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Graphene: Promises, Facts, Opportunities, and Challenges in Nanomedicine

Hong Ying Mao,[†] Sophie Laurent,[‡] Wei Chen,^{*,†,§} Omid Akhavan,^{#,||} Mohammad Imani,[⊥] Ali Akbar Ashkarran,[⊗] and Morteza Mahmoudi^{*,○,▽,◆}

[†]Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

[‡]Department of General, Organic, and Biomedical Chemistry, NMR and Molecular Imaging Laboratory, University of Mons, Avenue Maistriau, 19, B-7000 Mons, Belgium

[§]Department of Physics, National University of Singapore, 2 Science Drive 3, Singapore 117542, Singapore

[#]Department of Physics, Sharif University of Technology, P.O. Box 11155-9161, Tehran, Iran

^{||}Institute for Nanoscience and Nanotechnology, Sharif University of Technology, P.O. Box 14588-89694, Tehran, Iran

[⊥]Novel Drug Delivery Systems Department, Iran Polymer and Petrochemical Institute, Tehran, Iran

[⊗]Department of Physics, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran

[○]Nanotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

[▽]Department of Nanotechnology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran



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Author Information

- Corresponding Author
Present Address

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1. INTRODUCTION

Graphene, a two-dimensional (2D) sheet of sp^2 -hybridized carbon atoms packed into a honeycomb lattice, has led to an explosion of interest in the field of materials science, physics, chemistry, and biotechnology since the few-layers graphene (FLG) flakes were isolated from graphite in 2004.^{1–4} Apart from other carbon allotropes, i.e., fullerenes, carbon nanotubes, and graphite, graphene exhibits a myriad of unique chemical and physical properties. Because of its honeycomb lattice with two carbon atoms per unit cell, the valence and conduction bands touch the Brillouin zone corners, giving rise to a linear dispersion of the energy spectrum.⁵ As a result, charge carriers in graphene behave like massless relativistic particles or Dirac fermions. In addition, a strong ambipolar electric field effect along with ballistic conduction of charge carriers was observed.⁶ Due to the delocalized out-of-plane π bonds arising from the sp^2 hybridization carbon atoms, an unprecedented high carrier mobility of $\sim 200\,000\text{ cm}^2\text{ V}^{-1}\text{ s}^{-1}$ has been achieved for suspended graphene^{7,8} and $\sim 500\,000\text{ cm}^2\text{ V}^{-1}\text{ s}^{-1}$ for graphene based field effect transistor (FET) using hexagonal boron nitride as dielectric.^{9–12} Another notable electronic characteristic of graphene is the unusual half-integer quantum Hall effect for both electrons and holes at room temperature.^{5,13} Moreover, single-layer graphene is highly transparent toward visible light ($\sim 2.3\%$ absorption).¹⁴ It possesses superlative mechanical strength with a Young's modulus of $\sim 1.1\text{ TPa}$.¹⁵ An unparalleled thermal conductivity ($\sim 5000\text{ W m K}^{-1}$) and large surface area ($2630\text{ m}^2\text{ g}^{-1}$) have also been reported.^{11,12} Owing

Received: August 15, 2012

to these unique chemical and physical properties and the unique biocompatibility, graphene has attracted much attention in the scientific community for numerous potential applications of graphene in biotechnology, including biosensing,^{16,17} disease diagnostics,¹⁸ antibacterial^{19–23} and antiviral materials,²⁴ cancer targeting²⁵ and photothermal therapy,^{26–28} drug delivery,^{29,30} electrical stimulation of cells,³¹ and tissue engineering.^{32–34} To enlighten the significance of graphene, we have monitored the publication growth rate within the scientific community using Scopus database (date of search: 22 October 2012). The papers that consists of the exact “graphene” word in the title are counted in the period of 1992–2013. Figure 1 shows the

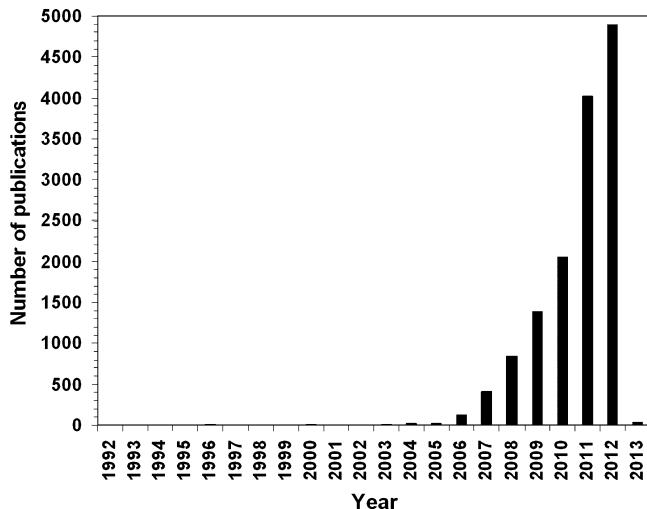


Figure 1. The number of papers containing the word “graphene” in the title published in the period of 1992–2013 (using Scopus database; date of search: 22 October 2012).

growth rate of publications in all these years. Specifically much attention has been drawn toward graphene in 2012 considering the time remaining in the year. This trend clearly reveals the global importance of graphene and the intense interest of the scientific community in this field.^{35,36}

For an extended search, derivatives of nanomedicine such as biosensing, biomedical, antibacterial, diagnosis, cancer and photothermal therapy, drug delivery, stem cell, tissue engineering, imaging, protein interaction, DNA, RNA, toxicity, and so on were also added. The papers that contain at least one of these words in the title, abstract, and keyword lists are automatically counted in the numbers during the mentioned period and the outcome is illustrated in Figure 2. As is clear from the results, the major portion of the statistics are devoted to the biomedical applications, indicating the great significance of graphene in nanobiotechnology.³⁷

These versatile applications also resulted in focused investigations on toxicity (including cyto- and very recently genotoxicity) of graphene.^{19,20} In addition, due to high potential demands for graphene and its derivatives (which can be mass-produced through reduction of the chemically exfoliated graphene oxide), environmentally-friendly as well as biocompatible reduction and functionalization of graphene oxide (GO) have greatly attracted the attention of researchers.³⁸

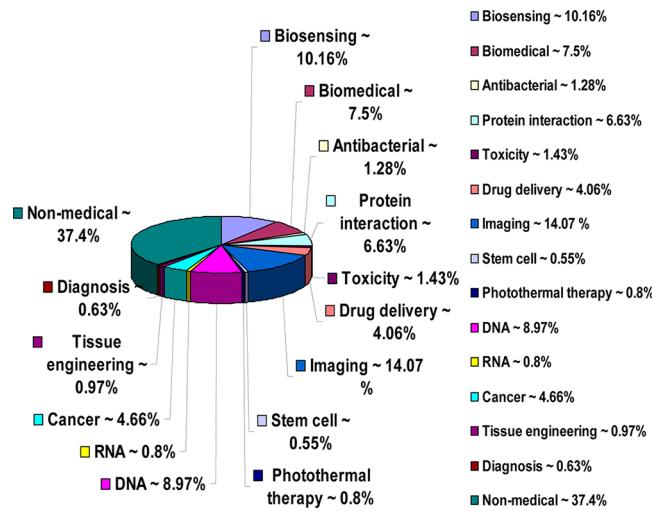


Figure 2. Distribution of different biomedical applications of graphene.

2. SYNTHESIS OF GRAPHENE AND GRAPHENE OXIDE

Up to now, various approaches have been developed for the synthesis of graphene and its derivatives, including mechanical exfoliation, epitaxial growth, unzipping carbon nanotubes, exfoliation of GO, liquid phase exfoliation of graphite, etc. The synthesis of graphene has been thoroughly reviewed elsewhere.^{4,39–42} In the following section, we will briefly discuss different methods for the synthesis of graphene with special focus on exfoliation of GO and liquid phase exfoliation of graphite, which exhibit great potentials in biological applications due to the easy accessibility for large-scale solution-based processes. Duch et al.⁴³ found that well-dispersed graphene at nanoscale and minimization of GO concentration in solutions can reduce their health risks; in fact, they explored various procedures, which could improve the biocompatibility of graphene in the lung, by applying different solutions of Pluronic-dispersed graphene and aggregating graphene into the mice lung. They observed that GO increases the rate of mitochondrial respiration and the generation of ROS, activating inflammatory, apoptotic pathways, which leads to persistent lung injury. On the other hand, in the case of pristine graphene the toxicity was mainly reduced after liquid phase exfoliation and was minimized when the unoxidized graphene was well-dispersed with the help of block copolymer Pluronic.

2.1. Mechanical Exfoliation

Despite the long history studies of graphite,⁴⁴ graphene was still a scientific model system until the few-layer graphene sample was isolated by Novoselov et al.¹ in 2004 with mechanical exfoliation of small mesas of highly oriented pyrolytic graphite. After fine-tuning, high quality single crystalline graphene samples with the lateral size up to millimeter-range was obtained.³ Graphene samples obtained by mechanical exfoliation are large enough for physicists to fabricate proof-of-concept devices, i.e., high-speed ambipolar field effect transistors and chemical sensor with single molecular sensitivity.^{45,46} However, it is low throughput and hence cannot be incorporated into practical applications which require the mass-production of graphene.

2.2. Epitaxial Growth

Epitaxial growth of graphene on single crystal silicon carbide (SiC) through high temperature annealing has also been reported.^{38,47–50} Graphene layers are formed by carbon

segregation after the evaporation of silicon atoms from the crystal surface. Chemical vapor deposition (CVD) is another thermal method for graphene growth by dissolving carbon atoms into a metal catalyst substrate, such as Ni, Cu, Co, Pt, Ir, and then forcing them to precipitate out after cooling to form large-scale graphene films.^{42,51–54} A unique advantage of CVD graphene is that after chemical etching of the metallic support, graphene films can be transferred to arbitrary substrates for practical devices fabrications.

2.3. Unzipping Carbon Nanotubes

Since carbon nanotubes are normally described as rolled-up cylinders of graphene sheets and the controllable synthesis of nanotubes is well developed, longitudinally unzipping carbon nanotubes represents a novel strategy to obtain graphene nanoribbons (GNRs) of precise dimensions.^{55–61} Taking advantage of potassium permanganate (KMnO_4) oxidation, a solution-based production of GNRs by unzipping multiwalled carbon nanotubes (MWNTs) has been reported.^{55,56,62–66} An extremely high yield of nearly 100% is achieved by this method. The electron confinement of the GNRs results in its interesting width-dependence electronic properties containing gradual transformation from semimetallic to semiconducting as the width decreases, particularly for sizes less than 10 nm.^{67,68} A similar property (transformation from semimetallic to semiconducting) was also observed in graphene nanomeshes.^{69,70} In addition, some other methods such as sonochemical⁷¹ and bottom-up⁷² approaches, lithography,^{68,73} and masking graphene by nanowires^{74,75} have been utilized for fabrication of GNRs. In order to obtain GNRs with smooth edges and controllable widths at high yields, argon plasma etching has been used to unzip MWNTs partly embedded in a polymer (PMMA) film.⁵⁶ High quality GNRs with a narrow width distribution of 10–20 nm are obtained by simply controlling the etching time.

2.4. Exfoliation of Graphite Oxide

The exfoliation of GO is the most developed method for the mass-production of graphene. In the presence of a mixture of KMnO_4 and concentrated sulfuric acid (H_2SO_4), the pristine graphite is converted into graphite oxide (GO sheets);^{76–79} however, one should note that high concentrations of some compounds (e.g., KMnO_4 in H_2SO_4 (i.e., around ~7 wt %/vol)) can be explored upon heating.⁸⁰ During the oxidation process, large numbers of oxygen-containing functional groups, i.e., carboxyl, epoxides, and hydroxyl groups, are attached onto the graphene basal plane and edges, making these GO sheets strongly hydrophilic. As a result, stable GO dispersions in water or polar organic solvents are obtained. Unfortunately, GO is an electrical insulator because a large proportion of sp^2 hybridized C–C bonds are converted into sp^3 hybridized C–C bonds.^{81,82} In order to restore the unique structural and electrical properties of pristine graphene, GO is reduced by strong chemical reductants (such as hydrazine⁸³ and sodium borohydride⁸⁴), thermal reduction,^{85,86} and/or microwave irradiation.⁸⁷ However, temperatures higher than 500 °C needed in the thermal reductions can restrict the efficacy of such methods in, e.g., fabrication of electronic devices. Meanwhile, strong chemical reductants such as hydrazine are found to be corrosive, highly explosive, and highly toxic.⁸⁸ For instance, hydrazine as a hepatotoxic and carcinogenic agent in kidney and liver damage can result in blood abnormalities, irreversible deterioration of the nervous system, and even DNA damage.^{89,90} In the recent years, reduction of GO by green and

biocompatible reductant agents such as melatonin,⁹¹ vitamin C,^{92,93} sugars,^{27,94} polyphenols of green tea,^{95,96} bovine serum albumin,⁹⁷ and even bacteria^{21,98} was also studied. Other green reduction methods (which some of them are also applicable at room temperature) have also been developed, such as flash photoreduction,⁹⁹ hydrothermal dehydration,¹⁰⁰ solvothermal reduction,^{101,102} catalytic,¹⁰³ and photocatalytic^{23,104–106} reductions. Although the extended π -conjugation of the graphene surface is largely recovered after reduction, the electrical conductivity of the reduced graphene oxide (rGO) is still several orders lower than that of pristine graphene due to the remaining oxygen-containing functional groups which disrupt the electronic structure of graphene. On the other hand, these remaining functional groups provide GO and rGO a tunability of chemical and electrical properties for different applications.^{107–109}

2.5. Liquid Phase Exfoliation of Graphite

In order to bypass the oxidation and reduction processes which result in the poor conductivity of rGO, liquid phase exfoliation of graphite has been developed recently.^{110–115} The most important factor for high yield and quality of the obtained graphene sheets is the surface energy of the solvent. When it matches that of graphene, a minimal energy cost of exfoliation is expected. Consequently, high-quality monolayer graphene is produced at significant yields.¹¹⁰ However, these particular solvents, such as N-methyl pyrrolidone, are expensive and require special care during handling, while for the most commonly used solvent, water, the surface energy is too high to work on its own as an exfoliation agent for graphene. An alternative way is to take advantage of water–surfactant solutions. Using sodium dodecylbenzene sulfonate (SDBS) as a surfactant, the dispersed graphitic flakes are stabilized against aggregation by Coulombic repulsion due to the adsorbed SDBS. After ultrasonication of graphite in water–SDBS solutions, FLG samples are obtained.¹¹¹ In addition, these surfactants tend to functionalize graphene sheets with tunable optical and electrical properties. The resulting graphene composites have been demonstrated to be suitable platforms for chemical sensors with specific selectivity.^{112,113}

2.6. Other Methods to Produce Graphene and Graphene Oxide

In addition to these approaches discussed above, there are many other methods for the synthesis of graphene. A mild, one-step electrochemical exfoliation of ionic-liquid functionalized graphite has been realized for the production of homogeneous graphene dispersion in polar aprotic solvents.¹¹⁶ Bottom-up organic synthesis is another approach. It has been reported that a graphene disc with carbon atoms up to 222 have been synthesized via this route.¹¹⁷

Another alternative for the synthesis of graphene as an appropriate, inexpensive, and less complicated technique is electrical arc discharge between two graphitic electrodes. Rao and co-workers¹¹⁸ synthesized graphene flakes including 2–4 layers in the inner wall region of the arc chamber using arc discharge between graphite electrodes under a mixture atmosphere of hydrogen and helium in different proportions without using any catalyst. In fact, they found that the presence of H_2 may minimize the formation of nanotubes and closed carbon structures. Similarly, Wu et al.¹¹⁹ observed considerable exfoliation and deoxygenation of GO, defect elimination and healing of exfoliated graphite during the hydrogen arc discharge exfoliation process. Wang and co-workers¹²⁰ produced large

scale graphene nanosheets using an arc evaporation of a graphite rod in air. They have found that the yield of graphene nanosheets depends on the pressure of the air; i.e., high pressure facilitates the formation of graphene nanosheets, but low pressure favors the growth of nongraphenic structures.

Recently Xu and co-workers presented a sonochemical approach for synthesis of polystyrene functionalized graphenes.¹²¹ They started with graphite flakes and a reactive styrene monomer for fabrication of polymer functionalized graphenes. They showed that styrene can serve both as a good solvent for exfoliation of graphite and as a monomer for the formation of reactive polymeric radicals.

3. PROTEIN–GRAPHENE INTERACTIONS

Protein adsorption on nanomaterials surface has received increasing attention for the past several years.¹ This phenomenon significantly affects the fate of these materials in biological systems including their cellular uptake and consequent toxic responses. Surfaces of nanomaterials are immediately covered by biomolecules (proteins, lipids, enzymes) upon their entrance into a biological medium.¹²² These coated surfaces confer a new “biological identity” to the nanosystem consisting of hydrophilicity/hydrophobicity, surface charges and energy, topography, etc. This new identity will determine the biological responses in the cellular/tissue level.^{123–129} Because of their high specific surface area, the carbon family of nanomaterials including graphene possesses potentially larger protein adsorption capacities than other nano-objects.^{130,131} After interaction with a biological entity such as cells, tissues, or organs, graphene sheets are completely different from their original pristine surface.^{132,133} Contamination of biomaterial surfaces with hydrocarbons, organic molecules, and elements was reported to change the surface composition and surface energy, which affected the protein absorption and cell attachment, proliferation, differentiation, and final osteointegration.^{132,133} Surface contamination of biomaterials is a thermodynamically driven process of surface energy reduction through the adsorption of adventitious air- or water-borne contaminants or renovation of the biomaterial surface through chemical processes.

Mao et al.¹³⁴ studied the protein adsorption profile on the surface of graphene sheets. Graphene-protein complexes were evaluated by atomic force microscopy (AFM), Raman spectroscopy, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-PAGE) and liquid chromatography mass spectrometry (LC-MS). The results clearly confirmed the existence of complex interactions between the proteins and the graphene sheet. The affinity of proteins to the surface of graphene sheets was significantly increased with a decrease in the molecular weight.

Lu and co-workers¹³⁵ decorated rGO sheets with β -lactoglobulin (BLG) which promote the reduced process of GO by hydrazine. They found that the BLG–rGO composite is an efficient platform for self-assembly of Au nanoparticles, and the resulting BLG-rGO/Au hybrid structures are promising candidates for surface enhanced Raman spectroscopy (SERS) applications. Recently, Mahmoudi et al.¹³⁶ showed that GO sheets can delay the amyloid beta fibrillation ($A\beta F$) process via adsorption of amyloid monomers due to the large surface active area of GO sheets. They found that the inhibitory effect of GO sheets was increased when the plasma protein concentration increases from 10% (*in vitro*; stimulated media) to 100% (*in vivo*; stimulated media).

For several applications, the existence of protein–graphene interactions can be quite useful. Zhang et al.¹³⁷ developed a graphene-based biosensing system for using peptides as probe biomolecules. They studied the feasibility for real time monitoring of protease activity based on fluorescence resonance energy transfer (FRET) between fluorescent peptides and graphene. Fluorescein isothiocyanate (FITC) labeled peptides are first adsorbed on the graphene surface, where they are in a quenched state. Protease activity cleaves the peptide, releasing the tagged tail to recover fluorescence. They choose a thrombin recognizing peptide as a model.

To show the sensibility of the FRET-based peptide-graphene biosensors for detection of thrombin activity, different concentrations of thrombin (between 0.002 and 0.3 μ M) were tested. The results indicated that the fluorescence intensity is linearly sensitive to thrombin concentration and increases with the increase of thrombin concentration. This novel probe offers many advantages for the simplicity of preparation and manipulation. This concept can be easily used for monitoring the hydrolytic activity of other proteases or kinases.

The authors supported this study further by measuring single amino acid adsorption on the graphene surface and found that positively charged moieties (like Arg, His, and Lys) and aromatic cycles (Trp, Tyr, and Phe) promoted adsorption. Very recently the authors found some evidence concerning size-, shape-, and concentration-dependent cytotoxicity and particularly genotoxicity of graphene to human mesenchymal stem cell (hMSC). The level of toxicities (particularly genotoxicity) increased and even significantly appeared at the ultralow concentration of $\sim 0.1 \mu$ g/mL after only 1 h time exposure as the lateral size of graphene decreased to nanometric scales (~ 10 nm)¹³⁸ or graphene nanoribbons (synthesized through unzipping MWCNTs) were used.¹³⁹ These results give some caution alarms about the potential risks in efficient applications of graphene-based nanomaterials (especially, nanosized graphene sheets) in future nanotechnology-based biomedicine including tissue engineering, drug delivery, and photothermal therapy.^{140,141}

Qin et al.¹⁴² used density functional methods to study the adsorption of L-leucine amino acid on graphene surface. They suggested that Van der Waals forces play a dominant role in the interaction, which is fully in agreement with the nonpolar surface of these nanomaterials lacking any functional groups except few (which are mostly on the edges) to provide other interactions like hydrogen bonds or polar interactions. However, evidently more research is required to study and completely understand the possible interaction mechanisms between proteins and graphene nanomaterials due to the emergence of new possible interactions like hydrophobic ones. These new interactions will arise due to the more complex structure of peptides, and consequently proteins, in comparison to simple amino acids and the presence of hydrophobic domains in the protein structure created by a sequence of amino acids. Interaction of proteins with graphene allows some advanced biological applications as biosensors, photocatalysts, drug delivery systems, and therapeutic modules but not without health risks.^{143–147}

The high active surface area of graphene, over other nanomaterials, is one of the main advantages of graphene based materials, which allows high-density biofunctionalization or drug loading. Due to the specific geometry of graphene (2D structure), both sides of a single layer graphene sheet can be

used as a substrate for the controlled adsorption of molecules and functional groups for surface modification. For instance, it is shown that covalent attachment of chitosan, folic acid, and polyethylene glycol (PEG) to GO produces a potential platform for the delivery of anti-inflammatory and water-insoluble anticancer drugs such as doxorubicin (Dox) and SN38, a camptothecin analogue.¹⁴⁸

A lot of biological applications based on the interactions of proteins with graphene or on its biofunctionalization have been described. Graphene and its derivatives can be functionalized with targeting species (e.g., peptides, avidin–biotin, proteins, and aptamers) through physical adsorption or chemical conjugation.¹³³ As previously explained, graphene can be used for the development of FRET biosensors. Compared to organic quenchers, graphene was shown to have a superior quenching efficiency for different organic dyes or quantum dots, with low background, high signal-to-noise ratio, and some advantages such as protection from enzymatic cleavage, due to the graphene itself.^{149,150} Graphene based biotechnology has also been used for living cell studies: (i) cellular probing and real-time monitoring,^{151,152} (ii) graphene field effect transistor (FET) for living cell detection,^{78,153} or (iii) drug delivery and cell imaging.³⁰

4. TOXICITY EVALUATIONS OF GRAPHENE AND ITS DERIVATIVES

Like other employed materials in nanomedicine, toxicity of the graphene is strongly dependent to its physicochemical properties (e.g., size and its distribution, surface charge, particulate state, number of layers, surface functional groups, and particularly shape).^{143,154–163} One of the most important issues for biomedical applications of graphene is its short- and long-term toxicity. Even though there are some valuable reports on the *in vitro* cytotoxicity effects of graphene, there is still no deep understanding on the involved mechanisms of cellular toxicity of graphene in the literature; therefore, this issue is yet to be explored in detail.^{19,164,165}

4.1. Bacterial Toxicity

Santos et al.^{166,167} have studied the bacterial interactions of graphene. The presence of bacterial toxicities suggests that graphene nanomaterials can be used in the future for applications such as antimicrobial coatings or products. Akhavan et al.²⁰ studied the bacterial toxicity of GO and rGO nanowalls against Gram-negative *Escherichia coli* (*E. coli*) and Gram-positive *Staphylococcus aureus* (*S. aureus*) bacteria. Both graphene derivatives were effective as antibacterial materials through direct contact interaction between extremely sharp edges of graphene sheets and cell wall membrane of the bacteria.²⁰ Hu et al.¹⁹ found that *E. coli* cell metabolic activity was reduced to approximately 70% and 13% after 2 h at concentrations of 20 and 85 mg/mL, respectively.¹⁹ It is notable that the BacTiter-Glo Microbial Cell Viability Assay was employed to measure the number of viable bacterial cells in cultures based on the quantification of adenosine triphosphate, which reflects the metabolic activity of cells.

These results were confirmed using transmission electron micrographs,¹⁶⁸ which revealed that the bacterial cells lost membrane integrity. However, the *Shewanella* family of bacteria, capable of metal reduction, has been shown to reduce GO in cultures with no inhibition of bacterial growth.¹⁶⁹ On the other hand, it was reported that *E. coli* bacteria can reduce GO to bactericidal graphene in a self-limiting manner.²¹

Intercalation of redox active metal ions such as Fe²⁺ between GO sheets may also be exploited for bacterial killing.¹⁷⁰ This fact could be exploited by designing metal-intercalated GO sheets for external application, e.g., to treat wounds infected with antibiotic-resistant bacteria.

Liu et al.¹⁷¹ showed an enhanced antibacterial activity of GO-Ag composites compared to Ag nanoparticles. Recently, Nguyen and Berry¹⁷² discussed results on graphene/cell interfacial biosensors and the principles of the modulation of charge-carrier properties in graphene derivatives via interaction with cellular membranes. Graphene's high sensitivity evolves from the π -carrier cloud confined within an atom-thick layer, quantum-capacitance-induced doping enhancement, closely spaced electronic bands, and a large surface area. They discussed the effect of the electronegativity of the cell wall and the dynamic changes in its chemical potential on doping specific carriers into graphene. Bacteria are trapped by the graphene sheets, and its edges damage the cytoplasmic membrane and inhibit bacterial activity.^{19,98,154} Bacteria and GO act as a redox system with electron transfer and electron mediator,^{98,169} but the exact mechanism is not clearly understood. The reactive oxygen species (ROS) generated in a concentration-dependent graphene is known as one of the important mechanisms describing cytotoxicity of graphene, especially at high concentrations (~100 μ g/mL),^{165,173} although it has been not checked for bacteria in detail. The direct contact interaction of extremely sharp edges of graphene nanowalls with wall membrane of cells is also proposed as one of the main mechanisms involved in cytotoxicity of graphene sheets.^{20,143} Trapping microorganisms within aggregating rGO sheets is also suggested as another effective mechanism for describing inactivation of microorganisms by graphene sheets, especially in a suspension.¹⁷⁴

4.2. Cellular Toxicity

The interaction between graphene or GO sheets and cells has been studied in monolayer cultures of lung epithelial cells,¹⁷³ fibroblasts,¹⁷⁵ and neural cells.¹⁶⁵ Single-layer GO sheets were internalized in cytoplasmic, membrane-bound vacuoles by human lung epithelial cells or fibroblasts and induced toxicity at doses above 20 μ g/mL after 24 h.^{143,175}

Huang et al.¹⁵⁶ employed surface-enhanced Raman spectroscopy to study the cellular internalization of gold nanoparticles-loaded GO using Ca Ski cells. In fact, the presence of gold NPs on the surface of GO enables detection of enhanced intrinsic Raman signals of GO inside the cell. They proposed that internalization of Au-GO into Ca Ski cells is happening mostly via clathrin mediated endocytosis and is an energy-dependent process. Yang and co-workers¹⁶² suggested that the exclusive 2D geometry with ultrahigh active surface area, small size (i.e., 10–50 nm in size), and biocompatible PEG coating may cause the enhanced permeability and retention effect of nanographene sheets for high tumor passive uptake. Very recently, Chowdhury and co-workers¹⁵⁸ exhibited dose (10–400 μ g/mL) and time (12–48 h)-dependent cytotoxicity of oxidized graphene nanoribbons. They observed that the degree of cytotoxicity was remarkably lower in MCF7 or SKBR3 cells compared to HeLa cells. They showed that water-solubilized O-GNR-PEG-DSPEs (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)]) have heterogeneous cell-specific cytotoxicity and mainly a different cytotoxicity profile, compared to graphene prepared by the modified Hummer's method. In this regard, much more investigations

should be done in order to fully clarify the toxicity and uptake mechanism of graphene-based materials.

Singh and co-workers¹⁷⁶ modified graphene with amine ($\text{G}-\text{NH}_2$). They found that amine-modified graphene structures had no stimulatory effect on human platelets, nor did it induce pulmonary thromboembolism in mice following intravenous administration. They have suggested that $\text{G}-\text{NH}_2$ is the safest graphene derivative with potential for biomedical applications due to its lack of thrombotic and hemolytic predisposition unlike other graphene derivatives. Chang et al.¹⁷³ studied the GO toxicity by examining its influence on the morphology, viability, and membrane integrity of A549 cells. Their results suggested a minimal toxicity at doses higher than $50 \mu\text{g/mL}$ and no cellular uptake. However, they demonstrated extracellular generation of reactive oxygen species (ROS) at high concentrations. It is noteworthy to mention that the oxidant-sensitive dye DCFH-DA was used for ROS detection. Thus, GO can be regarded as a relatively safe material at the mammalian cellular level, which is confirmed by the favorable cell growth on GO film.

Zhang et al.¹⁶⁵ also reported that a FLG increased intracellular generation of ROS and induced mitochondrial injury in neural cells after 4 and 24 h at a dose of $10 \mu\text{g/mL}$. Surface modification of graphene has been reported to alter its toxicity¹⁵¹ with reduced GO and carboxylated graphene reported to be less toxic than GO or native graphene.¹⁷⁷

Thin films made from graphene^{178,179} or GO¹⁸⁰ have been prepared via vacuum filtration of graphene plates through membrane filters. These graphene-based films have exhibited a combination of superior thermal, mechanical, and electrical properties.^{178–181} Biocompatibility of the graphene films was investigated using mouse fibroblast cell line (L-929)¹⁸² to assess the cytotoxicity of their surface used for cell growth comparing the results obtained for biocompatibility of carbon nanotubes.¹⁸³ The cells adhered and proliferated on graphene film well (see Figure 3) showing that the material is biocompatible.

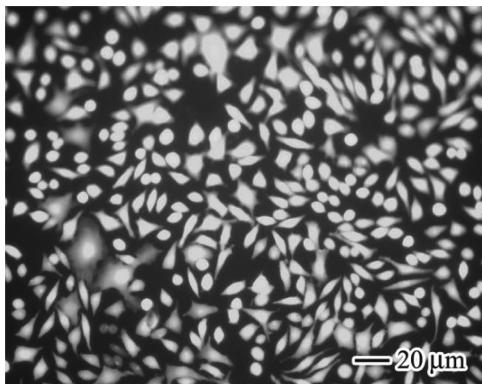


Figure 3. Fluorescence microscopy image of L-929 cells growing on graphene film. Reprinted from ref 182. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Some other studies have also shown the biocompatibility of the graphene derivatives in proximity of mammalian cells. Biris et al.¹⁸⁴ showed that osteoblast cells (MC3T3-E1) have a high ability to grow on graphene film. Agarwal et al.³³ reported that rGO is more biocompatible than single wall carbon nanotubes using different cell lines including neuroendocrine PC12 cells, oligodendroglia, or osteoblasts. Cells were cultured in parallel on carbon nanotubes and rGO films with the same initial

seeding density. For example, the proliferation of PC12 cells on the two types of substrates was monitored over 5 days after seeding. The cells proliferate well on rGO, whereas their proliferation is largely inhibited on nanotubes. Fe_3O_4 -GO nanocomposites presented a good physiological stability and showed low cytotoxicity to HeLa cells.¹⁸⁵ Prussian blue staining analysis showed that the Fe_3O_4 -GO nanocomposites can be internalized efficiently by HeLa cells, depending on the concentration of the composites incubated with the cells. Moreover, Fe_3O_4 -GO composites showed significantly enhanced cellular MRI, being capable of detecting cells at the iron concentration of $5 \mu\text{g mL}^{-1}$ with a cell density of $2 \times 10^5 \text{ cells mL}^{-1}$, and at the iron concentration of $20 \mu\text{g mL}^{-1}$ with a cell density of 1000 cells mL^{-1} .

The mechanisms of cellular uptakes of graphene are not exactly known. Its uptake possibly relies on direct penetration events such as endocytosis rather than energy-dependent pathways.¹⁸⁶ However, GO-gelatin is taken up through a nonspecific endocytosis way.¹⁸⁷

Ren et al.¹⁸⁸ described a one-step synthesis of superparamagnetic iron oxide/graphene composites by a hydrothermal method. These nanocomposites can be functionalized using a positively charged polyfluorene polyelectrolyte. They are characterized by their high super-paramagnetism, strong photoluminescence, and good water dispersibility.

Grafting quantum dots on graphene indicated a good biocompatibility to HeLa cells shown by MTT assays.¹⁸⁹ Compared to nanotubes, graphene has low toxicity as shown by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays.¹⁸⁴

Hu et al.¹⁴³ proposed that the cytotoxicity of GO is induced due to the physical damage that occurred in the cell membrane which can be attributed to the electronic interactions between negatively charged groups of graphene and positively charged phosphatidylcholine lipids.¹⁹⁰

Two possible mechanisms of graphene cytotoxicity have been proposed,¹⁷⁵ these studies were performed on human fibroblast cells: (i) graphene interacts on the cell surface and sends a stimuli signal which leads to a down-regulation causing cells to detach, float, and shrink or (ii) graphene enters into cytoplasm and the nucleus disturbing the cell metabolism and induces cell apoptosis and death.

It is increasingly being accepted that, in addition to protein adsorption, the cell “vision” effect, which is recognized as a crucial factor that should be considered for the safe design of any type of nanoparticles, must be considered in interpretation and generalization of cytotoxicity results.^{191–195} More specifically, the impact of exactly the same nanomaterials on various cells is significantly different and could not be assumed for other cells; the possible mechanisms that justify this cellular response relate to the numerous detoxification strategies that any particular cell can utilize in response to nanomaterials. Thus, what the cell “sees”, when it is faced with nanomaterials, is most likely dependent on the cell type. In this case, Mao et al.¹³⁴ showed that graphene-sheets can induce higher ROS production after interaction with Panc-1 cell (i.e., human pancreas type cell line) compared to HeLa (i.e., human cervix) cell line; more specifically, it was shown that the interaction of graphene sheets with plasma proteins appeared to have a higher toxicity for Panc-1 cell and the amplitude of toxicity is protein source- (cell line) and time-dependent.

Zhang and co-workers showed an increase of the activation of caspase 3, release of lactate dehydrogenase, and generation of

ROS, in neural pheochromocytoma-derived PC12 cells. Wang et al.¹⁹⁶ reported that GO would induce remarkable cytotoxicity of human fibroblast cells at a concentration above 50 mg/L. Hu et al.¹⁴³ reported that bare GO hardly prevented A549 cells proliferation, while PEGylated GO exhibited excellent stability in the presence of high-concentration salts and proteins and appeared to be less toxic *in vitro* and *in vivo*.

On the basis of the above descriptions, one can conclude the conflict in the toxicological results; there are two ignored factors in toxicity evaluations of nanomaterials, which caused the inconsistency of the cytotoxicity results. These two ignored factors are cell vision, which was discussed before, and variation of cell culture medium upon interaction with nanomaterials; in this case, the main problem with reliability of the toxicity assays, e.g., MTT method, is reduction of proteins/biomolecules in normal culture medium, which occurred by protein corona. In order to get reliable and reproducible data from the toxicity assays, e.g., MTT method, modified assays (i.e., using surface saturated nanomaterials) should be employed.^{197,198}

4.3. Graphene and Graphene Derivatives Toxicity to Simple Animals

Gollavelli and Ling¹⁹⁹ incubated multifunctional graphene (MFG) with HeLa cells and studied their interactions using confocal laser scanning microscopy. The results showed that MFG was rapidly internalized by the cells and localized in the cytoplasm region without entering into the nucleus. MFG was noncytotoxic and did not induce significant amounts of ROS or apoptosis in HeLa cells. The toxicity studies confirmed that MFG is biocompatible to zebrafish and does not induce any significant abnormalities nor affects the survival rate. Zhang et al.²⁰⁰ investigated *in vivo* toxic effects of GO and functionalized GO modified with PEGylated poly-L-lysine (PLL-PEG) (GO/PP) on *Caenorhabditis elegans* (*C. elegans*) worm (as a simple animal model), under both normal and stress conditions. They proposed that since under normal physiological conditions, cytochrome c (cyt c) protein is located on the inner membrane of mitochondria and H₂O₂ generation in cytoplasm and its decomposition to •OH were limited, GO/PP could not show significant toxic effects. However, under oxidative and thermal stress, •OH generation and cyt c/H₂O₂ electron-transfer systems may occur simultaneously. The accelerating effects of GO/PP on •OH generation would be highlighted because of H₂O₂ overproduction. In addition, cyt c release from mitochondria to cytoplasm provides more chance for GO/PP to contact directly with it. GO/PP in worms not only enhances and accelerates the electron transfer between cyt c and H₂O₂, but also damages the inherent antioxidant defense system of worms resulting in dramatic toxicity. Furthermore, the adverse effects of graphene incorporated in TiO₂ photocatalyst on minuscule animals (i.e., *C. elegans* nematode and *E. coli* bacteria) was recently reported.²⁰¹ It was found that the presence of graphene in TiO₂ can increase its photocatalytic efficiency so that minuscule animals such as *C. elegans* nematode inactivate under natural solar light irradiation. This result indicates caution on the destruction of, at least, simple animals in our environment due to extensive applications of the highly efficient graphene-TiO₂ photocatalysts which are extensively studied.

5. BIOMEDICAL APPLICATION OF GRAPHENES

Recently, some studies are reported in the literature on the subject of graphene nanomaterials that are very close to the

frontiers between biology and medicine: graphene can be used as a component in electrodes for neural stimulation,³² as a substrate for biomolecular imaging in TEM,²⁰² or as a platform for introducing nanopores for DNA sequencing.²⁰³ Graphene nanomaterials have been functionalized by the phage display technique.²⁰⁴ The major development of graphene nanomaterials is their application as molecular probes for fluorescence tracking of biological events. PEG-GO has been evaluated as a platform for *in vitro* and *in vivo* imaging. Cy7-PEG-GO passively accumulates into tumors.²⁵ Yang used the optical absorbance of GN in the near-infrared (NIR) region for *in vivo* photothermal therapy, allowing efficient tumor ablation after intravenous administration and NIR laser irradiation on the tumor. No side effects of PEG-GO were observed for the injected mice. Graphene nanomaterials have been suggested as biosensors,^{16,17,205} tissue scaffolds,^{206,207} carriers for drug delivery¹⁸¹ or gene therapy,²⁰⁸ antibacterial agents,¹⁹ and bioimaging probes.^{31,155,181} Their major advantage is their high specific surface area along with a high capacity for biofunctionalization or drug loading.

Both sides of a graphene plane can be used as a substrate for addition or adsorption of molecules and functional groups in a controlled manner. Covalent and noncovalent surface modifications have been performed to improve biocompatibility and colloidal stability. Generally, covalent modifications include oxidation by Hummers' method to make GO or rGO, conjugation of hydrophilic polymers, 1,3-dipolar cycloaddition, arylation, or amine coupling to carboxylic groups. Most of noncovalent modifications are achieved using hydrophobic forces or π–π interactions on the pristine graphene surface or unmodified graphenic compounds lying between functional groups on GO surfaces.¹³⁰

5.1. Graphenes Application for Drug Delivery

Graphenes have been proposed as a good material for attachment and delivery of drugs, such as anticancer agents.^{209,210} Several approaches to drug delivery have been evaluated *in vivo* and *in vitro*. To avoid the water insolubility of graphene, it is first oxidized to form a water-soluble GO derivative, but this form aggregates in biological buffered solutions such as cell culture media or serum.^{30,181} This aggregation is probably due to the interactions that occurred with salts and proteins. Functionalization of GO with PEG allows an increase of the aqueous solubility and the stability in biological media. GO-PEG was obtained by grafting PEG onto the carboxylic acid functional groups available on the GO planes. No toxic effect was identified and the biocompatibility of this material is good.²⁰⁸

Covalent attachment of chitosan,²¹¹ folic acid,²¹² or PEG³⁰ to GO thus produces a platform for the delivery of anti-inflammatory²¹¹ and water-insoluble anticancer drugs such as Doxorubicin (Dox).^{31,181,213} Functionalized GO becomes dispersible and highly stable in cell culture media, serum, and phosphate buffered solution. These nanomaterials showed negligible or no toxicity *in vivo*^{25,214} and *in vitro*^{30,181,211} at the usual doses. For some drug molecules, lowered pH increases their solubility and thus decreases their tendency to stay adsorbed, leading to potential controlled release in lysosomes following cellular endocytosis.^{181,212–214}

GO-PEG can rapidly form complexes with aromatic molecules such as SN-38,³⁰ a camptothecin (CPT) analogue (Figure 4). This new nanomaterial was highly effective in killing human colon cancer cells. CPT, used for colon cancer

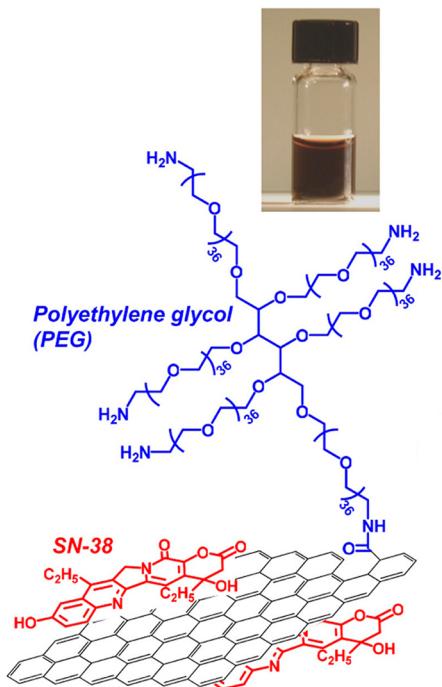


Figure 4. Structure of GO-PEG sheet loaded with SN-38. Reprinted from ref 30. Copyright 2008 American Chemical Society.

treatment, has a high excretion rate, and only a part of the administrated dose is converted to SN-38, its active form.²¹⁵ PEG-GO/SN-38 complexes are soluble and stable in water, PBS, and serum with negligible release. *In vitro* assays showed that PEG-GO/SN-38 is equally effective to free SN-38 in DMSO but approximately 1000 times more efficient compared to CPT incubated with HCT-116 cells, a human colon cancer cell line (see Figure 5).³⁰

GO has also been functionalized with sulfonic acid groups and then covalently bound with folic acid (FA)³¹ to specifically target human breast cancer cells with folic acid receptors. Simultaneous physisorption of Dox and CPT onto GO, covalently functionalized with sulfonate groups (SO_3^-) was achieved to increase drug stability and with FA for specific targeting.³¹ By using MCF-7 cells, a human breast cancer cell line expressing FA receptors, and A549 cells, a human adenocarcinoma alveolar basal epithelial cell line that does not express FA receptors, Zhang et al.³¹ showed specific uptake of FA-GO. Breast cancer cells were more sensitive to FA-GO/Dox/CPT than FA-GO/Dox or FA-GO/CPT.

FA-GO has been evaluated *in vitro* as a carrier for the photosensitizer chlorin e6 (Ce6). Photosensitizers are porphyrin-based molecules used in photodynamic therapy to induce cell death via the generation of ROS upon irradiation.²¹⁶ A significant reduction of the cell viability was reported with MGC803 cells, a human stomach cancer cell line, when exposed to FA-GO/Ce6 following irradiation.²¹⁷

GO was loaded with Dox, the weight ratio of the loaded drug to a single GO sheet can reach up to 200%.²¹³ The loading and release of Dox on GO showed strong pH dependence probably due to the hydrogen-bonding interaction between GO and drug. The fluorescent spectrum and electrochemical results indicate that strong $\pi-\pi$ stacking interaction exists between them.

Rituxan, a monoclonal antibody against the B-cell membrane surface marker CD20,²¹⁸ is often used in combination with

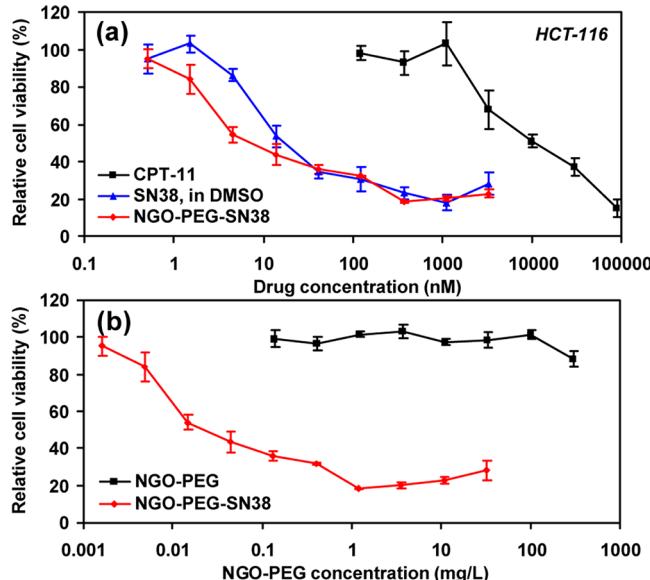


Figure 5. *In vitro* cell toxicity assay. (a) Relative cell viability data of HCT-116 cells incubated with CPT-11, SN38, and NGO-PEG-SN38 at different concentrations for 72 h. Free SN38 was dissolved in DMSO and diluted in PBS. Water-soluble NGO-PEG-SN38 showed similar toxicity as SN38 in DMSO and far higher potency than CPT-11. (b) Relative cell viability data of HCT-116 cells after incubation with NGO-PEG with (red) and without (black) SN38 loading. Plain NGO-PEG exhibited no obvious toxicity even at very high concentrations. Reprinted from ref 30. Copyright 2008 American Chemical Society.

chemotherapy drugs for the treatment of non-Hodgkin's lymphoma.²¹⁹ Rit-PEG-nano-GO/Dox, a nanocomposite obtained by covalent linking of Rituxan to PEG-GO with physisorption of Dox was evaluated *in vitro* for targeting of Raji B-cells, a human Burkitt's lymphoma cell line. Rit-PEG-nano-GO/Dox improved cell growth inhibition when compared to free Dox, PEG-GO/Dox, or PEG-GO plus free Dox with or without free Rituxan.¹⁸¹

5.2. Graphenes for Biosensing

rGO can be successfully used in order to develop highly efficient electrochemical and biological sensors thanks to their different functionalities which can be designed to be very sensitive to small changes in the chemical or biological environment.^{220–222} The responses have been obtained essentially by changes in conductivities or in capacities. Recently, Singh et al.²²³ summarized the different kinds of rGO based nanomaterials. Cash and Clark²²⁴ described the recent developments in nanosensors for glucose measurements.^{225,226} The use of glucose biosensors has been overviewed by other teams.^{18,19} Cash and Clark focused their review on electrochemical biosensors which can be applied to clinical samples.²²⁴

Kim and co-workers²²⁷ produced a glassy carbon electrode modified with graphene via a drop-casting method and evaluated its application for the electrochemical detection of dopamine. They have developed a sensitive sensor for the determination of dopamine without the interference by ascorbic acid. Similarly, Sun et al.²²⁸ developed a system for simultaneous electrochemical detection of ascorbic acid, dopamine, and uric acid using graphene/size-selected Pt nanocomposites. They showed that compared with glassy carbon and graphene electrodes graphene/Pt modified electro-

des provide superior performance in terms of peak potential and current in cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements. They believe that graphene–Pt nanocomposite-based electrodes can act as a novel platform for the next-generation of electrochemical biosensors.

Mohanty and Berry¹⁸ reported on the fabrication of novel rGO-based sensors: (i) single-bacterium device, (ii) label-free DNA sensor, and (iii) bacterial DNA/protein and polyelectrolyte transistor. The bacteria nanosensor was very sensitive with a single-bacterium attachment generating about 1400 charge carriers in the graphene structure. Similarly, single-stranded DNA tethered on graphene hybridizes with its complementary DNA strand to reversibly increase the hole density by $5.61 \times 10^{12} \text{ cm}^{-2}$.

Kuila et al.²⁰⁵ published a recent review on the advancement of graphene based biosensors. They discussed the use of graphene for the detection of glucose,^{205,229–233} Cyt-c,^{234,235} nicotinamide adenine dinucleotide (NADH),^{236,237} hemoglobin (Hb),²³⁸ cholesterol,²³⁹ ascorbic acid (AA),^{240,241} uric acid (UA),^{240,241} dopamine (DA),^{240,241} and H_2O_2 .^{242–244} GO and rGO have been also used for the fabrication of heavy metal ion sensors,^{88–91,245–248} gas sensors,^{220,221,249,250} and DNA^{17,205,251,252} sensors. There are three kinds of biosensors: graphene-based enzymatic, nonenzymatic, and nanoelectronic devices.²⁰⁵

Electrochemistry of enzymes involves direct electron transfer (DET) between the electrode and the active center of the enzymes without the participation of mediators or other reagents.^{16,253,254} The use of metal nanoparticle (NP) doped graphene has been reported to form exceptionally stable and cost-effective biosensors.^{255,256} One of the superior advantages of graphene (as compared to the other known nanomaterials) is its promising capability in the realization of simultaneous electrochemical sensing of all four DNA bases (that is, guanine, adenine, thymine, and cytosine) and so detection of single nucleotide polymorphisms (SNPs). The successful application of graphene in DNA sensing is assigned to its highly efficient 2D electrical conduction which results in a heterogeneous electron exchange between sharp edges of graphene sheets (rather than the basal plane of the sheets) and DNA molecules in a direct oxidation process. The first realization of simultaneous electrochemical detection of all our DNA bases using graphene was reported by Zhou et al.²⁴⁰ They showed that rGO modified glassy carbon electrodes can electrochemically detect the four DNA bases in both the single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) at concentration of $\sim 1 \mu\text{M}$ and physiological pH of 7.0 without a prehydrolysis step. Lim et al.²⁵⁷ then demonstrated that epitaxial graphene grown on SiC can yield a better electrochemical response to the bases of dsDNA ($3 \mu\text{M}$) by increasing the edge-plane-like defective sites of the epitaxial film through its anodizing. In this regard, Dubuisson et al.²⁵⁸ showed that the anodized epitaxial graphene electrode can even detect the four DNA bases of ssDNA at a low concentration of 1 nM (the lowest DNA concentration which has been detected by anodized epitaxial graphene). Very recently, rGO nanowalls with extremely sharp edges and preferred vertical orientations were successfully utilized for label-free electrochemical detections of SNPs at ultralow concentration of $\sim 20 \text{ zM}$ (corresponding to $\sim 10 \text{ DNA/mL}$).¹⁷ This achievement promises development of graphene-based DNA biosensors

with ultra high sensitive (single-DNA) resolutions, in upcoming nanotechnology-based biomedicine.

There is an increasing interest in using graphene for development of FRET biosensors.²⁵⁹ Recent studies have shown that graphene can be efficiently used as quenchers for various organic dyes and quantum dots.^{149,150,260–262} Graphene and GO interact strongly with nucleic acid via π - π stacking interactions. dsDNA cannot be stably adsorbed onto the surface because of the efficient shielding of nucleobases with the negatively charged phosphate backbone.^{263,264} Wang et al.¹³³ summarized the recent progress in biosensors that integrate the quenching property of graphene and the recognition properties of DNA.

Multicolor DNA analysis has been accomplished with graphene based FRET biosensors.²⁶⁵ The GO surface allows simultaneous quenching of multiple ssDNA probes labeled with different dyes. This phenomenon leads to a multicolor sensor for the detection of multiple DNA targets. Thus, a graphene-based FRET biosensor is able to detect different DNA targets with various sequences by using ssDNA probes with different sequences.

The ability of DNA adsorption, quenching capacity, and protection from enzyme cleavage have resulted in graphene being a new robust nanomaterial in biotechnology. This novel biosensor can be utilized in biomedical investigations, including *in situ* localization of mRNA, real time monitoring, cell imaging, and drug delivery.^{210,266}

5.3. Graphenes for Biomedical Imaging

Sun et al.¹⁸¹ developed functionalization chemistry techniques to increase solubility and biocompatibility of GO. They prepared different sizes of pegylated GO sheets that are soluble in buffers and stable in serum without agglomeration. The GO sheets are photoluminescent in the visible and infrared spectral regions. This photoluminescence of GO has been used for live cell imaging in the NIR region. GO functionalized with antibody against selective cancer cells were loaded with Dox. They observed a very good selectivity *in vitro* at Dox concentration of $10 \mu\text{M}$. The percentage of cell growth inhibition increased from 20%, using the GO-PEG/Dox, to 80% with the GO-PEG-rituxan/Dox showing a high potential of selective killing of cancer cells using GO-PEG-antibody/drug conjugates. Considering intrinsic optical properties, large specific surface area, and easy noncovalent interactions with aromatic drug molecules in account, GO is thus a promising new material for biological and medical applications.

Hong et al.²⁶⁷ explored the use of nanographene for *in vivo* tumor targeting and quantitatively evaluated the pharmacokinetics and tumor targeting efficacy by noninvasive positron emission tomography (PET) imaging. They demonstrated that nanographene can be specifically directed to the tumor neovasculature *in vivo* through targeting to CD105 (i.e., endoglin), a vascular marker for tumor angiogenesis. The grafting of the TRC105 targeting ligand (a monoclonal antibody that specifically binds to CD105) led to significantly improved tumor uptake of GO (see Figure 6). They suggested these GO conjugates for cancer-targeted drug delivery and photothermal therapy to enhance therapeutic efficacy.

5.4. Graphenes for Stem Cell Technology

Graphene and GO sheets can be used as biocompatible, transferable, and implantable platforms for stem cell culture.²⁶⁸ Lee et al.²⁶⁸ reported that the noncovalent binding capability of graphene allows them to act as a preconcentration platform for

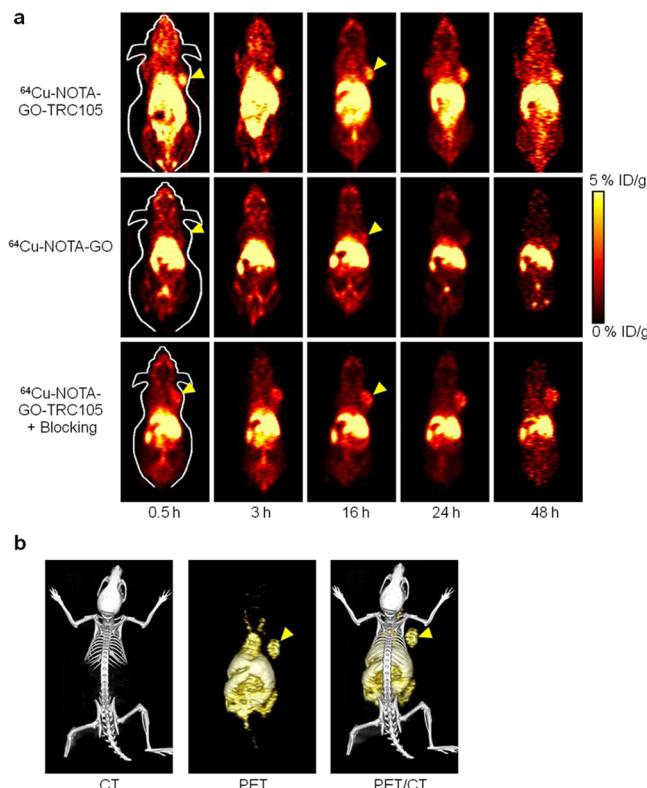


Figure 6. *In vivo* PET/CT imaging of ^{64}Cu -labeled GO conjugates in 4T1 murine breast tumor-bearing mice. (a) Serial coronal PET images of tumor-bearing mice at different postinjection time of ^{64}Cu -NOTA-GO-TRC105, ^{64}Cu -NOTA-GO, or ^{64}Cu -NOTA-GO-TRC105 after a preinjected blocking dose of TRC105. Tumors are indicated by arrowheads. (b) Representative PET/CT images of ^{64}Cu -NOTA-GO-TRC105 in 4T1 tumor-bearing mice at 16 h postinjection. Reprinted from ref 267. Copyright 2012 American Chemical Society.

osteogenic inducers, which accelerate mesenchymal stem cells (MSCs) growing on it toward the osteogenic lineage. The origin of accelerated differentiation was studied in the presence of different growth factor. The different binding interactions and their subsequent influence on stem cell growth and differentiation are attributed to different degrees of π - π stacking, electrostatic, and hydrogen bonding mediated by graphene and its derivatives.

Nayak et al.²⁰⁶ showed that graphene is an excellent biocompatible scaffold that does not hamper the proliferation of human mesenchymal stem cells (hMSCs) and accelerates their specific differentiation into osteoblasts. The differentiation rate is comparable to the one achieved with common growth factors, demonstrating the potential of graphene for stem cell research.

Park et al.²⁶⁹ observed the adhesion of human neural stem cells (hNSCs) on graphene. The differentiation of hNSCs was initiated simply by exchanging the culture media with media without the growth factors.²⁷⁰ After three weeks of differentiation, a difference in morphology was observed between the cells on graphene and those on cell culture flask (see Figure 7). Graphene was fully occupied within the differentiated hNSCs with neurite outgrowths, while many hNSCs were detached from the glass region during the differentiation process. They concluded that the graphene provided more favorable microenvironments for hNSC differentiation and promoted cell adhesion and neurite outgrowths than conventional substrates.

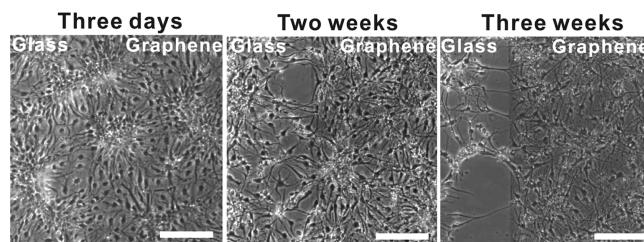


Figure 7. Enhanced neural-differentiation of hNSCs on graphene films. All scale bars represent 200 μm : Bright-field images of the hNSCs differentiated for three days (left), two weeks (middle), and three weeks (right). hNSCs on glass were gradually retracted and detached after two weeks, while those on graphene remained stable even after three weeks of differentiation. Reprinted from ref 269. Copyright 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Previous reports about the electrical properties of graphene allow considering various therapeutic applications such as neural stimulating electrodes for brain disease treatments.^{271–273} They also concluded that graphene had a good electrical coupling with the differentiated neurons for electrical stimulation. The neural activity of the differentiated cells was confirmed by electrical stimulation using a graphene electrode.

Chen et al.²⁷⁴ reported that G and GO can support the induced pluripotent stem cells (iPSCs) culture and allow for spontaneous differentiation. G and GO surfaces led to distinct cell proliferation and differentiation characteristics. Their data demonstrated that the different surface properties of G and GO governed the iPSCs behavior and implicate the potential of graphene-based materials as a platform for iPSCs culture and diverse applications.

5.5. Graphenes for Photothermal Therapy

Hybrid materials have also been successfully developed based on GO or graphene modified by magnetic nanoparticles. This composition is employed for simultaneous cancer therapy by loading an anticancer drug and magnetic resonance imaging (MRI).^{275–277} *In vivo* study of GO tumor uptake showed graphene as a potential candidate for photothermal therapy.^{25,278}

Photothermal therapy as a physical treatment approach to destruct cancer has emerged as an alternative of currently used cancer therapies. Yang et al.²⁵ showed that functionalized GO-PEG with strong optical absorption in the NIR region was a powerful photothermal agent for *in vivo* cancer treatment. These authors have also studied how sizes and surface chemistry affect the performance of graphene derivatives for *in vivo* photothermal cancer treatment.²⁶ Excellent *in vivo* treatment efficacy with 100% of tumor elimination has been observed after intravenous injection of rGO-PEG and followed laser irradiation. All mice after treatment survive over a time period of 100 days. It has been suggested that nanoscale PEG-GO can be a good candidate for photothermal cancer therapy due to its strong NIR absorption.²⁷⁹ After 24 h of intravenous injection, PEG-GO can passively accumulate in tumors of different xenograft tumor mouse models.²⁵ Upon irradiation, a significant increase in tumor temperature led to tumor ablation. Although PEG-GO accumulates in the reticulo-endothelial system after intravenous administration, there was no indication of any toxicological or pathological effects after 90 days postexposure.^{25,274} Yang et al.²⁶ studied *in vivo* fluorescent imaging and NIR photothermal therapy of cancer, by using nanoscale graphene-PEG. Excellent *in vivo* treatment efficacy

with 100% of tumor elimination has been observed after intravenous injection of rGO-PEG followed by NIR laser irradiation. All mice after treatment survive over a time period of 100 days. These authors²⁶ have also studied how size and surface chemistry affect the performance of graphene derivatives for *in vivo* photothermal cancer treatment using an ultralow laser power. Robinson et al.²⁸ examined *in vitro* fluorescence imaging and NIR photothermal therapy of cancer cells by using a low concentration (6.6 µg/mL) of reduced nanographene oxide-PEG. A synergistic effect of chemo-photothermal therapy utilizing GO-PEG was examined by Zhang et al.²⁹ Markovic et al.²⁷⁸ reported better *in vitro* photothermal cancer therapy performance of polyvinylpyrrolidone-coated graphene nanoparticles than DNA or sodium dodecylbenzenesulfonate-solubilized SWCNTs. Very recently, GO reduced and functionalized by glucose (without any PEGylation) was applied in photothermal cancer therapy and its therapeutic efficacy was compared with that of SWCNTs and MWCNTs.²⁷

6. CONCLUSION AND FUTURE PERSPECTIVES

This article highlighted the recent research progress in the production of pristine and functionalized graphene and graphene derivatives (e.g., graphene oxides), with particular emphasis on their biological and biomedical applications, encompassing drug delivery, biosensing, biomedical imaging, stem cell technology, and photothermal therapy. The interaction between protein and graphene or graphene derivatives has been thoroughly discussed. The toxicological profile of graphene and graphene derivatives in various biological and biomedical applications has been introduced. It is found that the presence of bacterial toxicities, deriving from the direct contact interaction between extremely sharp edges of graphene sheets and cell wall of the bacteria, makes graphene nanomaterials effective as antibacterial materials; on the other hand, both graphene and GO sheets are more biocompatible than single wall carbon nanotubes, and their toxicity can be further manipulated via surface modification. We also stressed that a proper cytotoxicity evaluation has to include the consideration of cell vision and variation of cell culture medium upon interaction with graphene based nanomaterials.

We summarized the recent progress on biological applications of graphene based nanomaterials. Functionalized GO has been demonstrated to be an efficient platform for the delivery of anti-inflammatory and water-insoluble anticancer drugs, arising from the extremely high specific surface area and hence a high capacity for drug loading. The application of graphene for biosensing is based on its extremely high sensitivity of the conductivity and capacity in responding to local electrical and chemical perturbations, while for graphene based FRET biosensors, it relies on the ability of the quenching of various organic dyes and quantum dots that are in the vicinity of graphene. GO based nanomaterials can also be employed for biomedical imaging as the GO sheets are photoluminescent in the visible and infrared spectral regions and hence can be utilized for live cell imaging in the NIR region. Moreover, the strong optical absorption in the NIR region allows functionalized GO and rGO to be powerful photothermal agents for *in vivo* cancer treatment. In addition, graphene based materials can be used for efficient stem cell growth and differentiation if a good electrical coupling between graphene and the differentiated neurons can be ensured.

In order to make further progress, great efforts should be devoted to the synthesis and functionalization of graphene with desirable physical and chemical properties. When graphene sheets are isolated from graphite, novel approaches with ease for mass production have to be developed for the separation of graphene layers and to prevent them from agglomeration during the biomedical applications. In addition, it is necessary to develop technologically and economically feasible approaches to functionalize graphene with desirable electrical, chemical, physical, and biological properties, such as increasing the solubility of graphene in biological media, tuning the toxicity (containing cyto- and geno-toxicity) of graphene to bacteria or cells, improving the specific selectivity of graphene for biosensing and biomedical imaging, and so on.

Very recently, it was reported that the presence of protein corona at the surface of nanomaterials, which is exactly what the cells and organs “see” *in vivo*, can change the reported diagnostic/therapeutic behaviors^{122,280,281} together with cytotoxicity assessments^{282,283} of pristine coated nanomaterials. Therefore, for graphene-based biomedical applications, it is crucial to consider therapeutic benefits from protein coated graphenes or graphene derivatives rather than pristine ones.

AUTHOR INFORMATION

Corresponding Author

*(W.C.) E-mail: chmcw@nus.edu.sg; (M.M.) Web: www.biospion.com; E-mail: Mahmoudi@Illinois.edu and Mahmoudi-M@TUMS.ac.ir.

Present Address

♦Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, United States.

Notes

The authors declare no competing financial interest.

Biographies



Dr. Hong Ying Mao received his Bachelor's degree from Zhejiang University in 2002 and Ph.D. degree from Department of Physics at Zhejiang University in 2011. He moved to Chemistry Department at National University of Singapore (NUS) in 2009 and did his postdoctoral research from 2011 to the present. His current research interests include the energy level alignments at organic–organic interface and interfacial engineering approaches for organic electronics and graphene-related devices.



Dr. Sophie Laurent was born in 1967. Her studies were performed at the University of Mons-Hainaut (Belgium) where she received her Ph.D. in Chemistry in 1993. She then joined Prof. R. N. Muller's team and was involved in the development (synthesis and physicochemical characterization) of paramagnetic Gd complexes and super-paramagnetic iron oxide nanoparticles as contrast agents for MRI. She is currently working on the vectorization of contrast agents for molecular imaging. She is lecturer and coauthor of around 120 publications and more than 200 communications in international meetings.



Immediately after receiving his Ph.D. from Sharif University of Technology (SUT) in 2003, Dr. Omid Akhavan was invited to join to SUT where he is currently Associate Professor. His research interests comprise physics of nanostructures, graphene, green synthesis of graphene-based materials, nanostructured photocatalysts, biophysics, biosensors, antibacterial nanomaterials, photothermal therapy, and stem cells.



Dr. Wei Chen received his Bachelor's degree from Nanjing University in 2001 and Ph.D. degree from Chemistry Department at National University of Singapore (NUS) in 2004 and did his postdoctoral research in Physics Department at NUS from 2004 to 2008. He has been an assistant professor in both the Chemistry Department and Physics Department at NUS since 2009. His current research interests include the atomic-scale investigation of interface problems for molecular electronics, organic electronics, graphene-related devices, and interface-controlled nanocatalysis. Dr. Chen is a recipient of the Lee Kuan Yew Research Fellowship, Hitachi Research Fellowship, Omicron Nanotechnology Award, NUS Young Investigator Award, and Singapore Young Scientist Award.



Mohammad Imani is an associate professor of pharmaceutics at Novel Drug Delivery Systems (NDDS) Department of Iran Polymer and Petrochemical Institute (IPPI). He majored in pharmacy (1996) at Isfahan University, Isfahan, Iran. He received a Ph.D. in pharmaceutics (2002) from Tehran University, Tehran, Iran, prior to joining IPPI. His research interests include developing new unsaturated aliphatic polyesters, application of polymeric materials to develop controlled-release implantable/injectable devices, and tissue engineering. Mohammad has been engaged in some international research focused on developing novel drug eluting cochlear implant devices and is advisor to some companies on drug eluting biomedical devices. Mohammad's research has been funded by government agencies and corporations. He is a coinventor on three U.S. patent applications owned by MedEl, Innsbruck, Austria, and IPPI.



Dr. Ali Akbar Ashkarran received his PhD in 2009 from Sharif University of Technology, Tehran, Iran. He is now an assistant professor of physics and Director of Nanotechnology Research Laboratory at University of Mazandaran. His current research interests include nanostructured materials, visible-light-active photocatalyst, liquid arc discharge, gas sensors, graphene-based materials, electro-spinning, biophysics, antibacterial nanomaterials and stem cells technologies.



Dr. Morteza Mahmoudi obtained his Ph.D. in 2009 from Sharif University of Technology with specialization on the cytotoxicity of superparamagnetic iron oxide nanoparticles. He is Director of NanoBio Interaction Laboratory at Tehran University of Medical Sciences (<http://www.biospion.com>). His current research involves the “ignored” parameters at nanobio interfaces.

ACKNOWLEDGMENTS

Authors acknowledge the support from Singapore ARF Grants R143-000-505-112, NUS YIA Grant R143-000-452-101, and NRF CRP Grant of “Graphene Related Devices and Materials”.

ABBREVIATIONS

2D	two-dimensional
1D-PAGE	one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
AFM	atomic force microscopy
$\text{A}\beta\text{F}$	amyloid beta fibrillation
AA	ascorbic acid
BLG	β -lactoglobulin
CVD	chemical vapor deposition
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
cyt c	cytochrome c
CPT	camptothecin
Ce6	chlorin e6

CV	cyclic voltammetry
Dox	doxorubicin
DPV	differential pulse voltammetry
DA	dopamine
DET	direct electron transfer
dsDNA	double-stranded DNA
<i>E. coli</i>	<i>Escherichia coli</i>
FLG	few-layers graphene
FET	field effect transistor
FITC	fluorescein isothiocyanate
FA	folic acid
GO	graphene oxide
GNRs	graphene nanoribbons
G-NH2	graphene with amine
GO/PP	GO modified with PEGylated poly-L-lysine
H_2SO_4	sulfuric acid
Hb	hemoglobin
hMSCs	human mesenchymal stem cells
hNSCs	human neural stem cells
iPSCs	induced pluripotent stem cells
KMnO_4	potassium permanganate
LC-MS	liquid chromatography mass spectrometry
LDH	lactate dehydrogenase
MWNTs	multiwalled carbon nanotubes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MFG	multifunctional graphene
MSCs	mesenchymal stem cells
MRI	magnetic resonance imaging
NIR	near-infrared
NADH	nicotinamide adenine dinucleotide
NP	nanoparticle
O-GNR-PEG-DSPEs	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)]
PEG	polyethylene glycol
PET	positron emission tomography
rGO	reduced graphene oxide
ROS	reactive oxygen species
SiC	silicon carbide
SDBS	sodium dodecylbenzene sulfonate
SERS	surface enhance Raman spectroscopy
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SO_3^-	sulfonate groups
SNPs	single nucleotide polymorphisms
ssDNA	single-stranded DNA
UA	uric acid

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