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

Antonina M. Monaco and Michele Giugliano

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 genotoxicity, 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 nanoplatelets and nanosheets [16] and graphene nanoribbons [17], increased levels of ROS, reduced viability and chromosomal aberrations

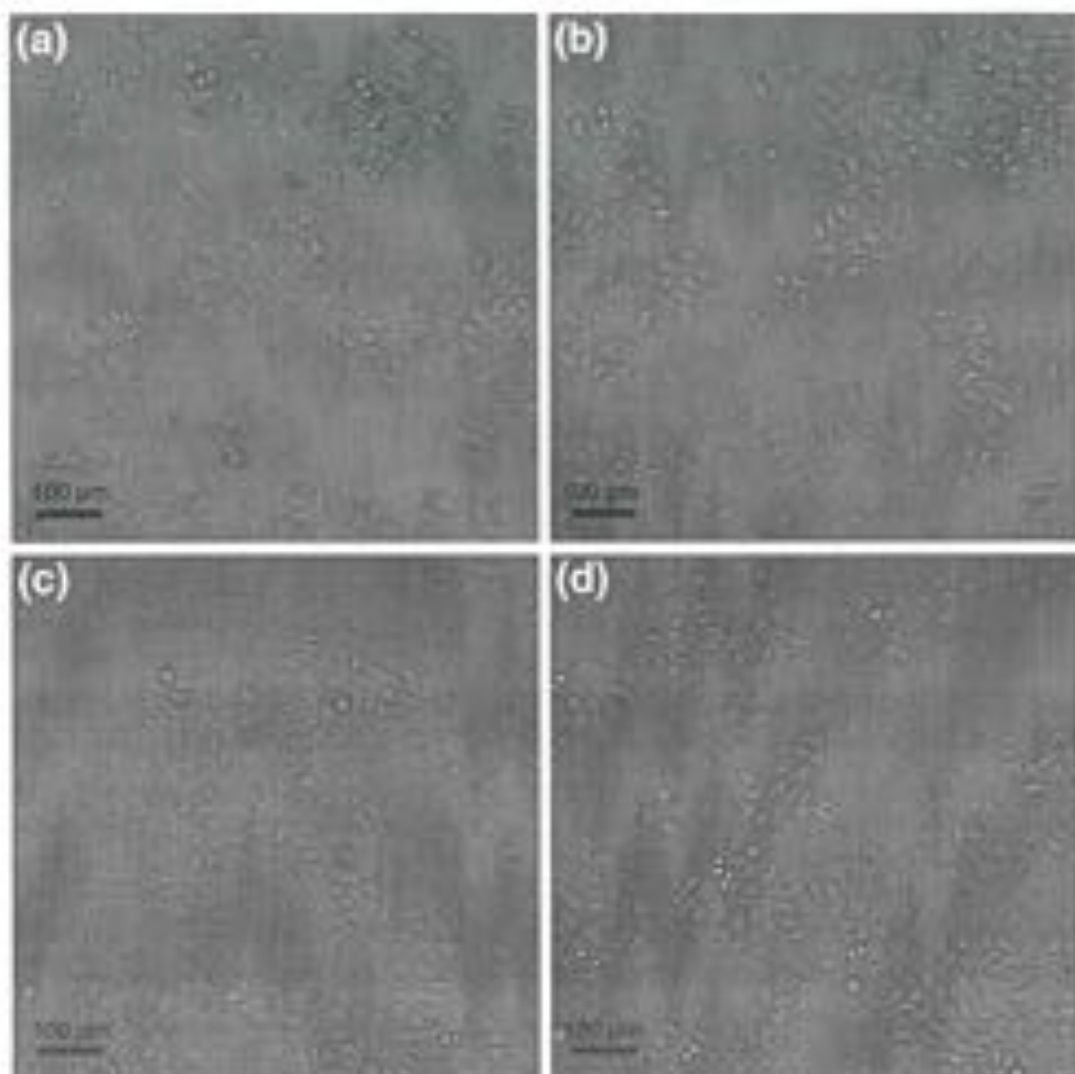


Fig. 1 Optical microscopy images of A549 cells grown on GO (a–c) and on control (d) substrates. Reproduced with permission from [109]

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-NH₂) 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



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

112 Lack of consensus in cellular viability is reflected also in evaluations of graphene
113 and its derivatives toxicity for bacterial cells; if, on one side, the use of GO, rGO,
114 graphene, and composites GO-Ag can be investigated for designing antimicrobial
115 coatings as pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Fusarium*
116 *oxysporum*, and *Aspergillus niger* are inactivated by the presence of these materials
117 [27–31], on the other side this antimicrobial activity has not been observed for both
118 same and different families of bacteria, such as *Shewanella* [32–34].

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

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

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

137 2 Biomedical Applications

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

144 This kind of applications can be divided in four main classes: (1) biosensors,
145 (2) substrates, coatings and scaffolds for implants and tissue engineering,
146 (3) biomedical imaging, and (4) drug delivery systems. Here we briefly review
147 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 tumors. 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 absorption 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 chemotherapeutic agent and heat. This nanocomposite was tested *in vitro* on a murine cancer cell line (EMT6) and *in vivo* on a Xenograft tumor



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

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

212 2.2 Bioimaging

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

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

225 The use of composites of GO and dextran-coated iron oxide nanoparticles
226 (Fe_3O_4 -GO) as T_2 -weighted contrast agent for MRI has been reported by Chen et al.
227 [48], who also highlighted that these composites exhibit significantly enhanced
228 cellular MRI signal.

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

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

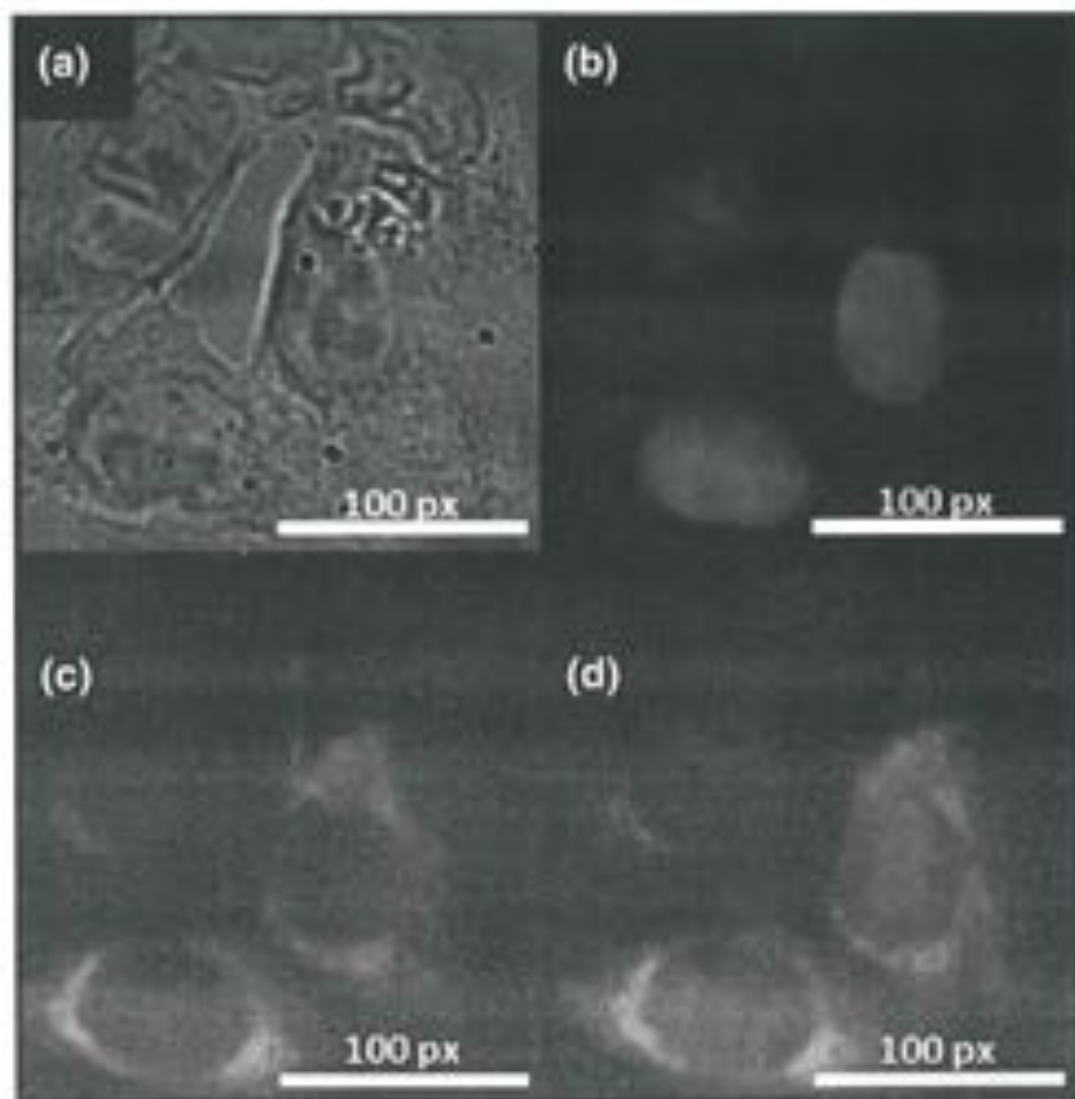


Fig. 2 Phase contrast picture (a) and fluorescent images (b-d) of human breast cancer cells incubated with green graphene quantum dots (GQDs). Nuclei are stained in blue (DAPI) and QDs have green fluorescence; panel d shows the overlay of panels b and c. Reproduced with permission from [49]. © 2012 American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

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



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. Bio-sensors 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



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



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

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

143 Another fabrication method has been recently proposed by Koerbitzer et al. [83],
144 who deposited a film of CVD-grown graphene on gold and on silicon dioxide
145 substrates to evaluate how graphene coating can influence the performance of,
146 respectively, conductive yet opaque and not conductive yet transparent electrodes.
147 Results of characterization of these devices showed that, when deposited on Au
148 electrodes, graphene does not significantly modify the electrochemical properties of
149 the electrodes themselves while, when deposited on SiO₂, it improves charge
150 injection capacity so that these electrodes display performances comparable to those
151 of TiN electrodes. These MEAs also showed good cell adhesion properties and
152 biocompatibility, as they were used to culture cryoconserved embryonic cortical rat
153 neurons for several weeks; however, the ability of these devices to detect extra-
154 cellular signals remains an open question, as authors did not perform recordings or
155 stimulation of the electrical activity in neuronal networks.

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

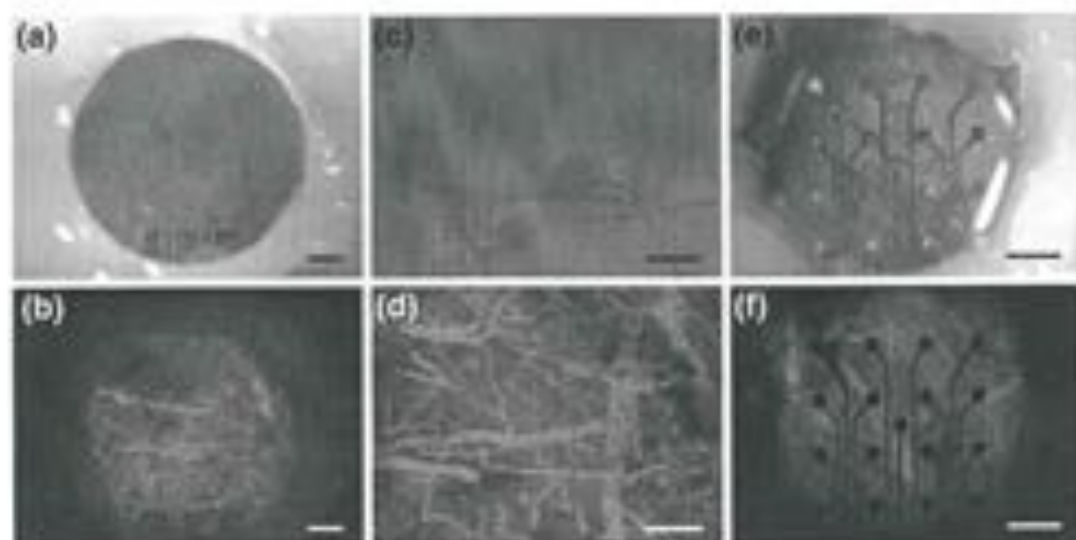


Fig. 3 *In vivo* cortical vasculature images through CLEAR device. Bright-field (a, e) and fluorescence (b, d) images of CLEAR device implanted on the cerebral cortex at different magnifications; scale bars 500 nm (a, b) and 250 nm (c, d). Bright-field (e) and fluorescence (f) images of standard micro-ECoG 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: $50 \times 50 \mu\text{m}^2$; Au: $500 \times 500 \mu\text{m}^2$) 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



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.

409 The same research group has also investigated the differentiation of hNSCs on
 410 GO nanogrids deposited on a substrate made of TiO₂ nanoparticles over a film of
 411 SiO₂ [93], on rGO/TiO₂ heterojunction films [94] and on GO and rGO films
 412 deposited by drop casting onto quartz substrates [95]. The particular design of these
 413 substrates, as well as their post-synthesis treatments, allowed for their use as bio-
 414 compatible flash photo stimulators for effective differentiation of hNSCs into
 415 neurons, which led to a more differentiation of hNSCs into neurons than glia, and to
 416 a more pronounced increase in cell growth and alignment along the geometrical
 417 pattern of the nanogrids. It has also been observed that, after pulsed laser stimu-
 418 lation, cells grown on rGO-coated substrates exhibit the self-organization of neu-
 419 ronal networks by elongation of the differentiated cells in the radial direction,
 420 probably due to the higher thermal conductivity of rGO (with respect to only quartz
 421 and GO-coated quartz substrates) that might induce on rGO surface, by thermal
 422 gradient, a sort of radial stress originating from the center of the laser spot.

423 Enhanced neuronal differentiation has been also reported by Solanki et al. [96]
 424 (Fig. 4), who designed a substrate composed by positively charged 300 nm Silica
 425 nanoparticles, known for promoting axonal growth for neuronal cultures *in vitro*,
 426 coated by GO nanosheets, because of the presence of Oxygen groups on GO
 427 surface. hNSCs were seeded on (a) glass (control condition), (b) only Silica NPs
 428 substrates, (c) only GO-coated substrates, and (d) on GO-silica nanoparticle (SiNP-
 429 GO) and the differentiation was induced; while in the first 5 days axons growth was
 430 randomly directed in all the substrates, from the sixth day hNSCs on GO and on
 431 SiNP-GO displayed an aligned axonal growth not observed in the other two
 432 conditions. 14 days after the induced differentiation, cultures on SiNP-GO were
 433 characterized by a higher average length of the axons compared to control condition
 434 (about 20 %) and to only GO one (about 10 %). Moreover, hNSCs differentiated on
 435 SiNP-GO substrates showed the highest expression levels for neuronal markers.
 436 These results are due exclusively to the unique chemical structure of GO, as they
 437 were not observed for hNSCs grown on Molybdenum disulfide (MoS₂), a
 438 two-dimensional material with physical structure similar to GO.

439 Tang et al. [97] demonstrated that culturing neural stem cells on CVD-grown
 440 graphene films leads not only to morphologically healthy, but also developed and
 441 active neuronal networks. Using both calcium imaging and whole cell patch clamp
 442 recordings, authors observed that cells grown on graphene films (a) exhibit higher
 443 frequency of Ca²⁺ basal oscillations and (b) generate both spontaneous (sPSCs) and
 444 miniature postsynaptic currents (mPSCs), hallmark of network's normal activity
 445 and of synaptic formation, with higher frequency and, only for sPSCs, higher mean
 446 current peak amplitude, with respect to the control condition. These results, though
 447 not shedding light on the mechanisms responsible for these observed features,
 448 suggest that graphene affects synaptic contacts, presynaptic events, and postsy-
 449 naptic features.

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

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

425 3.3 Graphene and Neuronal Growth: Primary Neuronal 426 Cultures

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

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



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

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

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

134 Another confirmation that graphene can be used as a nontoxic material for
135 interfacing neurons comes from the work of Bendali et al. [104], who successfully
136 cultured retinal ganglion cells (RGC) on glass coverslips, CVD-grown graphene
137 transferred on sapphire substrates and sapphire substrates, either bare or coated with
138 laminin and poly-D-lysine. Interestingly, retinal neurons were found viable, after
139 6 days *in vitro*, on both bare and peptide-coated graphene, though a statistically
140 significant reduction in the number of viable cells, as well a slight difference in cells
141 size, were observed for cells grown on bare graphene when compared to
142 peptide-coated substrates. Nevertheless, authors concluded that the observed
143 experimental evidences indicate that RGC can grow and survive on bare graphene,
144 though, in this condition, cells head for aggregation and formation of neurites
145 bundles, as confirmed by the fact that cell processes resulted to be thicker on
146 uncoated sapphire and graphene.

147 Luo et al. [105] designed and synthesized a biocompatible conducting
148 polymer-based nanocomposite through the electropolymerization of poly
149 (3,4-ethylene dioxythiophene) (PEDOT) in the presence of GO as dopant agent,

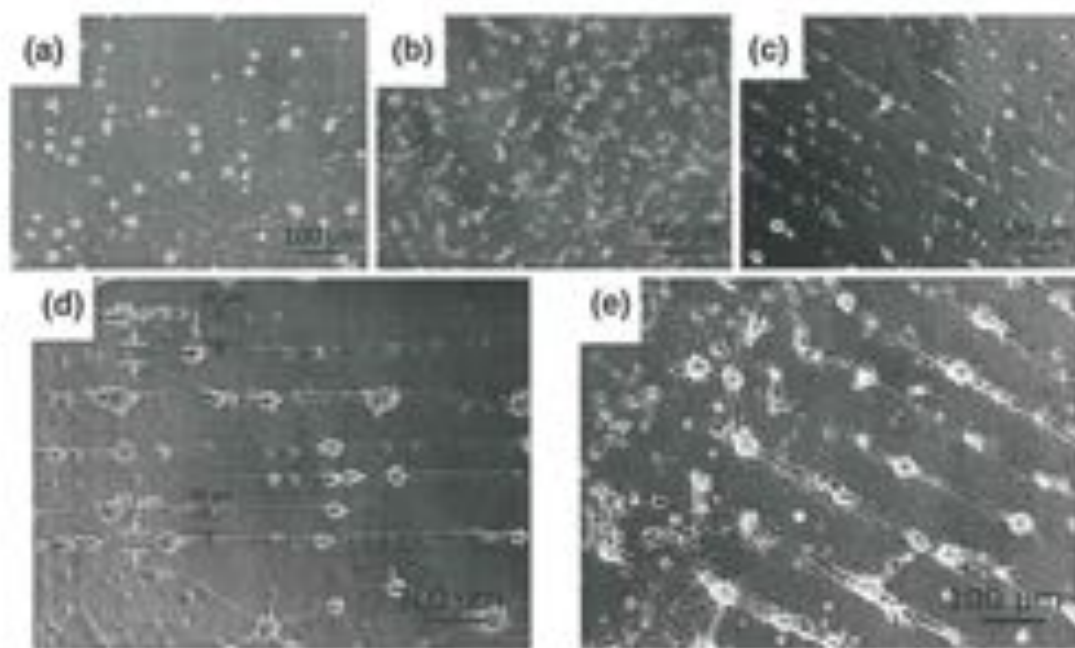


Fig. 5 Wide field transmission images of neurons seeded on different substrates: **a** bare glass/graphene (no neural network development observed); **b** PDL coated glass/graphene substrates (presence of neural networks). In **c-e** neural networks oriented along line patterns. Reproduced with permission 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	[71]
Layer-by-layer graphene on PLC nanofibrous scaffolds	3-D spines	Primary neuronal cultures	Good adhesion and neuronal networks development	[101]
GO nanoribbons on SiO ₂ films + TiO ₂ NPs		hNSCs	Biocompatibility; alignment along the geometrical pattern of the nanoribbons	[93]
rGO/TiO ₂ heterojunction films		hNSCs	Biocompatibility; enhanced differentiation of hNSCs into neurons than p18	[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]
PEODTGO films	Pristine graphene	Primary neuronal cultures	Neurotropic effects; longer neurites	[105]
CVD-grown graphene		Primary neuronal cultures	Good cell viability and adhesion	[102]
Silica NPs coated with GO nanoribbons		hNSCs	Aligned axonal growth; higher average length of axons; higher level of neuronal markers	[98]
CVD-grown graphene films		NSCs	Healthy and active neuronal networks; higher frequency of iPSCs	[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 pepsin-rGO	hNSCs	Higher cell proliferation on GO and pepsin-rGO; morphological changes in pepsin-rGO	[92]
CVD-grown graphene on polymeric substrates	Unidoped and Nitric Acid-dipped graphene; MEA	Primary neuronal cultures and <i>In vivo</i>	Recordings of neural activity <i>in vitro</i> and <i>in vivo</i> electrocorticography	[85] ✓ (a) ✓
Graphene-based carbon layer electrode arrays (CLEAR)	Transparent MEA	<i>In vivo</i> 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-PAB5	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 capability No recordings from cells	[83]

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