time, the influence of electrical stimulation on the differentiation of muscle cells on graphene-based materials, Ahadian *et al.* [58] presented a study where C2C12 myoblasts were cultured not only on ultrathin thermally reduced graphene (TRG) films but also on GO and glass slide substrates. After finding out that TRG films are more favourable for cell adhesion and spreading, they also demonstrated that myoblast cell differentiation was significantly increased after electrical stimulation (8V, 1 Hz and 10 ms for 2 continuous days). As a result, this work enabled the confirmation that graphene substrates are able to electrically regulate cells' behaviour, including its differentiation, leading to the development of numerous possible applications in the field of TE.

Meanwhile, several other studies have been exploring the capacity of graphene-based materials to control cells' behaviour via electrical stimulation. Concerning this, Meng *et al.* [59] used a large size non-functional graphene

nano-film (NGNF) as a substrate for the electrical stimulation and consequent behaviour modification of rat pheochromocytoma PC-12 cells. The graphene nano-film was fabricated by spray coating several high conducting graphene sheets on top of the polyurethane film. After performing numerous trials, they defined the intensity of 100 mV/mm as the optimized constant electrical stimulation condition, which revealed a significant enhance of PC-12 cells differentiation as well as neurite extension and growth. The effects of a programmed electrical stimulation was also investigated by applying 100 mV/mm at 1 Hz and 10 Hz to the cultured graphene substrates. When comparing the influence of these two types of stimulations, the authors observed a longer increase in the neuritis length with the programmed electrical stimulation, being also demonstrated that this type of stimuli have a much positive effect in the nerve behaviour than with the other one.

The promotion of PC-12 cells' differentiation via electrical stimulation on graphene substrates was confirmed also by the study of Sherrel *et al.* [78]. To fabricate graphene-biopolymer (GBP) electrodes, first, the CVD method was used to produce highly crystalline graphene layers on copper substrates. Then, these layers were transferred to biopolymer supports, including polylactic acid (PLA) and PLGA. The combination of graphene with these two copolymers allows the formation of a unique electrode structure with several benefits, including superior surface properties and the possibility of electrical communication with excitable cells. Electrical stimulation was applied by submiting the cells that were seeded in the GBP strutures to a biphasic current waveform with 250 Hz, which consisted in 100 µs pulses of ± 0.1 mA cm^{-2} with a 20 µs interphase gap, and a 3.88 ms rest period for each cycle, during 8 hours per day for 3 days. The results presented in this study corroborated that electrical stimulation enhances the differentiation of PC-12 cells when cultured on graphene conductive layers, since it was visible a great increase in the neurite length and neurites' connectivity when compared to unstimulated cells. The authors also demonstrated that graphene can be used as a coating for non-conducting, flexible biopolymer surfaces without hampering its biocompatibility, which is favourable for the creation of viable electrodes from non-conducting materials with optimized cell compatibility with graphene, that provides the suitable electrical properties for electrically stimulate cells.

Meantime, Guo *et al.* [79] suggested that an electrical stimulation-assisted culture process should enhance the ability of engineered conductive scaffolds to regulate the differentiation of MSCs towards the neural lineage. To obtain a self-powered electrical stimulation system for the enhancement of neural differentiation of MSCs on graphene substrates, an highly effective triboelectric nanogenerator (TENG), used to supply pulsed electric simulation signals, was combined with poly(3,4-ethylenedioxythiophene) (PEDOT)−rGO hybrid microfiber (80 μm in diameter), which allowed the enhance of the scaffold's electrical conductivity. Firstly, they found out that the conductive rGO-PEDOT hybrid microfiber exhibited enhanced MSCs proliferation as well as good neural differentiation tendency. Then, graphene microfibers were submitted to electrical stimulation by using the TENG, triggered by human walking steps, to apply 3000 pulses/day during 21 days with a frequency of about 120 times/min, resulting in a significant increase in the neural differentiation of MSCs. Their findings illustrate the potential of rGO-PEDOT hybrid microfiber scaffolds for neural TE applications and also demonstrate the viability of a human-motion-driven self-powered TENG to electrically stimulate stem cells cultured on graphene substrates.

In a more recent study reported by Akhavan *et al.* [80] GO foam layers were fabricated by precipitation of chemically exfoliated GO sheets in an aqueous suspension under ultraviolet (UV) irradiation. This is a very versatile method where 2D graphene layers can be manipulated in order to produce 3D scaffolds with the desirable shape and size. Hence, rolled laminin-functionalized GFs were developed as electrically conductive 3D scaffolds and applied in directional growth of neural fibres, demonstrating that these structures allow the differentiation of hNSCs into neurons under an electrical stimulation. It was established that under low voltages (<5 V), the electrical sheet resistance of GFs is low enough (~170 Ω/sq) to produce the electrical stimulation currents (~20 mA) necessary to allow the differentiation of the neural cells. Moreover, it was demonstrated that the rolled shape of the GFs was also an important factor since it led to the formation of cross-sections with superhydrophilic characteristics which induced the effective proliferation and differentiation of the hNSCs through the pores and scaffold's interfaces. Finally, it was investigated the influence of the electrical stimulation in the differentiation of the hNSCs

cultured on the GFs. A series of 100 ms cathodic voltage pulses were applied on the two ends of the graphene roll in intervals with duration of 10 seconds. This stimulation resulted in an increased cells' proliferation and in an accelerated differentiation of the hNSCs into neurons (rather than glia) through the pores of the foam. Therefore, the findings presented in this study validate the capacity of 3D GFs to act as a flexible and conductive scaffold for the regeneration of nervous systems and TE. This work excited further studies as the one presented by Ahadian *et al.* [81] in which they demonstrated the influence of electrical stimulation on the differentiation of cardiac stem cells that are seeded on graphene substrates. Here, graphene nanosheets were embedded into a 3D structure of mouse embryoid bodies (EBs) by using the hanging drop technique, in a ratio of 0.2 mg per mL graphene in the EBs, being posteriorly confirmed that this inclusion did not hamper stem cells' viability. Moreover, results showed that the inclusion of graphene in the EBs led to a decrease in the stem cell proliferation, which can be explained by the accelerated cell differentiation caused by graphene. In addition, it was demonstrated that the inclusion of graphene not only allowed the enhancement of the mechanical and electrical properties of the EBs, but also allowed to significantly increase the cardiac differentiation on the EB-graphene substrates. To assess the influence of electrical stimuli on the cells seeded on this structures, an electrical current with a voltage of 4 V, frequency of 1 Hz and a duration of 10 ms during 2 continuous days was applied. The results presented in this study reveal an enhanced cardiac differentiation of the EBs after electrical stimulation, which demonstrates once more that graphene is a promising material for the differentiation of several types of stem cells via electrical stimulation, revealing its potential for inumerous stem cell-based therapies.

3.2. Optical Stimulation

3.2.1. Laser Stimulation

For many years, the use of electrical current to stimulate neurons was the most appellative technique [64]. However, several limitations such as the necessity of having direct contact between the stimulating electrode and the tissue [82], the possible toxicity associated with the electrode's material [83,84] and the influence of tissue impedance and coupling on the effective stimulation [85] led researchers to explore other viable alternatives to effectively stimulate the differentiation of stem cells. Pulsed laser stimulation is already being used as an alternative to electrical stimulation for evoking neural activity in motor and sensory systems [82,86,87]. In fact, the use of lasers for inducing neural responses is becoming a more appealing method as it is not only able to stimulate the desired tissue without the necessity of having direct contact but also offers an improved spatial resolution of stimulation [88]. Regarding its influence in the behaviour of stem cells, as laser irradiation has the capacity of providing intensive electric fields to the culture media, it is expected that it can open the calcium ion channels of the cells and that its magnetic field component can control the released calcium ions, which in theory would result in an increased calcium ion concentration inside the neurons, therefore enhancing neural regeneration [28]. Previous works have already demonstrated the efficacy of lasers for modifying the behaviour of stem cells [89,90], however, until very recently there were no reported studies that investigated the influence of pulsed laser irradiation on the stem cells' behaviour when these are seeded on graphene sheets. In this regard, Akhavan *et al.* [57] reported for the first time the use of graphene in the self-organized differentiation of hNSCs into neurons under pulsed laser stimulation. After the preparation of the films, by drop-casting a GO suspension onto quartz substrates and reducing some of the GO films with hydrazine, hNSCs were seeded on the synthetized GO and rGO films to assess, firstly, the biocompatibility of these templates in the proliferation of the neural cells. The results revealed that GO films are able to induce a higher proliferation of the cells which was explained by GO's superior hydrophilicity. On the other hand, it was demonstrated that the better electrical conductivity of rGO sheets enable them to induce more differentiation of the stem cells into

neurons. To promote hNSCs differentiation into neurons and to rearrange their orientation towards the centre of the laser spot, GO and rGO templates seeded with hNSCs were submitted to nanosecond pulsed laser stimulation by using a ND-YAG nanosecond pulsed laser system (wavelength of 532 nm, pulse duration of 5 ns, repetition rate of 10 Hz, power of 0.3 W, maximum pulse energy of 300 mJ, and spot size of ≈ 0.5 cm^2) which was used at its second harmonic oscillation for 30 s at each hour to stimulate the cells through a photoexciting method. The results presented in this study clearly show that rGO sheets, as a 2D biocompatible scaffold, when stimulated by pulsed laser irradiation, not only provide an accelerated differentiation of hNSCs into neurons (rather than glia) but also cause the self-organization of a neuronal network on its surface by elongating the differentiated cells in the radial direction, which can be explained by the radial stress that is induced by the surface thermal gradient. Contrariwise, a decelerated differentiation was observed on the quartz substrates, which was assigned to the extra local heating produced by the irradiation on the quartz substrate. When comparing the efficacy of GO and rGO substrates it was visible that rGO films produced better differentiation results mainly due to its higher thermal and electrical conductivity, which allow it to transfer the extra heat generated by the laser irradiation outward from the laser spot. Hence, their findings not only demonstrate the excellent ability of chemically exfoliated rGO sheets for neural regeneration and repairing, but also encourage further investigations using graphene as the substrate for the promotion of stem cells' differentiation by applying pulsed laser irradiation.

3.2.2. Flash-Photo Stimulation

In order to reduce the possibility of causing cellular damages, which are usually induced by electric field stimulation, flash photo stimulation also started to be investigated. The use of this type of stimulation in graphene-based scaffolds is advantageous when these are combined with metal oxide semiconducting materials, such as titanium oxide (TiO_2) or zinc oxide (ZnO), once graphene and GO have the capacity of trapping extra electrons and the metals are able to effectively generate photoexcited electron-hole pairs with

those trapped electrons. When the conditions are favourable, appropriate chemical bonds, such as Ti-C and/or Ti-O-C, are formed at the existing interface between graphene and the metal oxide, leading to the relocation of the electrons present in the pairs to graphene materials. The increasing number of photoexcited electrons present on the graphene sheets ultimately induces local electric fields. The induction of this local electrical field is crucial once it allows the modification of cell's behaviour, such as its differentiation, by affecting cells' calcium ion channels. Therefore, graphene-based materials are a potential viable substrate for the photocatalytic-based stimulation of cells [28,91-93]. In this regard, Akhavan *et al.* [66] presented a pioneer work in which the influence of flash photo stimulation in the differentiation of hNSCs into neurons on graphene-TiO_2 heterojunctions was investigated. Herein, for purposes of comparison the differentiation behaviour of hNSCs was evaluated with and without flash photo stimulation on three different substrates (TiO_2, GO/TiO_2, and rGO/TiO_2). The hNSCs cultured on the graphene-based substrates were submitted to a flash photo stimulation by applying flash photo pulse trains (produced by Xenon lamp of a Canon camera) with pulse duration of 4 s and a frequency of 1 Hz (4 flashes/s) that would repeat after each 60 s in a total time of 30 min at each 12 hours. Moreover, in order to find the optimal conditions of stimulation, the concentration of the ascorbic acid in the cultured media was varied as well as the flash intensity, that was varied from 1–100 mW/cm^2 by changing the distance between the flash and sample. As shown in Figure 8, the results presented in this study show that flash photo stimulation induced different responses in the 3 substrates, since on the rGO/TiO_2 substrate the number of cell nuclei was increased by a factor of ~1.5, whereas on the GO/TiO_2 substrate and on the TiO_2 substrate a smaller increase was observed with rates of 48% and 24%, respectively. This increase observed under flash photo stimulation in all the materials was explained by the ability of this stimulation to prevent cell death in the absence of cell growth factors. Furthermore, when the influence of flash photo stimulation on the differentiation of hNSCs was analysed it was observed that the rGO/TiO_2 film was the substrate that presented better results. In fact, an 88% decrease in the ratio of glial cells and an 81% increase in the ratio of neuronal cells was observed, contrary to the other substrates (GO/TiO_2 and TiO_2) were only a 25% and a

15% increase in the neuronal cells rate was, respectively, observed, revealing that although photo flash stimulation enhances the differentiation of hNSCs it is also dependent on the chemical state of graphene sheets as well as the composition formed between the graphene sheets and the beneath TiO_2 layer. The enhanced differentiation verified on the rGO/TiO_2 films was assigned to the fact that the photoexcited TiO_2 films enable the injection of electrons from this substrate into the cells that are cultured on the rGO sheets through the Ti–C and Ti–O–C bonds formed at the interface of graphene and TiO_2 films.

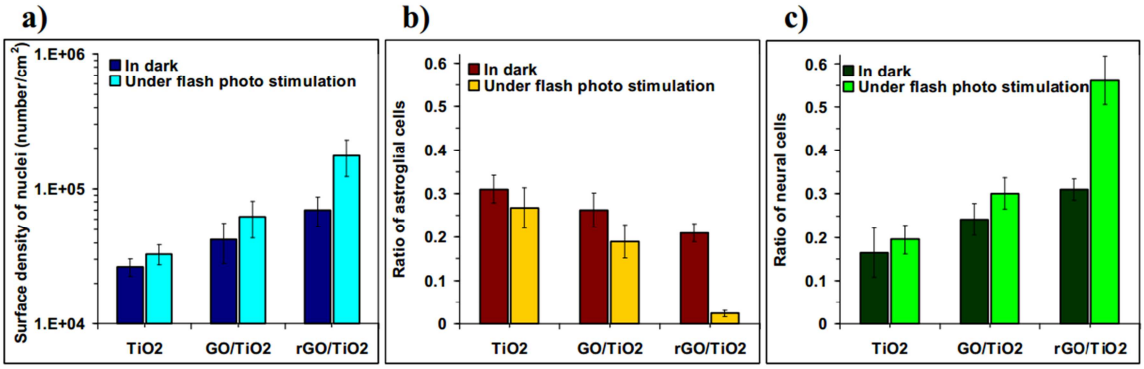

FIGURE 8- a) Surface density of cell nuclei and ratio of **b)** glial cells (GFAP-positivie cells) and **c)** neural cells (TUJ1-positive cells) on TiO2, GO/TiO2 annealed at 100°C and rGO/TiO2 after three-week differentiation in dark and under flash photo stimulation (n = 5, P< 0.01). Reproduced from Akhavan *et al.* [66] with permission from The Royal Society of Chemistry.

Finally, the optimal conditions for an efficient flash photo stimulation of hNSCs cultured on graphene-based substrates were assessed (Figure 9), being revealed that with a concentration of 15.0 mmol dm^{-3} of ascorbic acid in the cell culture medium and with an optimum flash intensity of 10 mW/cm^2 it is possible to not only increase the number of cell nuclei and differentiated neurons on rGO/TiO_2 (by factors of ~2.5 and 3.6), but also to decrease the number of glial cells (by a factor of ~0.28). These findings allowed introducing for the first time rGO/TiO_2 heterojunction film as a biocompatible flash photo stimulator for the effective differentiation of hNSCs into neurons, exciting further researches related with the photo stimulation of stem cells.

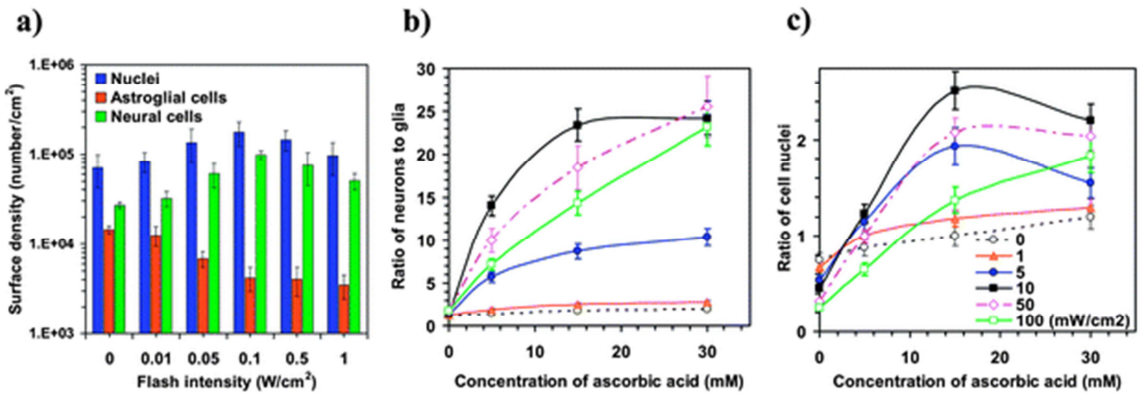

FIGURE 9- a) Surface density of cell nuclei, glia and neurons (n = 3, P< 0.05), **b)** ratio of neurons to glia (n = 3, P < 0.05) and **c)** ratio of cell nuclei (n = 5, P < 0.05) on rGO/TiO_2 film after three-week differentiation in a culture medium containing 15.0 mM ascorbic acid **(a)** and different concentrations of ascorbic acid **(b and c)**, under flash photo stimulation with various intensities. Reproduced from Akhavan *et al.* [66] with permission from The Royal Society of Chemistry.

Later on, in another work presented by Akhavan *et al.* [94] flash photo stimulation was used to induce the differentiation of hNSCs into 2D neuronal networks, which contained cell-to-cell and cell-to graphene electrical connections, on graphene nanogrids. To fabricate the nanogrids, graphene nanoribbons were synthesized by the oxidative unzipping of multiwalled CNTs and deposited on a SiO_2 matrix containing TiO_2 nanoparticles (NPs), being this structure proposed as a photocatalytic stimulator for the accelerated differentiation of hNSCs. Then, the hNSCs were stimulated to differentiate by applying, flash photo pulse trains (generated by a xenon lamp of a Canon camera) with 10 mW cm^{-2} of intensity, 4 s of pulse duration and 1 Hz of frequency (4 flashes per s), repeated after 60 s time intervals for 30 min at every 12 hours to the cultured substrates. The influence of flash photo stimulation on the differentiation of the cells was studied on both rGO nanoribbon (rGONR) grid/TiO_2 NPs/SiO_2 and quartz and rGO/TiO_2 substrates, in order to not only assess its effects but also to study the influence of the surface topography of the nanogrids on the differentiation of hNSCs. Thus, the results presented in this study show that the number of cell nuclei differentiated on rGONR grid/TiO_2 NPs/SiO_2 films increased 5.9 and 26.8 fold when compared with the number of cells on quartz substrates, in the dark and under

photo stimulation, respectively. In addition, the stimulation of the cells cultured on the rGONR grids, originated by the injection of photoexcited electrons from the TiO_2 NPs into the cells on the nanogrids, resulted in higher neural differentiation than differentiation of glial cells (1.8 and 0.17, respectively). The enhanced differentiation on the rGONR grids comparatively to the rGO sheets, which only had an increase of 1.6 and 3.1 (dark and under stimulation) was atributted to the physical stress induced by the surface topographic features of the nanogrids. Finally, the current-voltage properties of the neural networks that differentiated on the electrically disconnected rGONR grids were investigated with the purpose of analysing the formation of cell-to-cell and cell-to- rGONR electrical couplings after stimulation, which proved to be effective. Their findings, allowed confirming the ability of graphene-based materials to induce stem cell differentiation under photo flash stimulation, presenting rGONR grids as highly promising two dimensional scaffolds for applications in regenerative medicine and stem cell-based therapies, exciting further researches in the field of TE.

3.2.3. Near Infrared (NIR) Stimulation

Although flash photo stimulation has demonstrated to be an efficient method for the effective differentiation of hNSCs into neurons on graphene-based substrates [66], this is a method where the presence of a semiconducting photocatalyst material is mandatory, meaning that this stimulation will not produce favourable results when applied to substrates composed only by pristine graphene, once it acts like a zero band-gap semiconductor. Despite that, it has already been proved that is possible to induce band-gap opening and modulation of graphene not only by the fabrication of N-dope graphene sheets [95], but also by graphene nanoribbons [96] and graphene nanomeshes [28,68,69]. Although these structures have already been investigated for NIR photothermal therapies [23,24,97], up to now, there has been only one work that reported the study of the applicability of these graphene-based semiconductors in neural stimulation and differentiation. Therefore, in a more recent research presented by Akhavan *et al.* [98] the effects of applying NIR stimulation on hNSCs cultured on graphene nanomesh semiconductors were

assessed. To fabricate GO nanomeshes (GONMs), GO sheets were, firstly, photocatalytic degraded by using TiO_2 NPs embedded on the surface of a SiO_2 layer. For purpose of biological applications, several GONMs films were posteriorly reduced by hydrazine vapour, obtaining thereby rGO nanomeshes (rGONMs), which act as p-type semiconductors with a band-energy of approximately 1 eV. The biocompatibility of the graphene-based structures was first evaluated by analysing the adhesion and proliferation of stem cells in those substrates, being demonstrated that rGONMs have a similar biocompatibility in growth of hNSCs to GO sheets. Additionally, the number of cell nuclei and neural cells per unit area of the substrates (Quartz, GO, GONMs and rGONMs) studied was assessed, being established that GONMs are the most beneficial substrates for the proliferation of the hNSCs. These results were assigned to the excess of oxygen functional groups formed on edge defects of GONMs substrates, resulting in an enhancement of the surface's hydrophilicity. Afterwards, graphene-based nanomeshes were applied as semiconductor templates in NIR laser stimulation to induce the differentiation of hNSCs into neurons. In this work an 808 NIR laser with a spot size of ~0.5 cm^2 and a power density of 0.1 W/cm^2 was applied to the graphene-based nanomeshes for 10 min at each hour. In addition, the temperature of the samples was continuously monitored by using a thermocouple that was positioned inside the medium, since that for extended periods (10 min) of NIR irradiation the temperature of the incubated medium tends to increase to over than 45ºC, which can result in undesirable cell damages. Their observations demonstrated that under NIR laser stimulation rGONMs (with better chemical stability and higher electrical conductivity than GONMs) showed a higher level of cell differentiation, including elongation of the cells and increased differentiation into neurons than glia, when compared to quartz and rGO substrates. The higher biological activity of hNSCs cultured on the nonzero band-gap rGONM semiconductor than on the zero band-gap rGO sheets was attributed to the response of the low-energy (<0.5 eV) photoexcited electrons, which are injected from the rGONM semiconductors into the neural cells, to the NIR laser stimulation applied. In addition to that, the high-energy (~1.5 eV) photoelectrons present in the stimulated rGO could possibly inhibit cell proliferation and/or even cause cell damages. Finally, since the use of this laser irradiation produces thermal heating, the authors also

investigated the influence of conventional heating of the culture media up to ~ 43 ºC (the temperature typically reached under the irradiation) in order to assess the real influence of the NIR laser stimulation. Interestingly, they found out that no significant differentiation was induced under these conditions, which further confirmed that photoelectrons play an important role in the promotion of the hNSCs differentiation. Their findings not only suggest that rGONMs are promising scaffolds for neural regeneration and repairing, but also excite more researches related with the induction of stem cells' differentiation under NIR laser stimulation.

3.3. Mechanical Stimulation

Although it was already demonstrated that the behaviour of stem cells is influenced by the microenvironment in which they reside, the understanding of the mechanisms that regulate the fate decisions of stem cells is a subject that still attracts the attentions of many researchers [99]. In fact, it was just recently discovered that physical and mechanical factors have a significant influence in the modulation of cells' behaviour, being already demonstrated that the mechanical properties of the extracellular environment and the application of mechanical forces on cells can, indeed, trigger several cellular responses, including cells' self-renewal and its lineage specification [100-105]. As a result, the application of mechanical stimuli for inducing stem cell differentiation is becoming an increasingly recognized tool within the scientific community [99]. Since cells have the ability to sense forces, and transduce them into biochemical signals, when a perturbation on the cellular microenvironment occurs, including a change in the ECM stiffness, topography or composition, the cell immediately responds to those cues, adopting different shapes, generating traction stress or even producing other mechanical forces that can be transmitted to the neighbouring cells [102,105-107]. Therefore, when a mechanical stimuli, such as fluid shear stress, is applied to the cell surface several mechanosensitive ion channels, protein kinases and other membrane-associated signal-transduction molecules are activated, which triggers downstream signalling cascades that lead to changes in gene expression [108].

Graphene is a very versatile material that has the potential to act as an effective platform for inducing stem cell differentiation under mechanical stimulation. In a recent study, Kang *et al.* [109] reported the enhanced osteogenic differentiation of hMSCs by covalently conjugating the mechanical stiffness of GO flakes to 3D collagen scaffolds. The higher levels of osteogenic differentiation observed on the stiffer scaffolds were said to be mediated by MSCs mechanosensing, since the molecules that were involved in cell adhesion to stiff substrates were either up-regulated or activated. This study confirmed the importance of scaffold's mechanical properties and therefore, mechanical stimulation for promoting stem cell differentiation.

Furthermore, several theoretical and experimental studies have already suggest that mechanical strain influences the vibrational and electronic band structure of 2D graphene [110] and graphene nanoribbons (GNRs) [111]. In fact, in the work presented by Chen *et al.* [112] the effects of uniaxial strain in individual GNRs were for the first time successfully investigated. After applying uniaxial strains (0-6%) to individual GNRs with highly smooth edges by atomic force microscopy (AFM) manipulation, they were able to demonstrate that strain engineering GNRs allows to tune the bandgap of graphene in a non-monotonic manner, which could be beneficial for the modulation of cells' behaviour.

Despite of the unique properties of graphene which allow it to be a very advantageous platform for inducing stem cell differentiation, to this date there are not yet any reported investigation that demonstrates the effects of applying several mechanical strains on stem cells cultured on graphene-based substrates.

3.4. Chemical Stimulation

Due to its interesting chemical diversity, graphene-based materials have also been studied as substrates for controlling the differentiation of stem cells via chemical stimulation. Concerning this, Wang *et al.* [113] studied the effects of using modified graphene sheets on the MSCs neuronal commitment. Since cell growth is influenced by the surface chemistry of graphene templates [28], the authors produced a scaffold for stem cell growth composed by fluorinated graphene sheets. First, the CVD method was used to prepare graphene sheets

and then, to perform the fluorination of graphene several samples were exposed to a fluorinating agent. After studying the influence of cellular adhesion, morphology, gene expression and differentiation in terms of the surface chemistry, topography and mechanical properties of the substrate, they observed that fluorinated graphene substrates were able to induce a higher level of proliferation and also stronger polarization of MSCs when compared to substrates composed by graphene. To study the influence of chemical stimulation in the neuronal differentiation of these cells, the authors examined the expression of neuronal gene markers after the use of retinoic acid. When comparing the results of the cells cultured with and without the addition of the retinoic acid it was observed that neural differentiation can be significantly enhanced with the addition of the retinoic acid, which demonstrates that it can act as a neuron-inductive chemical agent.

The influence of the surface chemistry on the differentiation of stem cells cultured on graphene structures was also investigated by Akhavan *et al.* [114] which explored an alternative way to produce improved graphene sheets by using Asian red ginseng for the green reduction of chemically exfoliated GO into rGO. Concerning this, they analysed the influence of GO, hydrazine-rGO and ginseng-rGO films in the differentiation of NSCs into neurons. The results obtained show that ginseng-rGO films presented a better stability against aggregation when compared to the hydrazine-rGO ones in aqueous suspensions. Moreover, hydrazine-rGO films, which are hydrophobic, exhibited no toxicity against hNSCs whereas the hydrophilic GO and ginseng-rGO films proved to be more biocompatible since it allowed stem cells' proliferation after only 3 days. When evaluating the influence of these 3 substrates in the neural differentiation it was demonstrated that ginseng-rGO films were the substrates that allowed higher differentiation of hNSCs into neurons (rather than glia), followed by hydrazine-rGO substrates, being this explained by fact that rGO films have higher capability for electron transfer when compared to GO films. In the work already mentioned above, besides studying the influence of electrical stimulation, Tang *et al.* [72] also investigated the influence of chemical stimulation on the neural response of NSCs cultured on graphene films. Hence, high K^+ stimulation was applied to the cells by adding 50 mmol dm^{-3} KCl to the culture media. To assess the cell response the intracellular Ca^{2+} changes upon

stimulation were evaluated. The results presented, show that K^+ stimulation was able to induce a superior response (65% of fluorescence intensity) to electrical stimulation (30%) in the intracellular Ca^{2+} change, by allowing the depolarization of the cells and activating voltage-operated calcium channels which resulted in an increased extracellular Ca^{2+} influx. Furthermore, the authors also investigated the effects of this stimulation in the activation of C-jun, which is an inducible transcription factor that has the capacity to regulate other gene expression in response to extracellular stimuli [115]. Their examinations allowed to the conclude that K^+ stimulation induced the activation of C-jun in the neural networks on graphene substrates, once it was demonstrated that under stimulation the number of cells positive for phosphorylated C-jun was only 14%, whereas with stimulation the number significantly increased to 43%.

Meantime, in another investigation, Akhavan *et al.* [116] also tested the influence of using chemical inducers on the osteogenic differentiation of hMSCs cultured on graphene nanogrids. After demonstrating that the proposed hydrophilic graphene nanogrids were biocompatible, the authors investigated the influence of chemical stimulation on this type of substrate by adding known osteogenic inducers, such as dexamethasone and ascorbic acid. The results presented in this study reveal that the chemical stimulation of the rGONR grid induced an enhanced osteogenic differentiation of the hMSCs, revealing that the amount of osteogenesis in the patterned substrates was ~2.2 folds greater than the differentiation on the rGO sheets, which were uniform substrates. When comparing to the substrates that were not chemically stimulated, the authors observed lower levels of osteogenic differentiation on the patterned substrates and no differentiation on the graphene sheets. Hence, besides confirming the potential of chemical stimulation in the promotion of stem cell differentiation, this work also opened the path for further researches related with the surface topographic features since it was demonstrated that patterned substrates showed enhanced differentiation.

Later on, in the work reported by Weaver and Cui [117], a new nanocomposite composed by of conducting polymer PEDOT and GO nanosheets was fabricated and its NSC scaffolding performance evaluated. They found out that the biomolecules interferon-γ (IFNγ) and platelet-derived growth factor (PDGF) are able to selectively stimulate neuronal or

oligodendrocyte lineage differentiation, by covalently cross-linking to the surface of the GO or PEDOT nanocomposite, respectively, via carboxylic acid functional groups provided by GO. Additional, it was demonstrated that when the surfaces are stimulated with FNγ they have the capacity to support a larger population of neurons whereas when they are stimulated with PDGF they support a larger population of oligodendrocytes.

3.5. Topographical Stimulation

The microenvironment present on the cell imposes specific biophysical cues, including cells' topographical and mechanical properties, which can act through cell-substrates interactions and lead ultimately to the modulation of cellular behaviours such as differentiation [35]. In addition, since stem cells have the ability to sense the physical characteristics of their environment, specific biomechanical signals can be transmitted to them via various substrate topographical features such as pillars, grooves or pits [118]. Therefore, in the last years, researchers have started to further investigate the possibility of controlling stem cell fates by modulating some biophysical characteristics of the scaffolds, including the design of micro/nano-patterns, elasticity and scaffold's porosity [119-121].

The first work that studied the influence of morphological stimulation using graphene-based materials on the differentiation of stem cells was presented by Wang *et al.* [113], which investigated the possibility of inducing neural differentiation of MSCs by confining them into microchannels of fluorinated graphene surrounded by polydimethylsiloxane (PDMS) parallel lines in the absence of chemical inducers. To design the PDMS lines, these were ink-jet printed onto the fluorinated graphene substrates with the length and the width of respectively, 3 mm and 150 μm and a line spacing of ~50 μm, which resulted in a 30 μm wide fluorinated graphene microchannel in between the PDMS lines. The results presented in this study reveal that there is a correlation between the density of aligned MSCs and the coverage of fluorine on the graphene film and that the MSCs that were randomly seeded attached preferentially onto the fluorinated graphene microchannels, assuming a more elongated morphology as illustrated in Figure 10.

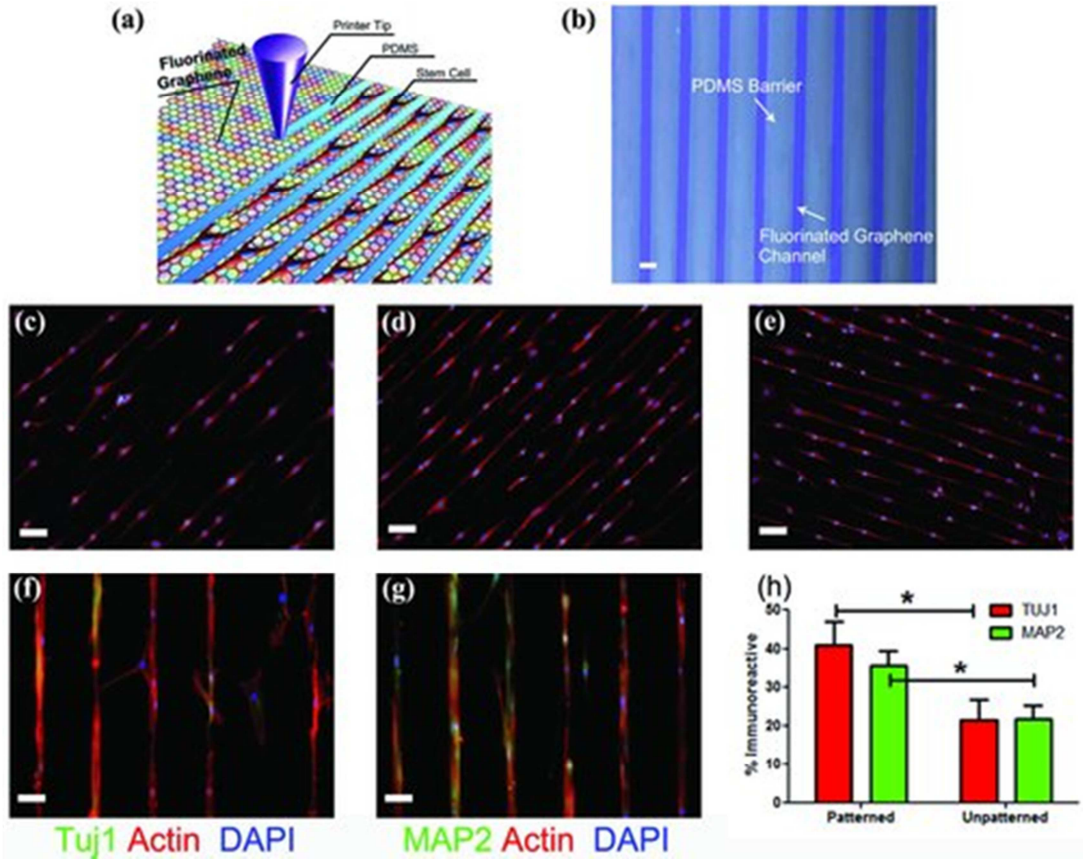

Figure 10- (a) Schematic drawing of patterning MSCs by printing PDMS barriers on graphene films directly. **(b)** Optical microscope image of printed PDMS on fluorinated graphene film (scale bar = 50 μm). **(c−e)** The aligned growth of stem cell on graphene, PFG and FG with printed PDMS pattern, respectively (scale bar = 100 μm). **(f, g)** MSCs preferentially attached on the FG strips and their F-actin aligned (red) and expressed neural specific markers - Tuj1 and MAP2 (green) (scale bar = 50 μ m). **(h)** Percentage of immunoreactive cells for Tuj1 and MAP2 on unpatterned and patterned FG strips. Note that the patterned FG strips induce higher expression of Tuj1 and MAP2 in the absence of retinoic acid. (n = 6, $p < 0.05$). Reproduced with permission [113] Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Moreover, they found out that not only the MSCs showed a preferential adhesion to the fluorinated graphene strips but also that their cytoskeleton aligned along the length of the pattern. To assess the effects on the neuronal differentiation, the expression of neuronal markers of the cells that were cultured on the micropatterns was analysed. When comparing with the results of the cells cultured under non-patterned fluorinated graphene, they observed

that the cells that were cultured under patterned fluorinated graphene demonstrated enhanced expression of the neuronal markers Tuj1 and MAP2 even without the presence of a neuron-inductive agent (Figure 10). This work allowed confirming that morphological changes in graphene-based substrates that induce variations in terms of cytoskeletal and nuclear alignment can, indeed, promote the differentiation of MSCs towards the neuronal lineage.

Afterwards, Kim *et al.* [122] proposed a simple method that uses graphene combined with chitosan to fabricate a nanotopographic substrate for stem cell engineering. The results presented showed that graphene-incorporated chitosan substrate was able to promote the adhesion and differentiation of hMSCs. Moreover, it was proved that the differentiation on rGO-chitosan substrate was higher than the ones obtained on the chitosan substrate and polystyrene regardless of the use of osteogenic of osteogenic induction media. Finally, they proved that nanotopographic cues of the substrate play a significant role in the enhancement of cell-to-cell and cell-to-material interactions, which results in an improvement of cells' functions.

Since it was already demonstrated in previous works [123], patterned geometries can play a significant role in the guidance of stem cell differentiation Akhavan *et al.* [94] proposed graphene nanogrids as a viable 2D patterned template for the proliferation and differentiation of hNSCs. The oxidative unzipping of multiwalled CNTs was applied to obtain graphene nanoribbons (length of 10 µm, width of 50-200 nm and thickness of 1 nm) and to posteriorly fabricate the graphene nanogrids. Then, the biocompatibility of rGONR grid/ TiO_2 NPs/SiO_2 films was investigated by using bright-field and flourescence imaging to analyse the adhesion and proliferation of hNSCs cultured on those substrates. For comparison, cells were also cultured on quartz, TiO_2 NPs/SiO_2, and GO nanoribbon (GONR) TiO_2 NPs/SiO_2 substrates. After analysing the fluorescence images, they observed that the hNSC were immunopositive for nestin (a green coloured marker used for staining NSCs). This observation allowed researchers to conclude that not only stem cells were able to proliferate on the films, but also that they exhibited the neural stem cell property as they induced positive results for nestin. Moreover, the hydrophilic graphene nanogrid coated films, in particular the GONR, demonstrated to have better cells' attachement as well as a increased proliferation of the hNSCs in the two

vertically preferred orientations, coincident with the pattern of the nanogrids, when comparing with the quartz substrate and the TiO_2 NPs/SiO_2 films, which exhibited lower cell growths with random orientations. Finally, they found out that elongated patterns of rGO nanogrids were able to demonstrate increased differentiation of hNSCs into neurons in the absence of any chemical inducer when compared with the SiO_2 substrate.

Meantime, Solanki *et al.* [124] reported the fabrication of a new substrate consisting of nanoparticle-based nanotopographical features modified with graphene. This substrate was studied for the enhancement of neuronal differentiation and growth. The arrays of graphene-silica NPs hybrid structures (SiNP-GO) were produced by coating GO sheets on the surface of positively charged silica NPs followed by packing on the surface of glass substrates. Since GO contains high concentration of oxygen functional groups attached to its basal plane (high electronegativity), they have a higher capacity to readily attach positively charged molecules or surfaces, which in this case was the surface of 300 nm silica nanoparticles (SiNPs). The substrates were then treated with the ECM protein laminin (10 μg/mL for 4 h) in order to provide further attachment and growth of the hNSCs on the GO. To investigate the influence of the proposed substrate (SiNP-GO) in the neuronal differentiation, other three substrates were used as control: a glass substrate with a positively charged surface; a glass substrate with a monolayer of positively charged NPs and a glass substrate with a positively charge surface coated with GO. Then the hNSCs were seeded in the four substrates and the influence of SiNPs, GO and SiNP-GO on the neuronal differentiation was analysed by performing immunocytochemistry and quantitative PCR on the differentiated cells after 14 days. Their findings revealed that in the first days of differentiation axons tended to be aligned only on the GO and SiNP-GO substrates and not on glass and SiNPs substrates. In fact, after 14 days of differentiation, these substrates exhibited very well aligned and well-extended axons, in opposing with the other substrates (glass and SiNPs) where although they demonstrated to have extended axons, they did not show any cell alignment. After analysing the images, the variation in the angle of orientation of the axons extending from differentiated hNSCs on substrates containing GO was quantified and compared with the orientation of the axons present on the control SiNPs and

glass substrates. Their results allowed the authors to confirm the observations made previously, since the angle of orientation of the axons from differentiated hNSCs on the GO and SiNP-GO substrates was ± 17.8°and ± 9.16°respectively, whereas in the other two substrates the axons extended randomly having a much higher variation of ± 42° and ± 46.11°, respectively. In addition to this, they also investigated the influence of nanotopographical features on the length of the axons extending from hNSCs that were differentiated on the different substrates on the day 14. It was confirmed that the length of axons present on the SiNPs was 20.76% more than the average length of those cultured on glass and 11-3% more than those cultured on GO, confirming that the alignment of the axons was exclusively induced by the presence of GO in the interior of the ECM while the SiNPs monolayer can induce an increase in the average length of the axons from hNSCs differentiated on SiNP-GO. Afterwards, to determine whether the alignment of the axons present on GO and SiNP-GO was due to the crowding of hNSCs and by consequence dependent on the seeding density, the authors reduced the cell density by 50% and after observing the cells' behaviour during two weeks, they confirmed that the axonal alignment of the differentiating hNSCs on the SiNP-GO substrates is not dependent of the cellular density, as the cells performed similarly. The effects of SiNP-GO on the neuronal differentiation of hNSCs were investigated by analysing the expression of immature and mature neuronal markers in the differentiated cells after two weeks. After performing immunostaining tests, it was demonstrated that a majority part of the aligned axons present in that substrate were characterized by the expression of several neuronal markers, including TuJ1, MAP2 and synapsin, and the axonal marker, GAP43. Moreover, it was demonstrated that the hNSCs differentiated on SiNP-GO substrates showed the highest expression levels for all neuronal markers. Next, for the first time the authors compared the axonal alignment of the differentiated cells on GO and CVD-grown graphene sheets. Although they observed axonal alignment on pristine graphene similar to the alignment observed on GO, they concluded that this was not sufficient for effective differentiation, being the GO substrates more advantageous for coating SiNPs, assembling ECM proteins and aligning the axons from differentiating hNSCs. Then, they further investigated the influence of the chemical structure of

graphene (hexagonal lattice carbon-based structure) in the axonal alignment. In this regard, a nanomaterial, molybdenum disulphide (MoS_2), with a physical structure similar to graphene was selected and deposited on glass substrates, which were already coated by laminin. Their observations allowed to conclude that the unique chemical structure of graphene caused the axons to align, once although the hNSCs have grown well and differentiated on these MoS_2 substrates, they did not reveal to have any axonal alignment. Hence, this work demonstrated the importance of morphological stimulation on the differentiation of stem cells, once it proved that is possible to enhance neuronal differentiation as well as axonal alignment by engineering specific microenvironments, which in this case consisted in nanotopographical features modified with GO.

In another work, Shah *et al.* [125] reported the use of graphene-nanofiber hybrid scaffolds for the guidance of NSCs differentiation into oligondendrocytes. In order to fabricate these hybrid scaffolds, GO was directly deposited on the surface of oxygen plasma-treated PCL electrospun nanofibers and then coated with the ECM protein laminin. They found out that GO-PCL nanofibers scaffolds have a higher capacity to promote the growth of more elongated cells when comparing to the simple PCL substrates. In addition, they demonstrated that the GO-PCL nanofibers scaffolds were able to induce a more selective differentiation of the NSCs into oligodendrocytes, which was confirmed by the presence of several oligodendrocyte markers, including CNP, PDGFR, Olig1 and Olig2, as well as mature oligodendrocytes markers, such as PLP, MBP, MAG and MOG. Moreover, it was observed that the use increased amounts of GO on the electrospun nanofibers further promotes the differentiation of NSCs into mature oligodendrocytes. Finally, they also demonstrated that GO contents of the nanofiber scaffolds have a significant influence on the overexpression of several key integrin-related intracellular signalling proteins, including focal adhesion kinase (FAK), Akt, integrin-linked kinase (ILK) and Fyn kinase (Fyn) proteins, known by promoting the oligodendrocyte differentiation.

In a more recent work, Kim *et al.* [35], hypothesize that the fabrication of combinatorial patterns of graphene-based nanomaterials could be a more advantageous approach to have a precise control of stem cell differentiation, in particular for the differentiation of human adipose-derived mesenchymal stem cells (hADMSCs). To generate the desired patterns over large surface areas the

microcontact printing (MCP) method was applied. They found out that GO line patterns were effective in the modulation of cells' morphology, which resulted in an enhanced differentiation of hADMSCs into osteoblasts. It was also confirmed that GO grid patterns are a promising method for the neuronal differentiation, once it allowed the highly efficient conversion of mesodermal stem cells to ectodermal neuronal cells, explained by its ability to mimic interconnected/ elongated neuronal networks.

4. CONCLUSION

Graphene and its derivatives shows to be emerging materials that holds great potential for biomedical applications, in particular on the development of stimulus responsive platforms for TE. Due to its unique structure and diversity of electrical, mechanical, chemical and morphological characteristics, graphene and its derivatives (GO and rGO) are now receiving increasing attention within the scientific community. In fact, its exceptional characteristics have already led many researchers to consider graphene as a promising platform for stem cell-based therapies, since graphene has already demonstrated to play an important role in the adhesion, proliferation and differentiation of several stem cells, demonstrating its potential not only for neuronal differentiation, but also for osteogenic and cardiac differentiation.

Moreover, besides of being an effective substrate for inducing spontaneous stem cell differentiation, graphene has already proved to be appropriated for inducing stem cell differentiation under several types of stimulation, including electric, NIR, flash photo, chemical, mechanical and morphological. Although the research with graphene for tissue regeneration is still in the early stages of development, this carbon nanomaterial may have a bright future in different clinical scenarios.

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