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Graphene-Based Smart Nanomaterials: Novel Opportunities for Biology and Neuroengineering

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Abstract In the last three decades, nanotechnologies have so deeply integrated themselves with medicine, that a new term, "nanomedicine," was specifically coined (Freitas in Nanomedicine, volume I: basic capabilities. Landes Bioscience, Georgetown, 1999, [110]) to indicate "the process of diagnosing, treating, and preventing disease and traumatic injury, relieving pain, and preserving and improving human health, using molecular tools and molecular knowledge of the human body. In short, nanomedicine is the application of nanotechnology to medicine." (Freitas in Nanomed Nanotechnol Biol Med 1(1):2–9, 2005, [1]).

As Freitas underlined in the same paper [1], though it has been formalized in the late 1980s [2, 3], the concepts themselves of nanotechnology and nanomedicine directly come from the famous visionary talk "There's plenty of room at the bottom" in which the Nobel prize winner Richard Feynman foresaw the great possibilities of the scale-down method: applying this method repeatedly he hypothesized the possibility of the construction of machines able to manipulate atoms and molecules [4].

Since then, so much progress has been made and so many goals have been achieved in several fields that, though not being completely aware of it, we can now consider nanotechnology as an integral part of our everyday life: Titanium dioxide nanoparticles are present in sunscreen lotions and in orthopedic implants; silver

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nanoparticles are used as antimicrobial agents in textiles and Iron oxide ones to improve the scratch resistance of paints; carbon fibers make our umbrellas lighter; nanosensors, nanometric drug carriers and *lab-on-chip* are more and more often used for theranostics at the nanoscale.

Nanoparticles and, more in general, nanodevices used in nanomedicine are synthesized from several elements, such as gold, silver, titanium, and carbon. The latter one, being the key element of life itself, has been intensively studied for biomedical and biological applications, in all its allotropic forms and, among them, researchers identified in nanocrystalline diamond, carbon nanotubes, and graphene extremely good candidates for drug and gene delivery systems, materials for coating electrodes for nervous system and cardiac stimulation, biosensors, and photothermal therapy.

Here we review some of the most important biological applications of graphene and its derivatives, such as graphene oxide (GO) and reduced graphene oxide (rGO), with emphasis on the applications in Neurosciences.

1 Toxicity

Interfacing new materials, regardless their being micro- or nano-, with biological systems requires in-depth biocompatibility evaluations, in terms of cyto- and genotoxity, generation of reactive oxygen species (ROS), interaction of the materials with cells constituents, biological media and organs, and depletion of essential nutrients for cell functions by absorption on surface's materials.

Though toxicity of nanomaterials based on carbon (C) (i.e. carbon nanotubes, carbon black, nanodiamonds, graphene, and its derivatives) has been extensively investigated, the debate in the scientific community is still quite heated given the controversial results and the remaining open questions about the precise mechanisms of internalization of these materials, as well as their localization once entered in the cytoplasm [5–8]. Furthermore, if on one side the most relevant limitations of CNTs in biological applications, such as the presence of metallic impurities and their asbestos-like shape [9, 10], can be overcome by graphene's morphology and synthesis methods, biocompatibility evaluations of graphene are made more difficult by different physicochemical characteristics of the forms employed, such as single-, few- or multilayer graphene, GO, rGO, nanosheets, nanoplatelets, and nanoflakes.

As reported for other C-based nanomaterials [11–13], surface functionalization of graphene reduces its unfavorable effects [14, 15], which are however due also to the size and shape of graphene itself.

Influence of lateral size of graphene and its derivatives on internalization and cell viability has been studied for several cell lines. Akhavan et al. observed and correlated, for the first time, in human mesenchymal stem cells (hMSc) treated with reduced graphene oxide nanoplateles and nanosheets [16] and graphene nanoribbons [17], increased levels of ROS, reduced viability and chromosomal aberrations

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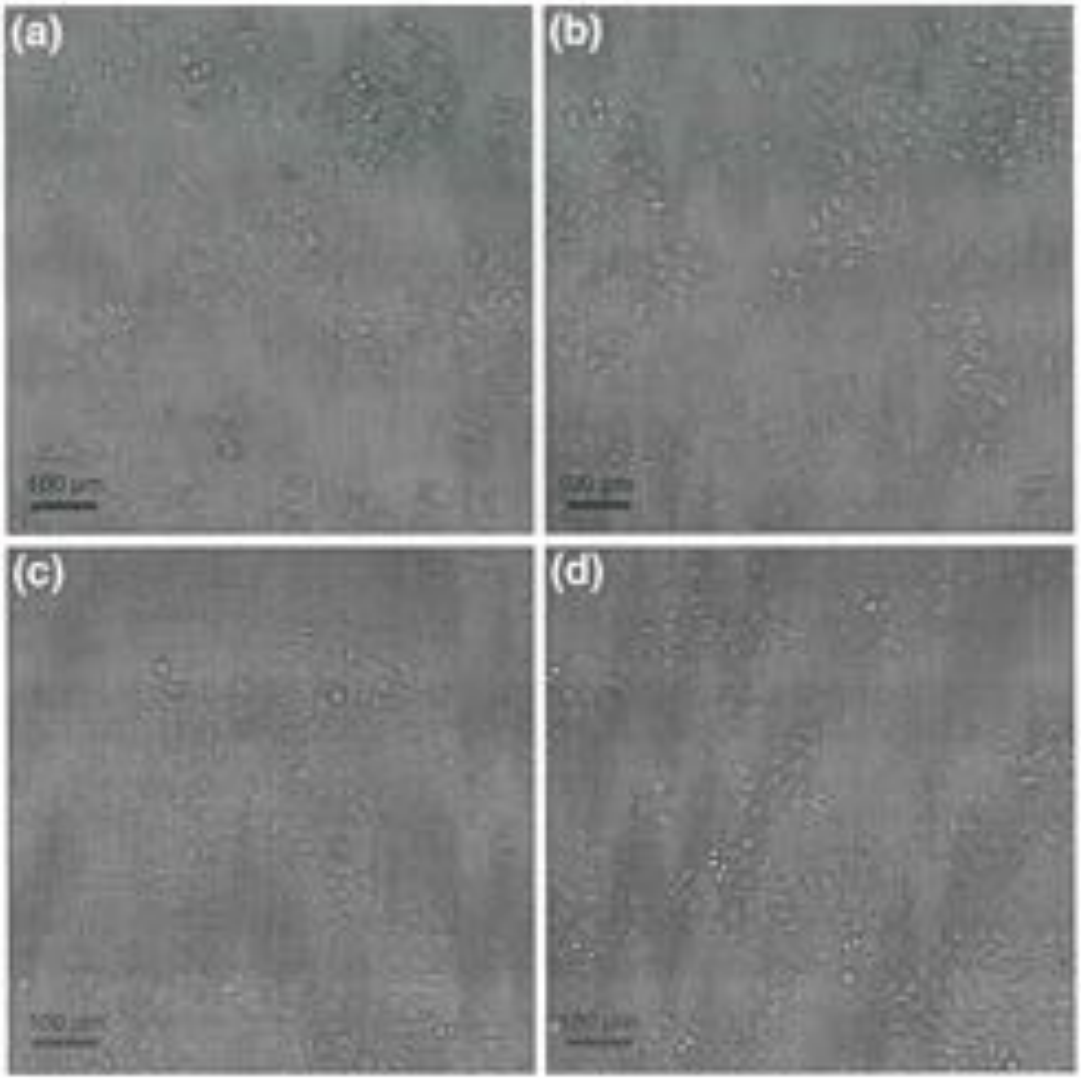

Fig. 1 Optical microscopy images of A549 cells grown on GO (**a**–**c**) and on control (**d**) substrates. Reproduced with permission from [109]

Another mechanism of GO toxicity, proposed by Hu et al. [23], suggests that physical damages observed in cellular membrane can be due to the electronic interactions taking place between the positively charged lipid layer and the negatively charged groups present on GO surface.

As already mentioned, surface modifications of graphene affect its toxicity; results of several studies show that carboxylated graphene and rGO exert less toxic effects than native graphene and GO [24], that chitosan coating of GO modulates its cytotoxicity [25], and that intravenous administration of amine-functionalized graphene ($G\text{-}NH_2$) to mice do not trigger any macrophages response leading to pulmonary thromboembolisms [26]. These studies also investigated haemocompatibility of graphene and GO [24, 25], which is a key aspect for drug delivery systems, which requires systemic route of administration. Graphene sheets resulted

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to be slightly more toxic than GO, and both materials did not affect coagulation pathways, though they induced a dose-dependent haemolysis of red blood cells.

Lack of consensus in cellular viability is reflected also in evaluations of graphene and its derivatives toxicity for bacterial cells; if, on one side, the use of GO, rGO, graphene, and composites GO–Ag can be investigated for designing antimicrobial coatings as pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Fusarium oxysporum*, and *Aspergillus niger* are inactivated by the presence of these materials [27–31], on the other side this antimicrobial activity has not been observed for both same and different families of bacteria, such as Shewanella [32–34].

In vivo assessments of contingent negative effects of graphene and its derivatives are less numerous than in vitro ones; in addition to the already cited study of Wang et al., the research conducted by Singh et al. on human blood platelets showed for the first time that intravenous injection of thin GO sheets in mice affects the release mechanisms of Ca^{2+} and the activation of Src kinases, this resulting in the formation of aggregates leading to pulmonary thromboembolism. These adverse effects showed a dependence on surface charge distribution of the material, as they were mitigated, albeit not completely removed, administrating rGO thin sheets [35].

Similarly to in vitro evaluations, material's functionalization leads to a modulation of its toxicity, as observed by Sahni et al. [36] studying the effects of PEGylated GO used as component for injectable hydrogels that resulted to be stable after administration to mice, without triggering sever toxic reactions.

Granuloma, pulmonary edema and rise of inflammations are reported for intravenous injection of GO at the dose of 10 mg/kg in mice, while no pathological changes of organs were found at 1 mg/kg. Lungs seem to be the preferred target organ for GO; although this makes it a good candidate for targeted drug delivery, its difficult excretion might lead, in a long-term scenario, to the same adverse effects observed for higher doses because of GO retention in lung tissues [37].

2 Biomedical Applications

The remarkable properties of graphene directly derive from its peculiar chemical structure, in particular its surface easy to functionalize, the ability to adsorb several aromatic biomolecules, and of being processed in aqueous solutions, their having both hydrophilic and hydrophobic groups as well as the fluorescence quenching ability, and make it and its derived nanomaterials extremely interesting for biomedical applications.

This kind of applications can be divided in four main classes: (1) biosensors, (2) substrates, coatings and scaffolds for implants and tissue engineering, (3) biomedical imaging, and (4) drug delivery systems. Here we briefly review some of the most relevant studies.

2.1 *Delivery Systems*

Graphene's chemical structure and surface area makes it extremely interesting material as drug carrier because of the possibility of binding pharmacological molecules on both sides of the graphene sheet. However, graphene is insoluble in water and this requires, as first step, the oxidation to its water-soluble form, graphene oxide and the subsequent functionalization with surfactants, mainly PEG, to avoid the clustering of the material once in contact with biological media.

These aspects were investigated by the group of Dai et al. [20, 21], who synthesized PEGylated nano-graphene oxide (NGO) loaded with Doxorubicin and SN-38 (7-ethyl-10-hydroxy-camptothecin), two water-insoluble anticancer drugs, and with an antigen to a specific activated-glycosylated phosphoprotein over expressed in cancer cells in order to target specifically the famers. Stability of these systems exhibited a pH-dependency and the efficiency of the SN-38 loaded NGO, tested on a human colon cancer cell line (HCT-116) resulted to be comparable with the free SN-38 in DMSO but remarkably more potent than a similar drug, camptothecin (CPT-11), incubated with the same cell line. Furthermore, the intrinsic photoluminescence of these NGO was used to image living cells in the near-infrared region (NIR) with very little background.

The pH-dependency highlighted by Dai et al. was investigated by other research groups, who observed that the increased release of drug molecules, related to their improved solubility for lowered pH, might eventually lead to a controlled release of the drugs themselves into lysosomes once the system "drug-carrier" is internalized in cells by endocytosis [38–40].

Binding GO, covalently functionalized with sulfonate groups, to folic acid (FA) allows the specific targeting of human breast cancer cells (MCF-7), as they express FA receptors; exploiting this specificity, Zhang et al. [41] demonstrated that FA–GO loaded with a controlled mix of CPT and Doxorubicin efficiently targets only cells expressing FA receptors and that it is more toxic to MCF-7 cells compared to FA–GO loaded with only a single drug. FA–GO has been also tested as carrier for Ce6, a photosensitizer used in photodynamic therapy; results of in vitro studies demonstrated that incubating human stomach cancer cell lines (MGC803) with FA–GO–Ce6 and then irradiating them significantly affects viability of cells [42].

Thanks to their strong optical adsoption in the NIR region, graphene and its derivatives have also been investigated as agent for photothermal therapy. Hu et al. [43] synthesized and tested in vitro a quantum-dot-tagged rGO nanocomposite (QD-rGO) covalently bonded with FA to be used for both cell/tumor bioimaging and photothermal therapy of MCF-7 cells, observing a selective uptake of QD-rGO in the targeted cells and their consequent death following 4 min of irradiation at 808 nm. Zhang et al. [44] explored the simultaneous use of PEGylated nano-GO as photothermal agent and as Doxorubicin carrier, thus obtaining a nanocomposite able to deliver both a chemotherapic agent and heat. This nanocomposite was tested in vitro on a murine cancer cell line (EMT6) and in vivo on a Xenograft tumor

mouse model, and it resulted to be more efficient than the two therapies singularly applied, leading to a complete destruction of tumors without any recurrence.

GO was also investigated for gene delivery and for the combined delivery of drugs and gene; Bao et al. [45] reported the use of chitosan-functionalized GO as a carrier to separately deliver CPT and pDNA into human liver and cervical cancer cell lines (HepG2 and HeLa cells), while Zhang et al. [46] designed polyethylenimine (PEI) functionalized GO loaded with Doxorubicin and short interfering RNA (siRNA) which, inhibiting the protein expression of targeted proteins, might overcome the problem of multiple drug resistance of cancer cells.

2.2 *Bioimaging*

Given their intrinsic photoluminescence in the VIS and in the IR spectral regions, graphene-based materials have been investigated to image living cells and several biomolecules inside living cells in the NIR via fluorescence, magnetic resonance (MRI), and positron emission tomography (PET) imaging.

Hong et al. [47] covalently functionalized nano-GO with a specific monoclonal antibody (TRC105) binding a vascular marker for tumor angiogenesis (CD105) and investigated its tumor targeting efficacy and pharmacokinetics in an in vivo model of murine breast cancer using PET and biodistribution studies. Results showed that nanocomposites are mainly excreted through renal and hepatobiliary pathways and that TRC105-GO effectively targets the tumor, this suggesting the possible use of this nanocomposite as combined agent for photothermal therapy and drug delivery system.

The use of composites of GO and dextran-coated iron oxide nanoparticles (Fe_3O_4-GO) as T_2-weighted contrast agent for MRI has been reported by Chen et al. [48], who also highlighted that these composites exhibit significantly enhanced cellular MRI signal.

Several research groups focused their attention on photoluminescence of graphene quantum dots (GQDs) and on how different preparation methods and surface functionalization can affect it. Peng et al. [49] obtained 1–4 nm sized GQDs by chemical exfoliation and acid treatment of carbon fibers, modulating the color of their photoluminescence by changing the temperature of the reaction. Green-photoluminescent GQDs were tested in vitro on human breast cancer cell lines and obtained results showed that GQDs can be used in high contrast bioimaging applications (Fig. 2).

Blue-fluorescent amino- and carboxy-functionalized GQDs were obtained by hydrothermal cutting of graphene sheets in presence of ammonia and/or water solutions [50, 51]; yellow-fluorescent GQDs were electrochemically synthesized by graphite rods and their uptake and toxicity were tested on three different kinds of stem cells—pancreas and cardiac progenitor cells (PPCs and CPCs), and neurospheres cells (NSCs)—observing that these GQDs can easily be internalized by cells without affecting their viability [52].

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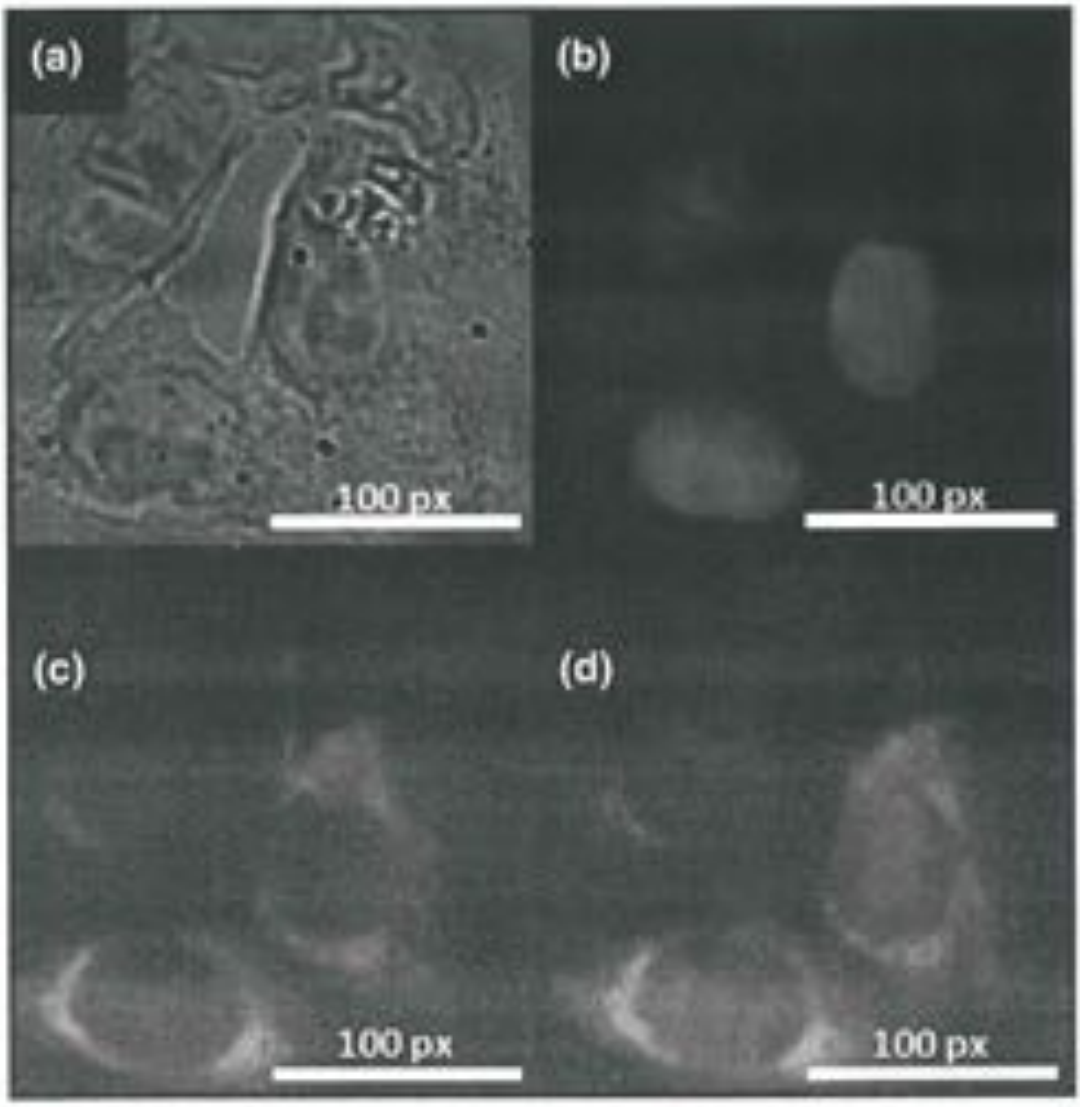

Fig. 2 Phase contrast picture (**a**) and fluorescent images (**b**–**d**) of human breast cancer cells incubated with green graphene quantum dots (GQDs). Nuclei are stained in *blue* (DAPI) and GQDs have *green fluorescence*; panel **d** shows the overlay of panels **b** and **c**. Reproduced with permission from [49]. © 2012 American Chemical Society

GO nanosheets, combined with DNA/RNA aptamers, were used as sensing platform for simultaneous, selective, and in situ detection of nucleotides involved in the regulation of several biological reactions, such as adenosine-5′-triphospahte (ATP) and guanosine-5′-triphosphate (GTP) [53]. This kind of detection is possible because no hydrolysis of ssDNA by deoxyribonuclease (DNase) has been reported ~~observed~~; this suggesting that, once adsorbed onto the surface of GO, ssDNA is protected from enzymatic digestion [54].

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2.3 Biosensors

Accordingly with the IUPAC definitions, a chemical sensor is a

> a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. Chemical sensors contain usually two basic components connected in series: a chemical (molecular) recognition system (receptor) and a physicochemical transducer. Biosensors are chemical sensors in which the recognition system utilizes a biochemical mechanism. [55]

Graphene, thanks to its excellent electrochemical properties, seems to be a promising material to be used in electrodes for detecting biomolecules.

Given the intrinsic fluorescence of GO from NIR to UV wavelengths [56], this material found use in the fabrication of fluorescence resonance energy transfer (FRET) sensors mainly interfaced with single-strand DNA (ssDNA), as the interaction between GO surface and the exposed bases leads to a strong adsorption of ssDNA to material surface. In this way it is possible to detect and quantify multiple ssDNA, as well as microRNA and double-strand DNA (dsDNA) [57–59]. A device composed by Silver nanoparticles as acceptor and GO, chemically treated with *n*-butylamine, as donor in a FRET sensor was developed and used to optically detect DNA, glutathione, cysteine, and immunoglobulin G [60].

So far, GO FRET biosensors have been used to detect a broad variety of biomolecules, such as insulin [61], proteins [62], and nucleotides [63], as well as metal ions [64, 65].

CVD-grown graphene was exploited in field effect transistor (FET)-based biosensors for detection of nucleic acids, growth factors and proteins [66, 67]; Loh et al. designed a graphene sensor integrated with microfluidic flow cytometry in order to detect red blood cells infected by malaria [19].

Wang et al. [68] developed a graphene modified electrode to be used for selective detection of Dopamine, overcoming the limitations of the simultaneous presence of ascorbic acid, whose oxidation potential is quite close to that one of Dopamine. A similar graphene/Pt-modified glassy carbon electrode was designed and successfully tested to simultaneously detect Dopamine, ascorbic acid and uric acid by Sun et al. [69]. This electrode was compared to only glassy carbon and to only graphene electrodes, and it resulted to provide better measurements of current and potential both using cyclic and differential pulse voltammetry.

2.4 Substrates, Scaffolds and Tissue Engineering

As other nanomaterials [70, 71], graphene and its derivatives have been used as substrates and scaffolds for differentiation of stem cells and antibacterial effects, as well as for culturing primary mammalian cells. Park et al. [72] reported an enhanced neuronal differentiation of human neuronal stem cells (hNSCs) grown on graphene films and measured the neural activity of these cultures using the graphene film

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itself as a stimulating electrode. A similar result was reported by Wang et al. [73], who observed that bone marrow derived mesenchymal stem cells (MSCs) cultured on fluorinated graphene showed neuron-like morphology with visible neurite protrusions and that they expressed neuronal gene markers.

Chen et al. [74] cultured induced murine pluripotent stem cells (iPSCs) on graphene and GO substrates, observing not only that both materials support iPSCs culture and allow for spontaneous differentiation into ectodermal and mesodermal lineages, but also that different substrates lead to distinct cell proliferation and differentiation characteristics. In particular, iPSCs proliferate and differentiate at a faster rate on GO than the control and the graphene condition.

The reasons of these enhanced growth and differentiation of stem cells on graphene and GO were investigated by Loh et al., who demonstrated that both graphene and GO act as preconcentration platforms for accelerated stem cell growth and differentiation through molecular interactions with growth agent [75].

3 Graphene in the Neurosciences

Their nanoscale dimensions, similar to those ones of the central nervous system (CNS), make nanomaterials ideal candidates for applications in neurosciences, and this drove researchers to investigate them (a) for developing both stimulating and sensing technologies to be interfaced with brain tissue and/or nerve cells to repair the brain on its own scale, (b) for refining brain imaging, (c) as a helpful tool in neurosurgery, and (d) for improving noninvasive diagnosis techniques allowing direct access to the CNS.

Graphene and its derivatives, as well as other C-based nanomaterials, has attracted great interest for applications in Neurosciences thanks to their chemical stability and electrically conductive properties. Three are the major applications: (a) as substrates and 3-D scaffold for neural growth, (b) as material for coating the electrodes of micro electrode arrays (MEAs), and (c) as material used for field effect transistors (FETs).

3.1 Graphene for Extracellular Stimulations and Recordings of Neuronal Activity: MEAs and FETs

Substrate-integrated microelectrode arrays (MEAs) are devices consisting of metallic electrodes (e.g., made of Pt, Au, and titanium nitrate) embedded in a planar substrate. Such devices allow the study of neuronal physiology, pathology and circuit-connectivity, both in vitro and in vivo, through extracellular recordings of

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neuronal activity. This methodology, though offering several advantages, such as the simultaneous detection of extracellular field potentials in a completely non-invasive way as well as the possibility of studying the very same neuronal networks over weeks and even months, is not (yet) able to detect synaptic potentials generated by single cells [76], and it has also to meet specific requirements, such as high spatial resolution, large signal-to-noise ratio (*S/N*), large charge injection limits, and great biocompatibility. If designing MEAs with smaller electrodes leads to improved spatial resolution, on the other hand this decreases the injected charge limits and worsen the *S/N* ratio, as a consequence of the reduced electrode's surface exposed to the electrolyte or to neuronal cell membranes. It is thus clear that the unique electrical properties of graphene make it a very interesting and promising material for the design of a novel class of (micro)electrodes whose use is not merely confined to electronic and material sciences applications [77–81].

An easy and relatively cheap technique to fabricate graphene-MEAs from a CVD-grown graphene films, deposited on quartz substrates, on which Au/Ti electrodes were patterned by lithography was developed by Du et al. [82]; such devices resulted to have good transparence and a *S/N* ratio comparable with that one of commercially available MEAs made of other materials and they were used to successfully detect extracellular spontaneous activity of cortical rat neurons from 14 to 40 days in vitro. Interestingly, once cleaned by means of conventional techniques (i.e., via mechanical washing and/or enzymatic digestion), graphene-MEAs exhibited a slightly higher value of impedance with respect to the value before their use, this indicating a long-term stability of the devices.

Another fabrication method has been recently proposed by Koerbitzer et al. [83], who deposited a film of CVD-grown graphene on gold and on silicon dioxide substrates to evaluate how graphene coating can influence the performance of, respectively, conductive yet opaque and not conductive yet transparent electrodes. Results of characterization of these devices showed that, when deposited on Au electrodes, graphene does not significantly modify the electrochemical properties of the electrodes themselves while, when deposited on SiO_2, it improves charge injection capacity so that these electrodes display performances comparable to those of TiN electrodes. These MEAs also showed good cell adhesion properties and biocompatibility, as they were used to culture cryoconserved embryonic cortical rat neurons for several weeks; however, the ability of these devices to detect extracellular signals remains an open question, as authors did not perform recordings or stimulation of the electrical activity in neuronal networks.

The possibility of designing transparent and flexible graphene-MEAs is extremely intriguing, as it might open new horizons in the investigation of electrical properties of populations of neurons such as the simultaneous optical imaging, Optogenetic modulation and electrophysiological recordings. Park et al. [84], for instance, developed an implantable graphene-based, carbon-layered electrode array (CLEAR) allowing high-resolution neurophysiological recordings. Characterization of these MEAs by means of cyclic voltammetry and electrical impedance

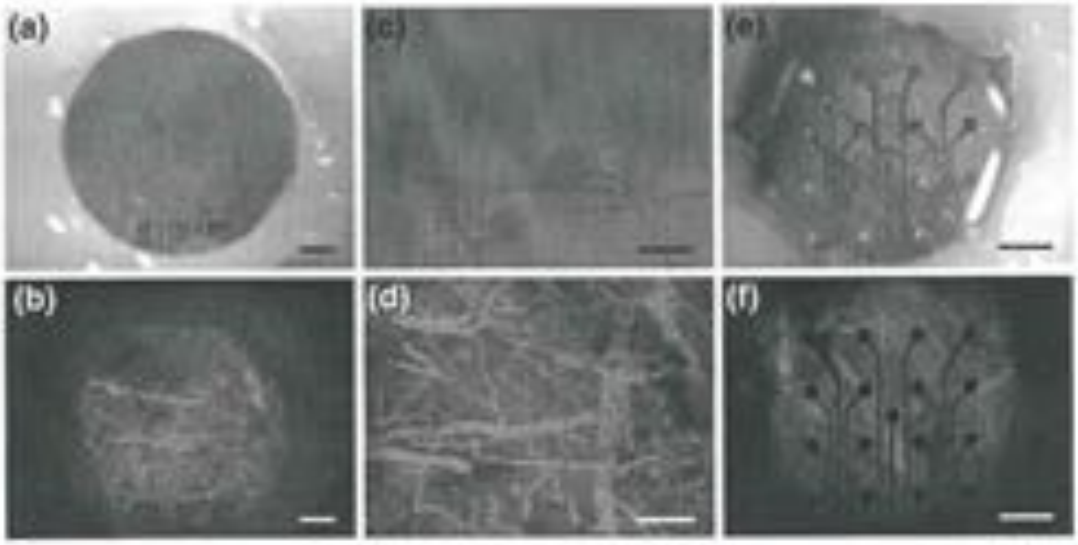

Fig. 3 In vivo cortical vasculature images through CLEAR device. Bright-field (**a**, **c**) and fluorescence (**b**, **d**) images of CLEAR device implanted on the cerebral cortex at different magnifications; *scale bars* 500 nm (**a**, **b**) and 250 nm (**c**, **d**). Bright-field (**e**) and fluorescence (**f**) images of standard micro-ECoG arrays; *scale bars* 750 nm. Reproduced with permission from [84]. © 2014 American Chemical Society

spectroscopy revealed a slightly higher impedance value, compared to conventional Pt microelectrodes arrays, which is thought not to affect recordings of neuronal activity, and similar CV curves were observed for the CLEAR device and Au microelectrodes. Efficiency of these CLEAR MEAs was then tested in vivo, by implanting them in both mice and rats, in comparison to conventional Pt devices; results show that CLEAR MEAs allow to record neuronal signals without difference from Pt MEAs, but with the significant advantage to allow optogenetic stimulation, as well as fluorescence and OCT imaging, directly through the electrode sites, made possible thanks to graphene transparency (Fig. 3).

Similar transparent devices were developed by transferring undoped or nitric acid-doped graphene grown by CVD onto flexible polyimide substrates, previously patterned with Au contacts, and single electrodes (doped and undoped graphene: $50 \times 50\ \mu m^2$; Au: $500 \times 500\ \mu m^2$) were tested for in vitro recordings from brain slices and in vivo electrocorticography recordings. Graphene and doped graphene electrodes showed lower impedances than Au electrodes, especially for frequency lower than 1 kHz, and they allowed in vivo recordings of neural activity with high *S/N* ratio, as well as calcium imaging in hippocampal slices by both two-photon and confocal microscopy [85].

Heo et al. [86] had also investigated the use of graphene for in vitro or in vivo stimulator devices; their research led to the design of a graphene/PET film to test the effects of non-contact field stimulations on cell-to-cell coupling. The electrical stimulation delivered through this film, whose biocompatibility and suitability for cell proliferation were demonstrated, affected the regulation of cytoskeleton protein

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related to cellular mobility, such as actin, this reflecting in morphological changes in cellular edges.

FETs are another kind of arrays used to record electrical activity of tissue and electrogenic cells and, if on one side they offer some advantages with respect to MEAs (i.e., easier fabrication of high-density structures, intrinsic amplification and better *S/N* ratio for structures of similar dimensions), on the other side they present the relevant setback of low stability of Silicon (their major component) in aqueous solutions, as well as the sharp edges and poor flexibility of crystalline structures needed in order to achieve a high *S/N* ratio; these drawbacks thus set limitations to the use of such devices for in vitro, but especially for in vivo, investigations [87–89].

Graphene, by virtue of its extraordinary electrical and optical properties and chemical stability, has attracted the interest of many researchers working in this field, and this led to the design and development of flexible graphene solution-gated FETs (graphene-SGFETs) with better gate sensitivity than common FETs and interesting *S/N* ratio, that were successfully used to record action potentials in electrogenic cells [78, 90, 91].

3.2 *Graphene and Neuronal Growth: Neural Stem Cells*

As shown in the previous paragraph, several studies have demonstrated that graphene and its derivatives enhance, though in a nonspecific way, cellular growth and the differentiation of different kind of stem cells—such as human neural stem cells (hNSCs) and mesenchymal stem cells (MSCs)—into neurons.

The important role of substrate's surface chemistry in the differentiation of MSCs into neurons has been investigated by Wang et al. [73], who reported stronger polarization and higher proliferation of MSCs seeded on fluorinated graphene substrates. This specific surface functionalization of graphene-induced morphological changes and promoted the differentiation of MSCs into neurons both in presence and in absence of neuron-inductive chemical inducers, such as retinoic acid.

Akhavan et al. [92] compared the contingent effects of GO and rGO, reduced by both conventional hydrazine-based and by an innovative green ginseng-based methods, on hNSCs; they highlighted a better attachment and a higher proliferation for cells grown on GO and ginseng-rGO when compared with both control and hydrazine-rGO condition, probably due to the higher presence of Oxygen group on the surface of ginseng-rGO and GO. Moreover, 3 weeks after the induced differentiation of hNSCs, by means of culture medium lacking growth factors, cells grown on rGO, and especially on ginseng-rGO, displayed significant morphological differences. These results seem to be related to the rGO higher capability for electron transfer and to the already mentioned higher hydrophilicity, and thus to a better biocompatibility, of GO and ginseng-rGO.

The same research group has also investigated the differentiation of hNSCs on GO nanogrids deposited on a substrate made of TiO_2 nanoparticles over a film of SiO_2 [93], on rGO/TiO_2 heterojunction films [94] and on GO and rGO films deposited by drop casting onto quartz substrates [95]. The particular design of these substrates, as well as their post-synthesis treatments, allowed for their use as biocompatible flash photo stimulators for effective differentiation of hNSCs into neurons, which led to a more differentiation of hNSCs into neurons than glia, and to a more pronounced increase in cell growth and alignment along the geometrical pattern of the nanogrids. It has also been observed that, after pulsed laser stimulation, cells grown on rGO-coated substrates exhibit the self-organization of neuronal networks by elongation of the differentiated cells in the radial direction, probably due to the higher thermal conductivity of rGO (with respect to only quartz and GO-coated quartz substrates) that might induce on rGO surface, by thermal gradient, a sort of radial stress originating from the center of the laser spot.

Enhanced neuronal differentiation has been also reported by Solanki et al. [96] (Fig. 4), who designed a substrate composed by positively charged 300 nm Silica nanoparticles, known for promoting axonal growth for neuronal cultures in vitro, coated by GO nanosheets, because of the presence of Oxygen groups on GO surface. hNSCs were seeded on (a) glass (control condition), (b) only Silica NPs substrates, (c) only GO-coated substrates, and (d) on GO–silica nanoparticle (SiNP–GO) and the differentiation was induced; while in the first 5 days axons growth was randomly directed in all the substrates, from the sixth day hNSCs on GO and on SiNP–GO displayed an aligned axonal growth not observed in the other two conditions. 14 days after the induced differentiation, cultures on SiNP–GO were characterized by a higher average length of the axons compared to control condition (about 20 %) and to only GO one (about 10 %). Moreover, hNSCs differentiated on SiNP–GO substrates showed the highest expression levels for neuronal markers. These results are due exclusively to the unique chemical structure of GO, as they were not observed for hNSCs grown on Molibdenum disulfide (MoS_2), a two-dimensional material with physical structure similar to GO.

Tang et al. [97] demonstrated that culturing neural stem cells on CVD-grown graphene films leads not only to morphologically healthy, but also developed and active neuronal networks. Using both calcium imaging and whole cell patch clamp recordings, authors observed that cells grown on graphene films (a) exhibit higher frequency of Ca^{2+} basal oscillations and (b) generate both spontaneous (sPSCs) and miniature postsynaptic currents (mPSCs), hallmark of network's normal activity and of synapse formation, with higher frequency and, only for sPSCs, higher mean current peak amplitude, with respect to the control condition. These results, though not shedding light on the mechanisms responsible for these observed features, suggest that graphene affects synaptic contacts, presynaptic events, and postsynaptic features.

Another interesting result has been recently published by Kim et al. [98], who reported the neurogenesis of hMSCs even in absence of any external neurogenic factors. Authors considered this "spontaneous" neurogenesis to be due to the enhanced formation, on graphene substrates, of three-dimensional clusters of hMSCs that, by mimicking an in vivo-like situation, might promote the secretion of cytokines and chemotaxic factors. This hypothesis seems to be confirmed by the fact that no remarkable differences were found for the body and nuclei shapes of hMSCs grown on glass and graphene-coated substrates, thus suggesting that graphene has a specific, yet still unclear, effect on the formation of these 3D spheroid structures and on the regulation of the growth and the neural differentiation of hMSCs.

The importance of developing innovative methods that, overcoming limitations of conventional cell culturing techniques leading to 2-D networks, allow the formation of 3-D neuronal networks where cells exhibit closer features to the complex in vivo conditions in terms of network morphology and gene expression, is at the basis of the work of Li et al. [99], who designed a 3-D graphene foam scaffold for neural stem cells. Such a scaffold resulted to be not only an extremely good substrate for cell proliferation and adhesion, allowing the formation of 3-D neural networks, but also to be able to up-regulate the expression of a protein, Ki-67, associated with cellular proliferation. Furthermore, the electrochemical properties of these scaffolds were investigated by cyclic voltammetry, in order to test the possibility of using them as neural stimulation electrodes; 3-D graphene foam scaffolds exhibited an increased electrical stimulation via a capacitive charge injection when compared conventional graphene film electrodes, probably due to the larger specific surface area of the 3-D scaffolds themselves.

3.3 *Graphene and Neuronal Growth: Primary Neuronal Cultures*

One of the first papers reporting on successful use of graphene films, synthesized by CVD, for culturing murine hippocampal neurons is that one of Li et al. [100]; in this works it was observed that cells viability is not altered by the presence of the graphene, that neurons grown on graphene films exhibit similar density and morphology with longer average length of neurites, when compared with control condition. Furthermore, it was also found an overexpression of the GAP43 protein, associated with neurites growth; authors hypothesized that this improved neurites sprouting and, consequently, the GAP43 overexpression might be due to both the nanoscale morphology of graphene films and to its high electrical conductivity.

As already highlighted, graphene's unique conductive properties make it one of the best candidates for interfacing with electroactive cells, as several physiological

functions involve electrical or charge transfer. This aspect was studied by Zhou et al. [101], who coated poly-ε-caprolactone (PCL) nanofibrous scaffolds with a graphene layer-by-layer self-assembly, in order to obtain electronically conductive tridimensional architectures with specific surface chemistry, that were successfully used as 3-D scaffolds for neuronal growth in vitro.

Sahni et al. [102] investigated the biocompatibility of CVD-grown graphene films interfaced with neuronal cultures, in terms of viability and of neurites outgrowth of cortical neurons on bare, graphene- and poly-D-lysine (PDL)-coated plastic polymer dishes. Remarkable differences were found in neuronal viability, higher on graphene and PDL substrates than in the bare ones, in their adhesion on graphene films, probably due to Van der Waals forces between the material surface and cell membranes, as well as in neuronal morphology, with neurons cultured on graphene displaying more linear dendritic structures compared to the other two conditions.

In order to investigate the properties of smaller isolated neuronal networks, both in terms of cell morphology and electrical properties, neurons can be forced to grow on an ordered pattern, obtained by a variety of techniques and using several materials and/or proteins to be patterned on the substrates. Results obtained by Lorenzoni et al. [103], place themselves in this very context: CVD-grown graphene deposited on glass and on silicon wafers were irradiated by single KrF excimer laser pulses to obtain series of stripes with higher surface roughness than the underneath glass, exposed by the laser, length of 800 μm and width variable from 30 to 60 μm, that were used, after coating with poly-D-lysine, to culture primary hippocampal embryonic neurons. After 7 days in vitro, neurons were found to grow and develop only on the graphene stripes, showing a healthy morphology despite the formation of cell clusters, and stayed healthy up to 3 weeks (Fig. 5).

Another confirmation that graphene can be used as a nontoxic material for interfacing neurons comes from the work of Bendali et al. [104], who successfully cultured retinal ganglion cells (RGC) on glass coverslips, CVD-grown graphene transferred on sapphire substrates and sapphire substrates, either bare or coated with laminin and poly-D-lysin. Interestingly, retinal neurons were found viable, after 6 days in vitro, on both bare and peptide-coated graphene, though a statistically significant reduction in the number of viable cells, as well a slight difference in cells size, were observed for cells grown on bare graphene when compared to peptide-coated substrates. Nevertheless, authors concluded that the observed experimental evidences indicate that RGC can grow and survive on bare graphene, though, in this condition, cells head for aggregation and formation of neurites bundles, as confirmed by the fact that cell processes resulted to be ticker on uncoated sapphire and graphene.

Luo et al. [105] designed and synthesized a biocompatible conducting polymer-based nanocomposite through the electropolymerization of poly (3,4-ethylene dioxythiophene) (PEDOT) in the presence of GO as dopant agent,

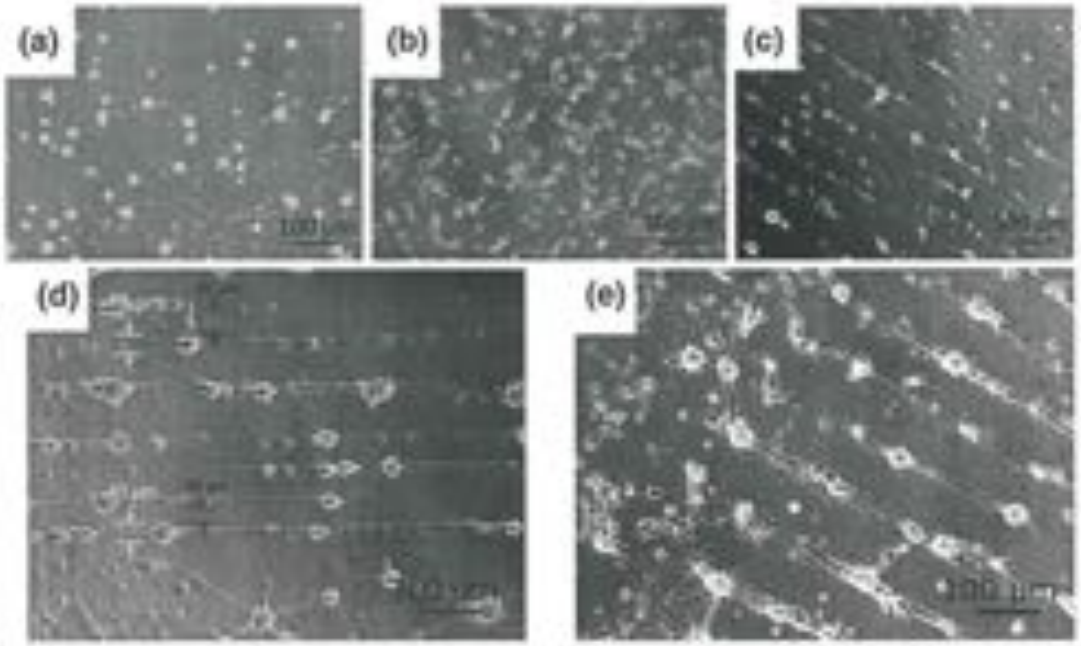

Fig. 5 Wide field transmission images of neurons seeded on different substrates: **a** bare glass/graphene (no neural network development observed); **b** PDL coated glass/graphene substrates (presence of neural networks). **In c–e** neural networks oriented along line patterns. Reproduced with permission from [103]

and used these PEDOT/GO films as substrates for neuronal growth, reporting no remarkable toxic effects and the development of neuronal networks with significantly longer neurites than control condition, even in absence of protein commonly used to increase cell adhesion.

As mentioned above, surface functionalization of graphene and C-based nanomaterials in general improves their biocompatibility; when it comes to neurons, this is not the only effect of chemically modifying graphene surfaces, as shown by Tu et al. [106]. In their study, they demonstrated that adhesion and outgrowth of neuronal cells, seeded onto graphene substrates, feel the effects of surface charges; rat hippocampal neurons were grown on carboxylated GO (GO–COOH) as control condition with negative surface charge, and on GO–COOH whose surface had been functionalized with three different functional groups: (a) methoxy ($-OCH_3$), with almost neutral surface charge, (b) amino (NH_2), with positively charged surface, and (c) poly-*m*-aminobenzene sulfonic acid ($-NH_2/-SO_3H$, PABS) which resulted to be zwitterionic. After 7 days in vitro, almost the 90 % of neurons were still viable on all the four substrates and neurons cultured on amino functionalized GO substrates showed a greater number of branches per neurite and of neurites per neuron, as well as a longer length of neurites, even without exhibiting relevant differences in cell morphology. However, it is difficult to comment these findings in

terms of contingent applications of these substrates as scaffolds and/or as electrodes material for neural stimulation, given the lack of a direct comparison with the conventional control conditions, such as glass or plastic culture substrates.

One of the key questions when novel materials are interfaced with neural cells for future translational applications is whether these materials allow the formation of fully developed and active neural networks. In this framework, we investigated the properties of GO and rGO as substrates for neuronal growth, with a particular attention to their biocompatibility and to the contingent alterations of the electrical properties of neurons and networks; we observed that, though no remarkable differences were found for the percentage of living cells of out the total across the three conditions, the total density of neurons grown on GO was reduced to almost the 35 % of the initial seeding density, while it was almost the 50 % for both control and rGO conditions. We explained this difference taking into account the fact that although GO, being atomically rougher than rGO, should promote neuronal adhesion, its superficial charge is more negative than rGO, and this aspect might have been then predominant, under our culture conditions. We also reported that both passive (i.e., input resistance; membrane capacitance, time constant, and resting potential) and active (i.e., action potential threshold; the peak of AP amplitude) neuronal properties did not significantly across the three conditions, with the only exception of the AP width at half amplitude that was slightly, yet significantly larger, on GO and rGO, compared to control; this can be attributed to differences in ionic channels expression, (e.g., KV), as their density and membrane distribution are known to affect AP shape. Furthermore, neurons grown on GO and rGO substrates exhibited a slightly higher spontaneous activity than control conditions, thus suggesting an earlier formation of synaptic connections or a stronger synaptic connectivity; this enhanced activity can be explained in terms of increased length and number of neurites, as previously reported, and in terms of the efficacy of excitatory synaptic connections and their number [107].

Similar results in terms of viability and ability of developing functional neuronal networks have been recently reported by Fabbro et al. [108], who grown hippocampal neurons on graphene substrates obtained by ball milling or liquid phase exfoliation of graphite. Such substrates resulted to be inert neuron-interfacing materials and they supported the development of neuronal networks in absence of any protein or polymer promoting adhesion; however, no perturbation of neuronal network synaptic performances has been observed with respect to control condition. Differences in impact on neuronal activity between graphene and CNTs might be due, according to the authors, to morphological differences between the two materials, especially in terms of their roughness which is, for CNTs, significantly higher (Table 1).

Table 1 Graphene and its derivatives in neurosciences

Material	Note	Model	Observed effects	References
CVD-grown graphene		Primary neuronal cultures	Good cell viability; longer neurites; overexpression of GAP43 protein	[100]
Graphene/PET films	Extracellular stimulation	Primary neuronal cultures	Biocompatibility; morphological modifications	[86]
Fluorinated graphene		MSCs	Morphological changes; promoted differentiation into neurons	[73]
Layer-by-layer graphene on PLC nanofibrous scaffolds	3-D structures	Primary neuronal cultures	Good adhesion and neuronal networks development	[101]
GO nanogrids on SiO_2 films + TiO_2 NPs		hNSCs	Biocompatibility; alignment along the geometrical pattern of the nanogrids	[93]
rGO/TiO_2 heterojunction films		hNSCs	Biocompatibility; enhanced differentiation of hNSCs into neurons than glia	[94]
CVD-grown graphene on Sapphire substrates		Retinal ganglion cells	Reduced viability and neurites bundles on pristine graphene; no remarkable difference with the control when treated with laminin	[104]
CVD-grown graphene	Solution-gated FETs	Retinal ganglion cells	Biocompatibility	[78]
3-D CVD-grown graphene scaffolds	hNSCs		Biocompatibility; upregulation of Ki-67 protein	[99]
Patterned CVD-grown graphene		Primary neuronal cultures	Ordered growth along the patterned stripes	[103]
PEDOT/GO films	Pristine graphene		Neurotoxic effects; longer neurites	[105]
CVD-grown graphene		Primary neuronal cultures	Good cell viability and adhesion	[102]
Silica NPs coated with GO nanosheets		hNSCs	Aligned axonal growth; higher average length of axons; higher level of neuronal markers	[96]
CVD-grown graphene films		NSCs	Healthy and active neuronal networks; higher frequency of sPSCs	[97]

(continued)

Table 1 (continued)

Material	Note	Model	Observed effects	References
GO and rGO		hNSCs	Effective differentiation into neurons; self-organization of neuronal networks	[95]
GO and rGO	Hydrazine-rGO and ginseng-rGO	hNSCs	Higher cell proliferation on GO and ginseng-rGO; morphological changes in ginseng-rGO	[92]
CVD-grown graphene on polymeric substrates	Undoped and Nitric Acid-doped graphene; MEA	Primary neuronal cultures and in vivo	Recordings of neural activity in vitro and in vivo electrocorticography	[85]
Graphene-based carbon layer electrode arrays (CLEAR)	Transparent MEA	In vivo implantation in rodents	Recordings of neural signals; optogenetic stimulation	[84]
GO-COOH + different functional groups leading to different surface charge	GO-COOH; GO-COOH-NH_2; GO-COOH-OCH_3; CO-COOH-PABS	Primary neuronal cultures	Better cell adhesion; more branches per neurites; more and longer neurites per neuron on GO-COOH-NH_2	[106]
CVD-grown graphene on quartz	MEA	Primary neuronal cultures	Detection of extracellular spontaneous activity	[82]
Graphene		hMSCs	Spontaneous neurogenesis without neurogenic factors; 3-D clusters of MSCs	[96]
GO and rGO	PEI treatment necessary	Primary neuronal cultures	Biocompatibility; higher frequency of spontaneous activity on GO and rGO	[107]
BM and LPE graphene	Pristine materials	Primary neuronal cultures	Formation of neuronal networks; no alterations of synaptic activity	[108]
CVD-grown graphene on Au and SiO_2	MEA		More charge injection capacity No recordings from cells	[83]

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