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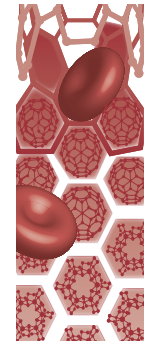
Nanomedicine



# Graphene-based nanomaterials: biological and medical applications and toxicity

Graphene and its derivatives, due to a wide range of unique properties that they possess, can be used as starting material for the synthesis of useful nanocomplexes for innovative therapeutic strategies and biodiagnostics. Here, we summarize the latest progress in graphene and its derivatives and their potential applications for drug delivery, gene delivery, biosensor and tissue engineering. A simple comparison with carbon nanotubes uses in biomedicine is also presented. We also discuss their *in vitro and in vivo* toxicity and biocompatibility in three different life kingdoms (bacterial, mammalian and plant cells). All aspects of how graphene is internalized after *in vivo* administration or *in vitro* cell exposure were brought about, and explain how blood–brain barrier can be overlapped by graphene nanomaterials.

**Keywords:** biodiagnostics • biodistribution • blood–brain barrier • drug delivery • gene delivery • graphene • graphene derivatives • graphene oxide • tissue engineering • toxicity

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## A brief history of graphene

Around 750 BC, in his major work 'Works and Days,' the Greek poet Hesiod described the five ages of Man: Golden Age, Silver Age, Bronze Age, Heroic Age and Iron Age. Based on the advances of the last decades, the 21st century has become known as the Carbon Age. Researchers from all over the world have worked extensively to: characterize, develop and produce new materials built on the many allotropes of carbon. These carbon-based materials such as fullerene [1], graphite [2], graphene [3] and carbon nanotubes [4], have special properties due to their nanoscale dimensions.

Graphene is one of the crystalline forms of carbon, best described as a one-atom thick planar layer of carbon atoms that are bonded together to originate a π-conjugated material in a hexagonal pattern, forming a honeycomb crystal lattice [5]. In other words, graphene is a monolayer sheet of $sp^2$-hybridized carbon atoms, produced either by mechanical or chemical exfoliation of graphite or through chemical vapor deposition (CVD) methods [6]. The properties of graphene and the primary structure of carbon nanotubes (CNT) and graphite allow them to be used in nanoelectronics [6], opto-electronics [6], sensors [6], electric and photovoltaic [7] and biotechnology [8].

The first well-known example of graphene came in 1859, when the British chemist Benjamin Collins Brodie successively treated graphite flakes in an oxidative environment. It resulted in an increase in its overall mass and in a change in its characteristics; the material was latter called as Graphon [9]. Only in 2004, graphene was isolated by simple mechanical exfoliation [3] by Novoselov, Geim and coworkers, and its enormous potential could begin to be explored.

Graphene displays a large theoretical specific surface area (~2630 $m^2.g^{-1}$), high intrinsic mobility (~200,000 $cm^2v^{-1}s^{-1}$) [10], a Young's modulus of approximately 1.0 TPa [11] and a thermal conductivity of approximately 5000 ($Wm^{-1}K^{-1}$) [12]. Since 2004, numerous studies have confirmed that graphene has excellent electrical, mechanical and thermal properties. A myriad of protocols have been subsequently developed for

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the production of graphene from a variety of sources using techniques such as CVD [12,13] 'graphitization of silicon carbide (SiC) surfaces' [14,15], intercalation/exfoliation methods [16] and other reductive chemistry reactions [17].

In order to obtain large-scale graphene production, the use of CVD and SiC methods using vacuum annealing is more commonly applied. Using CVD, David and co-workers have reduced the time needed for graphene synthesis from 140 to 30 min via rapid heating and quenching at ambient pressure. However, these methods generate not only monolayer graphene (MLG) (which possess excellent electrical transport properties). Few-layers graphene (FLG) is also produced since MLG tends to restack, compromising this graphene material properties [18]. FLG of 1–20 µm thickness and with 1 to 12 graphene layers [18–20] was obtained by CVD on Ni (nickel) films.

The newly synthesized graphene layers are separated by a distance of 3.35 Å, and by 6.8 Å in graphite oxide layers. To obtain a graphene layer, the distance must be increased by physical or chemical approaches to up to 12 Å [21]. For several years, researchers did not think it would be thermodynamically stable for a graphene sheet to exist naturally as a 2D crystal with such few atomic layers. In 2007, Meyer showed that graphene possesses deformations that stabilize the occurrence of monolayer sheets thus forming a 3D structure [22] (Figure 1). Single layer graphene foils produced by CVD are rolled with self-positioned layers of InGaAs/Cr forming compact multiturn tubular structures that consist of successive graphene/metal/semiconductor heterojunctions on a radial superlattice [23]. Using elasticity theory and Raman spectroscopy, it was demonstrated that it is possible to produce homogeneously curved graphene with curvature radius on the 600–1200 nm range [23].

A growing number of publications are dedicated to uncovering new potential application of graphene nanomaterials, as well as to highlight the benefits of their use and the need to perform more research on these nanomaterials. The fields of photonics, electronics and the biomedical arena have being taking advantage of their potential. In the latter, for example, they have been used to perform gene, drug, peptide and protein delivery in addition to developing biosensors and new composites to tissue regeneration. Furthermore, their cytotoxicity is being exploited to induce selective cell death and to create new biotechnological strategies to study living cells.

In this article, we summarize the latest progress in graphene and its derivatives used in tissue engineering, drug delivery, gene delivery and biosensing; we also discuss their toxicity and biocompatibility for bacterial, mammalian and plant cells and their possible *in vivo* applications and toxicology, all together for a concise review.

## Important graphene derivatives

By using graphene as a starting material, it is possible to obtain some biologically and medically important derivatives (Figure 2) such as ultrathin graphite, graphite oxide, graphene oxide (GO), reduced graphene oxide (rGO) and nano-graphene oxide (nano-GO). The following is a brief discussion of structure and synthesis of these derivatives.

### Ultrathin graphite

The adjacent graphene sheets in graphite are separated from each other by 0.335 nm and are held together by weak van der Waals forces. In 1996, Kotov produced an ultrathin graphite (width ~43 Å) with diminished resistance using the hydrazine reduction method [24]. In 1998, Kovtyukhova and co-workers, by electrostatic self-assembly, obtained graphite monolayer films (sheets) ranging from 11 to 14 Å in thickness, with lateral dimensions between 150 nm and 9 µm [25]. Graphite films have also been synthesized by catalytic decomposition of C-containing gases on metals [26], or carbides [15]. By the precipitation of several layers of graphite onto Ni foam, the 3D structure of ultrathin graphite foam (UGF) shows excellent electrochemical stability with great industrial potential [24]. Recently, Sun and co-workers synthesized graphite layers 0.7–1.5 nm thick and 50–300 nm width by microbial oxidation of dispersed graphite by nitrifying bacteria [27].

### GO & graphite oxide

Graphite (Figure 3A) can be used to originate the GO (Figure 3B) or graphite oxide.

Graphite oxide is an inorganic multilayer system, highly hydrophilic, formed by a layered structure of 'graphene oxide' sheets [28]. GO, on the other hand, is normally obtained by acid/base treatment of graphite oxide followed by sonication. Besides oxygen epoxide groups, several other functional groups are also found in this structure such as carbonyl (=CO), hydroxyl (-OH) and phenol groups attached to the sheet edges [29]. Additionally, GO also contains a range of negatively charged [30] reactive oxygen functional groups on its surface [3,29] conferring solubility in water or other polar solvents such as ethylene glycol, dimethylformamide (DMF), *N*-Methyl-2-pyrrolidone (NMP) and tetrahydrofuran (THF) at a range of 0.5 mg/ml [31]. GO presents diminished electrical conductivity that can be improved by treatment with a reducing agent. However, chemical modification

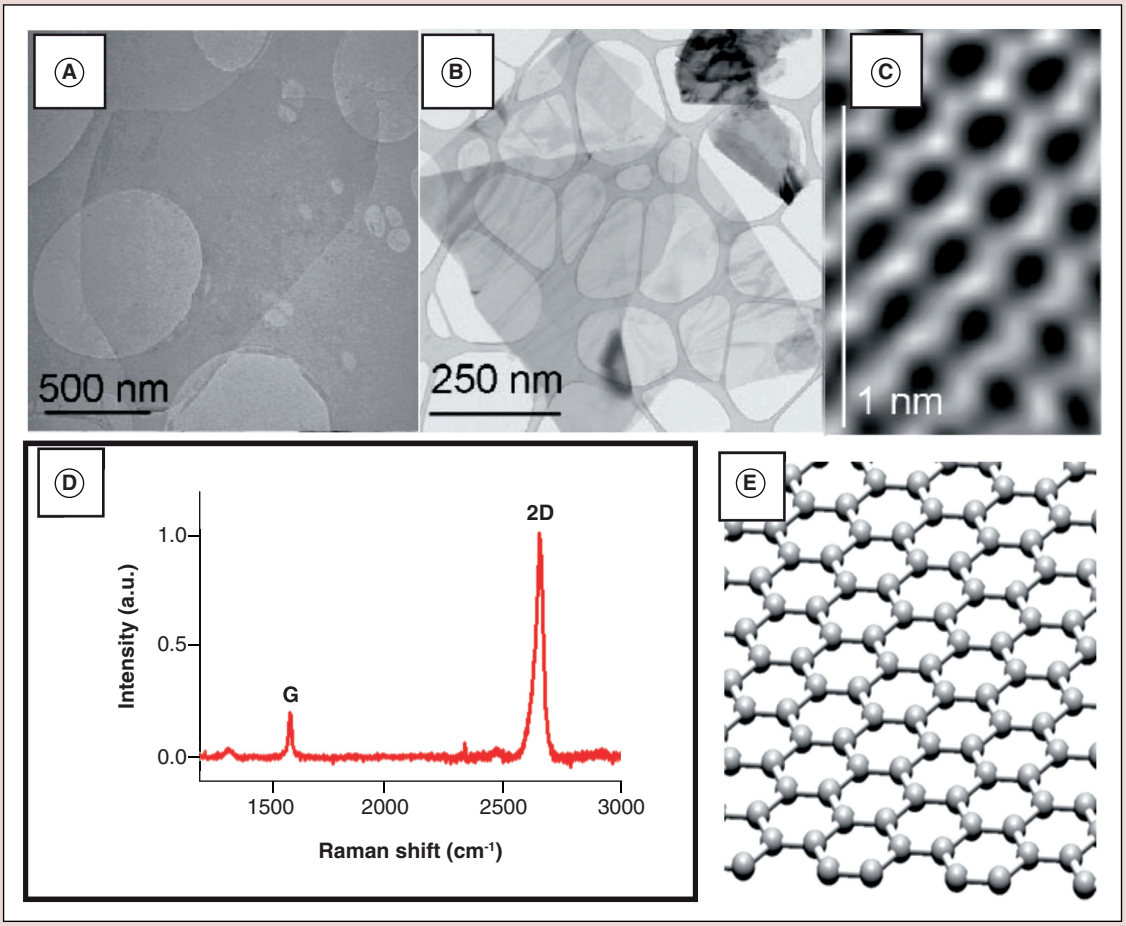


**Figure 1. Monolayer graphene.** A transmission electron microscope image of **(A)** a graphene monolayer and **(B)** a folded graphene monolayer. **(C)** An atomic resolution image of a portion of a graphene monolayer. **(D)** Raman spectrum indicating monolayer graphene and **(E)** schematic representation of graphene.

increases GO solubility in organic environments [31].

Although subjected to extensive investigation, the atomic structure and composition of the GO has still not been completely elucidated. Different atomic models have been proposed, the most notably being the Klinowski-Lerf's model which is based on NMR spectroscopy data. This model describes GO as an assembly of pristine aromatic 'islands' that are separated from each other by aliphatic 6-membered rings containing C-COH groups, epoxide groups and double bonds [21]. This model has undergone some changes in 2006, but it is currently the most accepted one [32].

## Reduced graphene oxide

As mentioned before, graphite oxide is a 3D structure formed by stacking several GO layers containing epoxide and hydroxyl functional groups [32]. In 2007, Stankovich S *et al.* [33] obtained reduced graphene oxide (rGO) by reducing graphite oxide with hydrazine at 100°C for 24 h. The chemical reduction of GO makes it more hydrophobic and can thus result in the formation of precipitates. This method of reducing graphite oxide with hydrazine results in delamination and pyrolysis of the oxygen-containing functional groups yielding graphene layers [34]. The chemical reduction of these colloidal suspensions can be obtained with several other reducing agents, such as hydroquinone [35], sodium borohydride [36] ($NaBH_4$) and ascorbic acid [37]. Recently, a high-quality rGO (Figure 3C) with great electrical properties was produced using thiophene [38,39]. The sulfur atoms (that present strong electron donor ability) contribute to conductivity enhancement that makes possible that dye-sensitized solar cell containing photoanode of this material, exhibit much better performance than the ones containing common rGO photoanode [39].

## Nano-GO

The stability of GO is size dependent. Smaller GO sheets are more hydrophilic due to the higher density of


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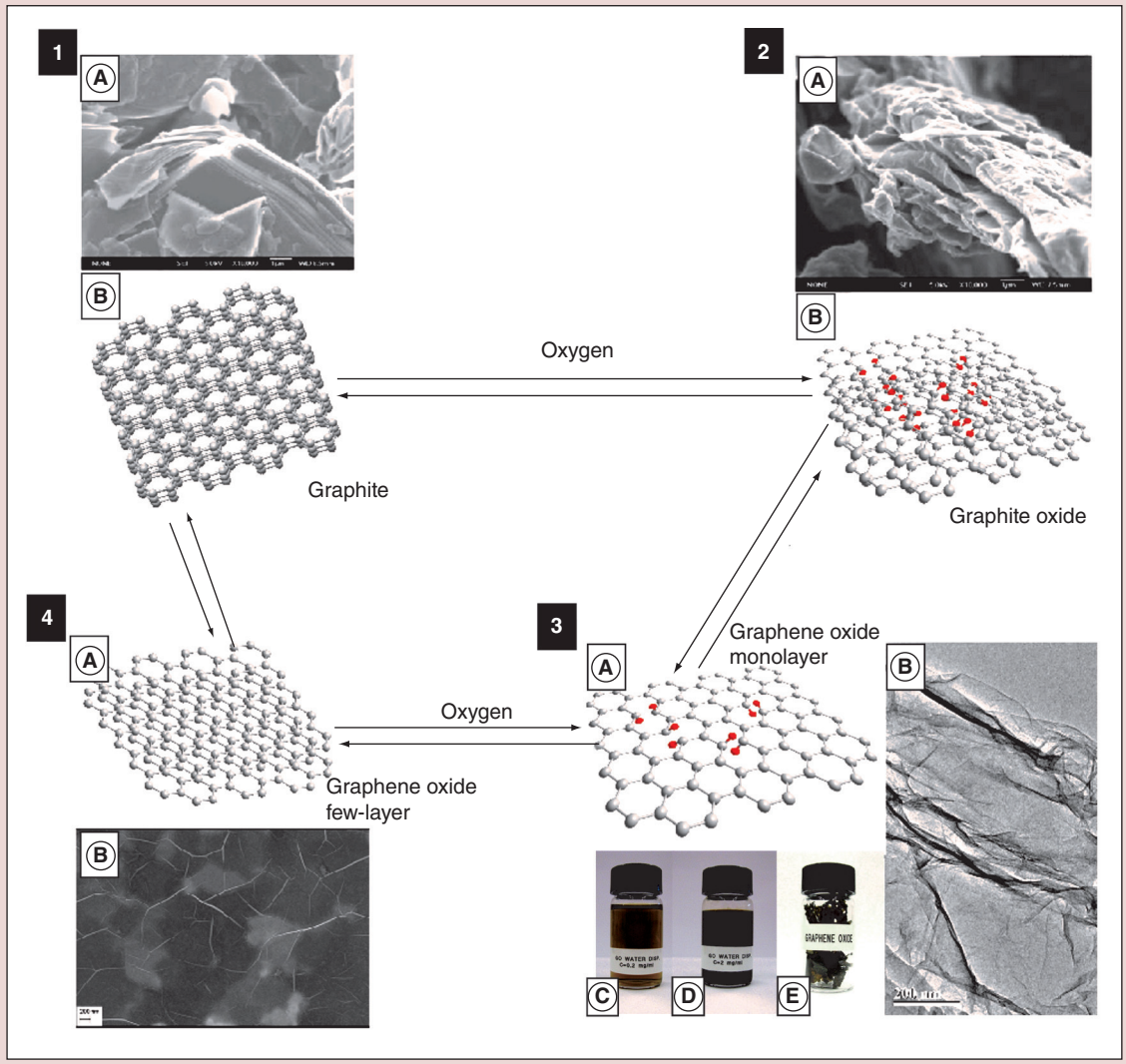


**Figure 2. There are many different graphene nanomaterials types, and they can be categorized among the limiting cases of graphene oxide, graphene, graphite oxide and graphite.** The properties differ depending on where the graphene sits within this space. Box 1: **(A)** represents the structure of graphite captured by transmission electron microscope (TEM) and **(B)** is the scheme of a molecular model. Box 2: **(A)** TEM image obtained for the graphite oxide and **(B)**, the scheme of a molecular model. Box 3: **(A)** represents the molecular structure of graphene oxide (GO) monolayer; in **(B)**, TEM image of single-layer graphene; GO aqueous solution in lower concentration **(C)**, high concentration **(D)** and solid GO **(E)**. Box 4: GO few-layer's molecular model in **(A)**, and TEM image in **(B)**.

charges originating from the ionized –COOH groups on their edges [40]. In addition, due to its enhanced surface-to-volume ratio, high dispersibility in water and organic solvents and a wide range of reactive surface-bound functional groups, nano-GO exhibits visible and near-infrared (NIR) fluorescence (being used in biosensors and cell imaging). It is therefore an excellent candidate for conjugations with other polymers or nanomolecules for several biomedical applications [41]. Nano-GO conjugation, up to approximately 5–50 nm (graphite oxide sheets 50–500 nm), exhibits excellent stability in a wide range of biological solutions at approximately 1 mg/ml concentration. [42] Combining nano-GO with PEG yields a versatile substrate, and PEGylated-Nano-GO (Figure 4) applications have been proposed for photodynamic therapy (PDT) and tumor photothermal therapy [43].

## Graphene as an advantageous carbon-based material for biological & medical use

Nanotechnology is a field of interdisciplinary research involving physics, chemistry, engineering, biology and medicine. One of the emerging branches of nanotechnology is nanomedicine, with tremendous potential

not only for developing rapid methods for the detection, diagnosis and treatment for an array of diseases, but also for gene transfection, and *in vitro* and *in vivo* bioimaging [8]. Currently, several different types of nanomaterials are available for biological and medical purposes, such as those derived from carbon [44], gold [45–48], semiconductor quantum dots (QDs) [48] and polymeric nanoparticles [49]. Moreover, these nanomaterials are being further studied for their unique physicochemical characteristics and novel functional properties that allow them to be conjugated with biomolecules such as proteins, peptides and antibodies for biological and medical applications. Among the carbon-based nanomaterials are graphene [3] and its derivative materials. Graphene derivatives include CNT [50], fullerene derivatives [51] and graphite (Figure 5), all of which have the potential to possess invaluable biological and medical applications.

## Why are graphene and its derivatives suitable for biological & medical applications?

The pioneers of graphene derivatives, first described in 2004, were awarded the 2010 Nobel Prize in physics because of their tremendous application potential. GO, like graphene, has an extremely high specific surface area. Besides that, it also exhibits visible and NIR fluorescence: a feature exploited in biosensors and cell imaging. GO is reactive due to its several chemical groups that can form covalent or noncovalent interactions [52]. Its edges have been shown to be more reactive than its surface because of the additional free reactive oxygen functional groups.

Graphene and its derivatives possess outstanding mechanical, electronic, optical, thermal and chemical properties [53] and are also biocompatible. In single layer graphene, the electrons travel as if they carry no mass, which means it has a high electron mobility [54], vital to field-effect transistor based biosensors [55] and can transfer energy to nearby molecules. They can also exhibit some interesting properties that can be useful in sensor or separation methodologies in example we can mention the anomalous quantum Hall effect. When graphene is attached to magnetic materials, for example, it acquires ferromagnetic properties [56,57]. The low density states at the Fermi level has been shown to be useful in preventing thrombosis on stents [58]. Its thermal conductivity is isotropic, and it is much higher than the value observed for all other carbon structures [12]. Mechanically, graphene also appears to be one of the strongest materials ever tested, with high elasticity, flexibility and adaptability to flat or irregular surfaces [59]. These properties not only make it useful in cell culture [60], but also makes it possible to build structurally adaptive and self-supporting scaffolds [61]

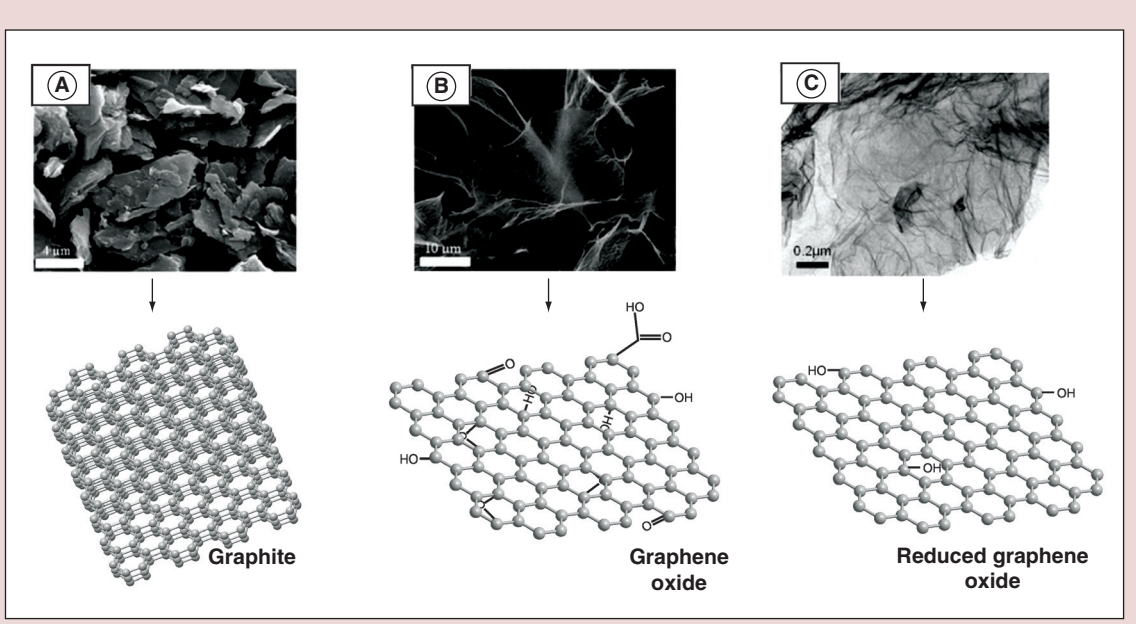


**Figure 3. Oxidation of graphite and reduction of graphene.** Graphite is a 3D structure **(A)** formed by graphene layers; graphite oxide is a 3D structure formed by graphene oxide (GO) layers. **(B)** GO contains epoxide and hydroxyl functional groups (Klinowski-Lerf's model). GO's reduction, for example, by pyrolysis, leads to the production of monolayer graphene without functional groups containing oxygen **(C)**. In **(A)** the structure of graphite was characterized using scanning electron microscope. In **(B)**, the same technique was used to characterize a monolayer of graphene oxide. In **(C)**, transmission electron microscopy was used to characterize a monolayer of reduced graphene.


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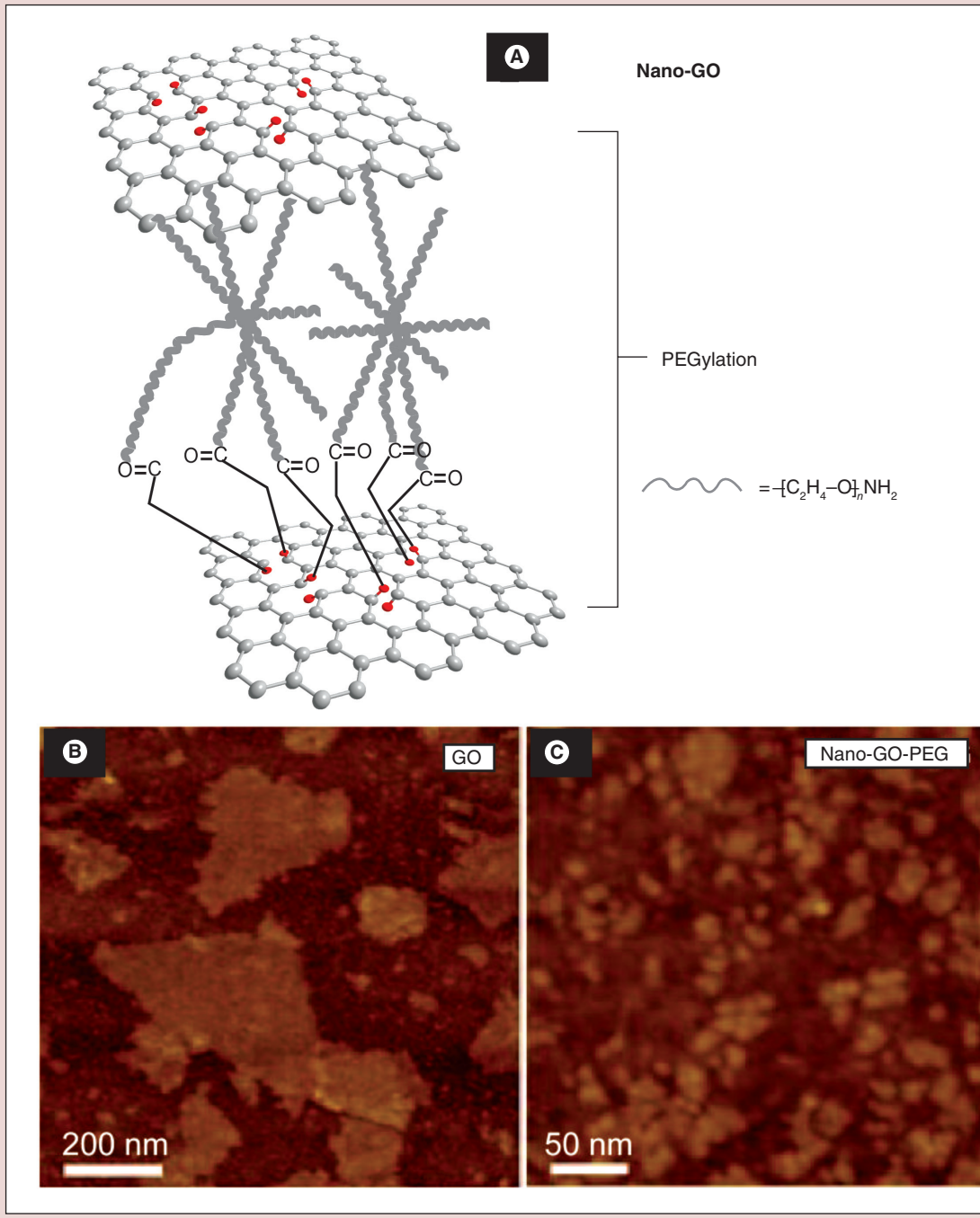


**Figure 4. PEGylation of nano-graphene oxide. (A)** Schematic illustration of PEGylation of nanographene oxide by PEG–stars polymers. **(B & C)** Atomic force microscopy images of GO and nanoscale GO-PEG, respectively.
GO: Graphene oxide.

and to use GO together with silk fibroin to make flexible reinforced films for tissue regeneration [62]. Besides, it is highly transparent to visible light, having an intrinsic optical absorbance in the NIR [59] contributing to photothermal therapeutic uses [63] because the potential for noxious side-effects is reduced since NIR is largely harmless to cell components (Figure 6).

Recently, there have been extensive efforts to take advantage of graphene and its derivatives' ability to allow different chemical modifications. The final goal is to develop new analytical technologies and innovative biotechnological applications involving these nanomaterials. For example, in the field of electrochemical sensors and biosensors, inconsistencies in sig-

nal amplification and problems related to the presence of electrochemically active impurities in electrodes (e.g., those made of CNT), could be overcome thanks to graphene electrodes. Fewer graphene impurities and consistent signal amplification enabled the development of graphene-based enzyme electrodes, nonenzymatic graphene-based electrodes and graphene-based nanoelectronic devices that are vital to electrochemical detection of cytochrome c, cholesterol, glucose, horseradish peroxide, nicotinamide adenine dinucleotide (NADH) and hemoglobin (using graphene-based enzyme electrodes); hydrogen peroxide ($H_2O_2$), dopamine, uric acid and ascorbic acid (using nonenzymatic graphene-based electrodes); heavy metal ion, gas and DNA (using the graphene-based nano-electronic devices) [17].

## Tissue engineering with graphene-based nanomaterials

Tissue engineering is a multidisciplinary field focused on the application of engineering and life sciences for the development of biological substitutes that can repair, maintain or improve tissue's function [65]. One of the most relevant principles in tissue engineering involves the development of bioactive degradable substrates, known as scaffolds, with biocompatible surfaces and favorable mechanical properties suitable for cell culture, which not only allow cellular attachment, proliferation, differentiation, but also support for new tissue formation [66].

To achieve this purpose, scaffolds must possess specific properties for tissue reconstruction and biological response. The main characteristics include: biodegradability: to avoid the necessity of surgical removal, the scaffolds must be made from materials with controlled biodegradability or bioresorbability by the surrounding tissues; high porosity: to facilitate cell adhesion and diffusion, the scaffold must have high porosity and suitable interconnecting pore size to favor tissue integration and vascularization; surface: surface chemical modification to favor cell attachment, differentiation and proliferation; mechanical properties; biocompatibility: not induce adverse response and pliability: able to be produced into a variety of shapes and sizes [66].

Biocompatible polymers have been used to fabricate biodegradable scaffolds and are classified as natural or synthetic polymers. The natural-based materials include polymers made of polysaccharides (chitosan, hyaluronic acid or alginate) or proteins (collagen and fibronectin). However, the use of natural-based materi-

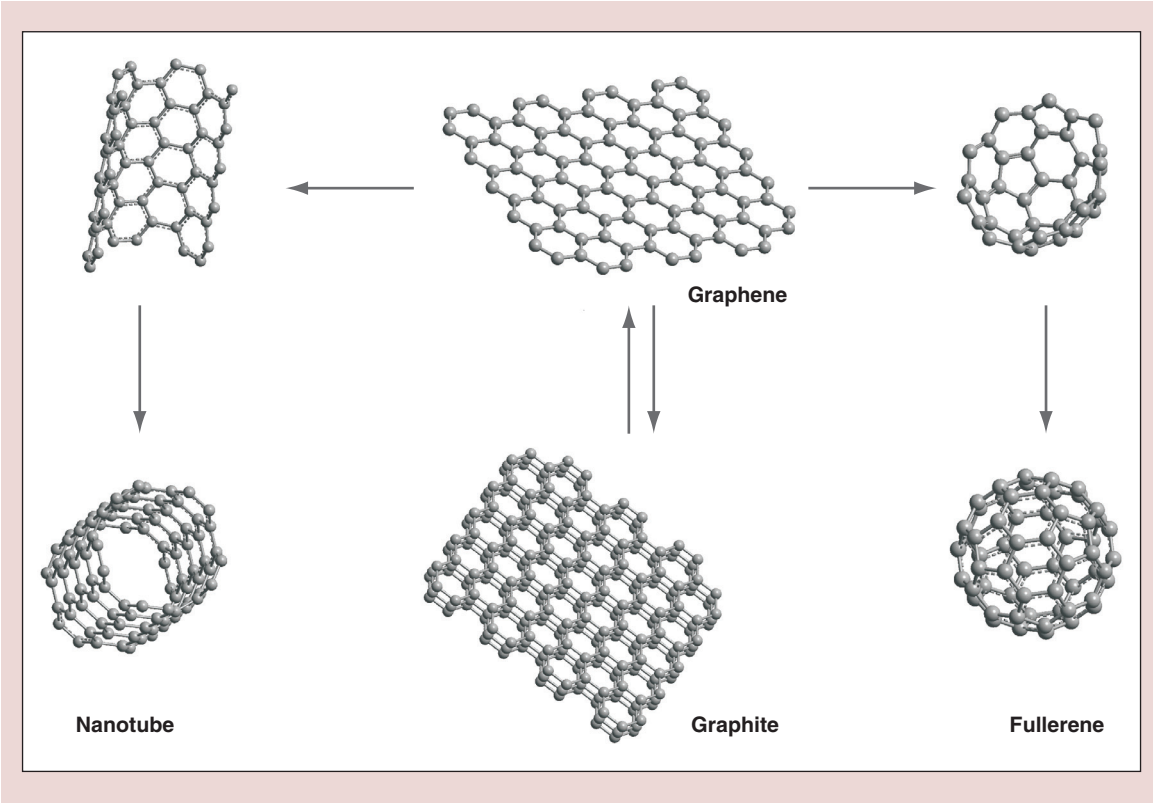


**Figure 5. The epitome of graphite forms.** Graphene is a 2D material, with carbon atoms arranged in the form of honeycomb. That is the 'mother' of all other allotropic forms of carbon: 0D fullerenes or buckyballs, 1D carbon nanotubes and 3D graphite.


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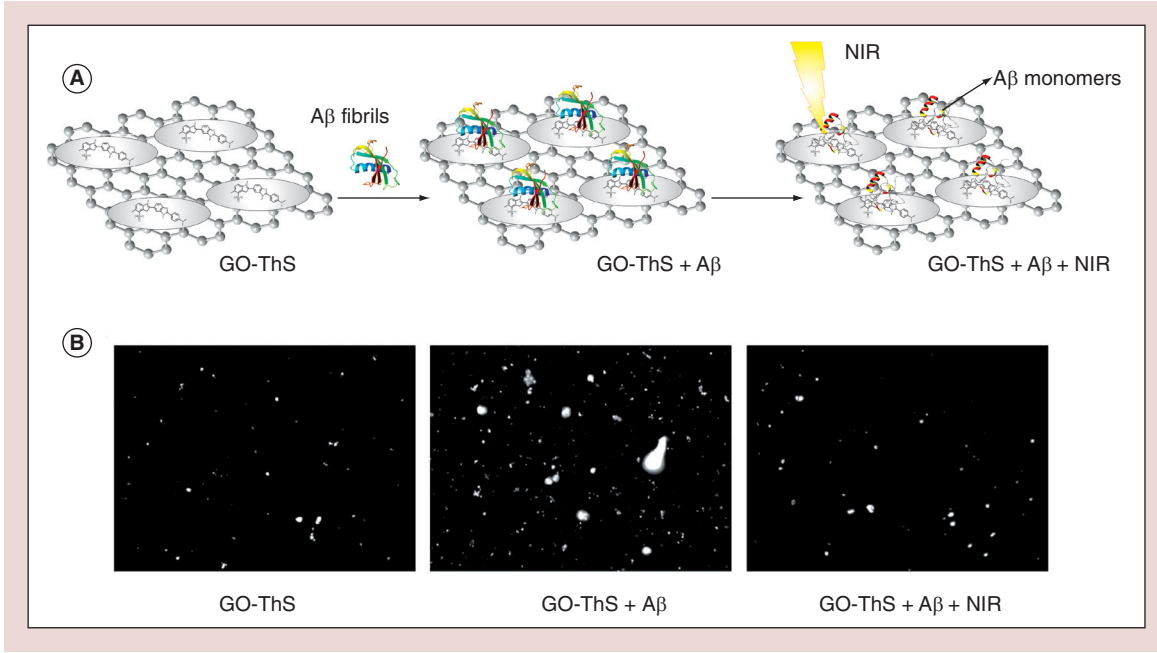


**Figure 6. Graphene oxide intrinsic optical absorbance in the near-infrared and its therapeutic use. (A)** Schematic representation of ThS-modified GO with high NIR absorbance used for Alzheimer's disease treatment. GO was covalently linked to ThS, which can selectively attach to Aβ aggregates forming the conjugated GO-ThS + Aβ. Strong NIR optical absorption ability of nano-GO was used to generate local heat-dissociating attached Aβ fibrils through low-power NIR laser irradiation. **(B)** (Left image) fluorescence images of Aβ incubated with GO-ThS in mice cerebrospinal fluid. (Middle image) under irradiation of GO-ThS + Aβ for 8 min, the fraction of ThS-positive deposits decreased, confirming that GO-ThS under irradiation can dissociate amyloid plaques. This photothermal therapy treatment shows remarkably reduced side-effects and improved selectivity since only the lesion exposed to the light is treated, while other tissues that have not received irradiation are not affected.
Aβ: Amyloid β; GO: Graphene oxide; NIR: Near-infrared; ThS: Thioflavin-S.
Adapted with permission from [64].

als presents some risks. If they are to be used in tissue engineering, they are not suitable because they possess weak mechanical strength. There is a risk of pathogen transmission and immunorejection when using material that comes from animals or cadavers. Also, the biodegradability and the mechanical properties of nonsynthetic materials are harder to control. Therefore, the use of synthetic polymers has been found to be more suitable over the naturally occurring ones. Synthetic polymers including poly(lactic acid), poly(glycolic acid), poly(3-caprolactone), poly(hydroxyl-butyrate) lack cell-recognition signaling and strength, degradation rate and microstructure [55,67] can be controlled.

Moreover, the incorporation of carbon-based materials such as CNT and graphene into polymers can enhance their physical properties and the performance of the polymer matrix not only by imparting novel properties such as electrical conductivity, but also by playing an important role in reinforcing artificial scaffolds. In fact, carbon-based materials can be considered a physical analog of the components of the extracellular matrix due to their similar dimensions [68]. It has also been demonstrated that dispersing a small fraction of CNT into a polymer can significantly improve its mechanical strength and also promote osteoinduction, which further stimulates the growth of new bone tissue as well as provides a suitable interface between the native bone and the implant [61,62].

Graphene-based materials possess mechanical properties such as high elasticity, flexibility and adaptability to flat or irregular surfaces that are suitable for the structural reinforcement of materials frequently used for tissue engineering [69]. In fact, compared with single-component scaffolds, the incorporation of graphene presents higher mechanical strength, stability, lubricity, water retention and most importantly, can improve adhesion, differentiation and cell function [61,62].

The use of graphene-based nanomaterial as a support for adhesion and proliferation of mammalian cells has been recently investigated. The incorporation of graphene into synthetic hydrophilic polymers such as polyvinyl alcohol [70] and poly(methyl methacrylate) [71] demonstrated an overall increased tensile strength and elasticity modulus in a concentration-dependent manner and no alteration in cell compatibility. Also, hydrogels synthesized by chemical covalent linkage of the carboxyl groups present in GO with the amide groups of chitosan have been recently used to

allow control over mechanical properties and biodegradability. In addition, the GO-chitosan hydrogel was able to maintain size and shape, reduce degradation rate compared with chitosan alone, as well as enhance mechanical strength. Moreover, recently, cellular adhesion, proliferation and differentiation of a mouse pre-osteoblast cell line was significantly improved using this carbon nanomaterial [69].

Also, a recent report demonstrated that the osteogenic potential could be improved by using graphene-coated substrates. In this report, it was demonstrated that graphene can control and accelerate the osteogenic differentiation of human mesenchymal stem cells and pre-osteoblasts into osteoblasts by enhancing the expression of osteocalcin and accelerating calcium deposition in human mesenchymal stem cells cells due to graphene's unique surface physicochemical properties [69]. Moreover, due to the unique electrical conductivity properties of graphene, it has great potential in neural tissue engineering and therefore can influence the behavior of electro-active neural cells (Figure 7). An enhanced differentiation of human neural stem cells into neurons on graphene was recently demonstrated by Park and co-workers [72]. In this study, graphene served as an excellent cell-adhesion layer during a long-term differentiation process and induced the differentiation of human neural stem cells more toward neurons than glial cells. They also found that graphene had a good electrical coupling with the differentiated neurons for electrical stimulation; neural activity of the differentiated cells was confirmed by electrical stimulation using the graphene electrode [72].

The development of graphene and GO-coated biomaterials for attachment, proliferation and differentiation of induced pluripotent stem cells (iPSCs) holds great promise for iPSCs culture and can act as a biocompatible platform for differentiation of iPSCs. Graphene-based materials exhibit varying binding interactions with different growth factors and hence have a differential influence on the growth of stem cells and their subsequent differentiation into specific tissue types. Chen and coworkers, in a recent study, used mouse cells to induce iPSCs on graphene and GO substrates, wherein cells displayed distinct proliferation and differentiation characteristics. According to the authors, the GO enabled better iPSCs attachment and proliferation than graphene, which can be explained by the presence of abundant oxygen atoms (e.g., OH) on GO surface. GO was found to promote differentiation of iPSCs toward endodermal lineage better compared with graphene. The differentiation toward ectodermal and mesodermal lineages was the same for both substrates. Thus, it indicated the importance of surface properties of graphene-based substrates in controlling the differentiation of iPSCs [73].

## Drug delivery with graphene-based nanomaterials

Drug delivery systems using graphene-based nanomaterials have been studied since 2008 (summarized in Table 1). Zhu S *et al.* [74], were one of the first to describe a GO-PEG-Rituxan system for targeted drug delivery. Nanographene materials can be prepared at low cost and have specific surface properties that enable then to interact with various molecules, which allow then to be used in drug delivery. Furthermore, these nanocarriers have mainly been used for cancer treatment mainly, to improve effectiveness and reduce side-effects. These nanocomplexes can also increase the aqueous solubility of some aromatic drugs that alone are insoluble.

Since GO forms negatively charged sheets, chemical modifications with positively charged substances could be used to prepare these complexes, which are maintained via electrostatic interactions. For example, chitosan modifications enhance the stability of the nanocarriers in aqueous medium by means of hydrophilic and cationic interactions [75]. In 2011, Bao H *et al.* [76] and Depan D *et al.* [77] prepared a nanocomplex of chitosan and anticancer drugs camptothecin [76] and doxorubicin (Dox) [77], respectively. Moreover, the folic acid conjugated chitosan used by Depan *et al.* made possible the generation of a pH-responsive system for drug delivery.

Several other modifications, besides the ones using chitosan, can be made to graphene sheets to improve drug delivery. In fact, these have been used even before 2011; since the graphene structure itself contains delocalized π–electrons it makes it possible to bind various aromatic molecules via π–π stacking interactions [78]. In 2009, Yang *et al.* used $Fe_3O_4$ in order to obtain nanoparticles that could be used for delivery of doxorubicin. This nanosystem presented interesting characteristics such as pH sensitivity and magnetic properties; moreover, it also allowed controlled targeting and release, which makes it an attractive delivery vehicle for cancer treatment [79]. Furthermore, Yang *et al.* synthesized a pH-sensitive nanocarrier by binding $Fe_3O_4$ and folic acid to GO nanosheets [79], whereas Ma *et al.* used iron oxide nanoparticles associated to partially reduced GO, for *in vitro* magnetic targeted drug delivery and *in vivo* photothermal therapy [80]. rGO nanocomposites combined with other metal-nanomaterials such as gold nanocluster-reduced GO systems were used not only for drug delivery but also for imaging of cancer cells, by Wang and co-workers [81].

Zhang *et al.* also achieved the targeting of drugs using GO nanocarriers covalently bound to folic acid molecules, which were able to deliver the load to MCF-7 cells overexpressing the folic acid recep-


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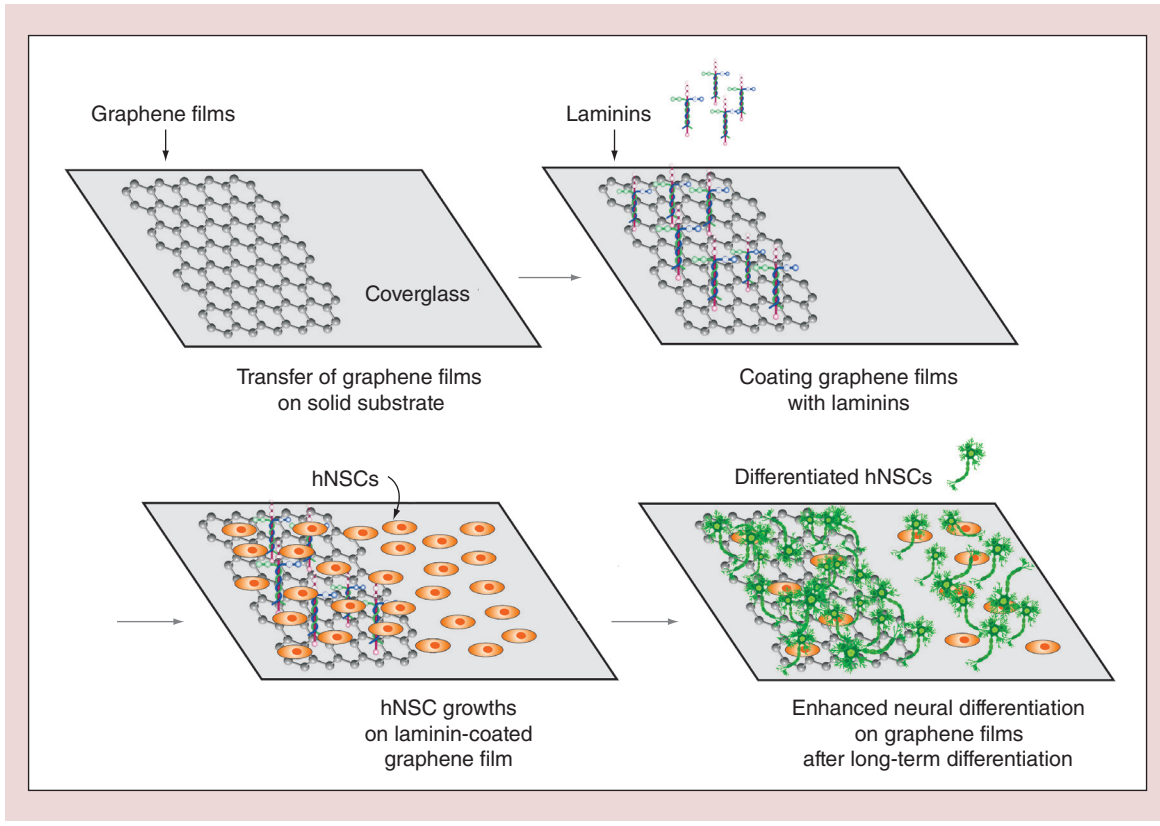


**Figure 7. Tissue engineering with graphene.** A schematic diagram depicting growth and differentiation of hNSCs on graphene coated with laminin.
hNSC: Human neural stem cell.

tor. Moreover, nanocarriers loaded with two anticancer drugs, camptothecin and Dox, demonstrated a synergistic dose-dependent cancer cell killing effect *in vitro* [82].

Besides the synergic effects of different anticancer drugs, the synergistic effect of chemotherapy (drug delivery) and photothermal therapy using graphene-based nanocarriers *in vitro* and in a mouse model has already been evaluated [70].

In 2011, Sahoo *et al.* functionalized GO with polyvinyl alcohol to deliver camptothecin to MDA-MB-231 human breast cancer cells [83]. One year later, Yang *et al.* improved this system's ability to deliver Dox by binding folic acid-modified β-cyclodextrin (as a target unit) and adamantanylporphyrin (as a linker unit) to the GO. These assays were performed *in vitro* and in a BALB/c nude mice model that were transplanted with HeLa cells. Drug delivery using these nanocarriers caused significantly higher tumor-growth inhibition than Dox alone [84].

Drug delivery systems responsive to environmental stimulations hold tremendous potential in targeted killing of cancer cells. Recently, Wen and collaborators using PEG-modified GO, generated a redox-responsive nanocarrier: that could detach the anticancer drug at tumor areas, with elevated glutathione levels and a reducing environment [85]. Besides the redox status of the environment, temperature can also trigger the release of drugs from graphene-based nanocarriers. The poly(N-isopropylacrylamide) that is thermo-responsive, was conjugated to GO by Pan *et al.* to efficiently deliver camptothecin to cancer cells [86].

Another inducer of drug release from the nanocarrier, as previously mentioned, is pH [87]. A pH-dependent release of drugs from graphene-based nanomaterials was also demonstrated by Hu and coworkers, wherein they used a pH-dependent release mechanism for Dox with PF127, a nonionic surfactant polyol associated with graphene nanosheets [88].

Graphene-based nanocarriers alone are also useful to treat cancer in spite of the controlled drug release systems. GO and the anticancer drug hypocrellin A (a photosensitizer) complex can induce the generation of singlet oxygen molecules which can kill the impregnated cells after exposed to light irradiation [89]. Photosensitizers such as Chlorin e6 (Ce6) can also be loaded in functionalized folic acid-GO complex for targeted PDT [90].

Diverse anticancer compounds beside Rituxan, doxorubicin, hypocrellin A and camptothecin, have

already been conjugated with graphene nanomaterials for delivery. In addition, SN38 (an analog of CPT) [91], ellagic acid [92], β-lapachone [93], 5-fluorouracil and 1,3-bis(2-chloroethyl)-1-nitrosourea [94] have also been conjugated to the nanosystems for targeted killing of cancer cells (see Table 1). More recently, Liu *et al.* developed a nanosystem using naphthalene-terminated PEG and the anticancer drug Dox integrated onto oxidized graphene. This system not only provided efficient cellular entry of the drug and sustained drug release, but also induced negligible *in vitro* cytotoxicity related to the nanovehicle [95].

Although the classes of anticancer drugs are most studied in combination with carbon nanomaterials, there are also examples of other drugs loaded onto these graphene nanomaterials (Figure 8); for example,

Table 1. Drug delivery systems using graphene-based nanomaterials.

| Drug delivery system | Drug loaded | Target cells in the study | Ref. |
|---|---|---|---|
| Graphene oxide-PEG- Rituxan (B-cell specific antibody: anti-CD20) | Doxorubicin | B-cell lymphoma line (Raji) | [96] |
| Graphene oxide-PEG | SN38 | Human colon cancer cell line HCT-116 | [91] |
| Graphene oxide-chitosan | Camptothecin | HepG2 and HeLa cell lines | [84] |
| Graphene oxide-folic acid-chitosan | Doxorubicin | † | [87] |
| Graphene oxide-$Fe_3O_4$ | Doxorubicin | † | [97] |
| Graphene oxide-$Fe_3O_4$- folic acid | Doxorubicin | SK3 human breast cancercells and HeLa cells | [84] |
| Graphene oxide partially reduced by PEG-iron oxide nanoparticles | Doxorubicin | † | [80] |
| Reduced graphene oxide-gold nanocluster | Doxorubicin | HepG2 hepatocarcinoma cells | [81] |
| Graphene oxide-folic acid | Doxorubicin + camptothecin | MCF-7 human breast cancer cells | [82] |
| Graphene oxide-PEG | Doxorubicin | EMT6 cells | [70] |
| Graphene oxide-poly(vinyl alcohol) | Camptothecin | Human breast cancer MDA-MB- 231 cells | [83] |
| Graphene oxide- adamantanylporphyrin-folic acid-modified β-cyclodextrin | Doxorubicin | HeLa and OCT-1 cell lines | [84] |
| Graphene oxide-PEG | Doxorubicin | HeLa cell line | [85] |
| Graphene oxide- poly(N-isopropylacrylamide) | Camptothecin | A-5RT3 cells | [86] |
| Graphene nanosheets-PF127 | Doxorubicin | MCF-7 human breast cancer cells | [88] |
| Graphene oxide | Hypocrellin A | HeLa cells | [89] |
| Graphene oxide | Chlorin e6 (Ce6) | Human stomach cancer MGC803 cells | [90] |
| Graphene oxide-tween 80 | Ellagic acid | Human breast carcinoma cells (MCF7) and Human colon adenocarcinoma cells (HT29) | [92] |
| Graphene oxide-maltodextrin | Ellagic acid | Human breast carcinoma cells (MCF7) and Human colon adenocarcinoma cells (HT29) | [92] |
| Graphene oxide-pluronic F38 | Ellagic acid | Human breast carcinoma cells (MCF7) and Human colon adenocarcinoma cells (HT29) | [92] |
| Graphene oxide- $Fe_3O_4$ | β-Lapachone | MCF-7 human breast cancer cells | [93] |
| Graphene oxide-chitosan | Ibuprofen and 5-fluorouracil | CEM cancer cells | [98] |
| Graphene oxide-chitosan | 5-Fluorouracil | MCF-7 cancer cell lines | [98] |
| Graphene oxide-polyacrylic acid | 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) | Mouse glioma GL261 cancer cells | [94] |
| Graphene oxide-naphthalene-terminated PEG | Doxorubicin | HeLa cell line | [99] |

†The study did not involve cells or did not specify a cell line.


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the anti-inflammatory drug Ibuprofen was released in a controlled way by a chitosan-functionalized GO nanocarrier [98].

An important feature of drug delivery performed by graphene materials is that they can allow a different release rate when compared with nanotubes based on the π–π interactions. By attaching to graphene materials different drugs (mainly aromatic ones) it is possible to observe π–π interactions stronger than when using nanotubes to perform the delivery. As a result, the drugs are released much more slowly from graphene allowing sustained drug release [100].

## Gene delivery with graphene-based nanomaterials

Graphene-based nanosheets, that have a large $sp^2$ hybridized carbon area, are able to interact not only with drugs, but also with other molecules like the nucleic acids DNA and RNA. Thus, they can be used for gene delivery or as carriers and protectors of probes involved in identifying miRNAs (Figures 9 & 10) [101] .

One of the obvious questions that arise is how can DNA, that is a polyanion, interact with GO? DNA is not completely electrostatically repelled from GO surfaces because its bases can establish hydrophobic and π–π stacking interactions thus overcoming the electrostatic repulsion and binding to the nanomaterial surface [103].

Hence, this type of interaction occurs preferentially in ssDNA molecules that have their bases free to interact with the graphene surface. However, dsDNA may also interact with graphene-based nanomaterials [104]. Adsorption of DNA can be optimized using small fragments at low pH and at high ionic strength [105], in which they are sterically protected from attack by nucleases (DNases) [103].

DNA's protection is one of the desirable characteristics that a safe gene delivery vector should have besides being capable of efficient cellular uptake. Therefore, graphene is an interesting option to deliver plasmids, especially when functionalized with cationic polyethylenimine that interacts well with DNA's phosphate groups. An obstacle that exists in polyethylenimine-graphene carriers is the former's poor biocompatibility and high cytotoxicity that increases with increasing molecular weight [104].

Kim *et al.* conjugated low-molecular-weight branched polyethylenimine to GO to deliver a plasmid with the cytomegalovirus promoter, controlling the expression

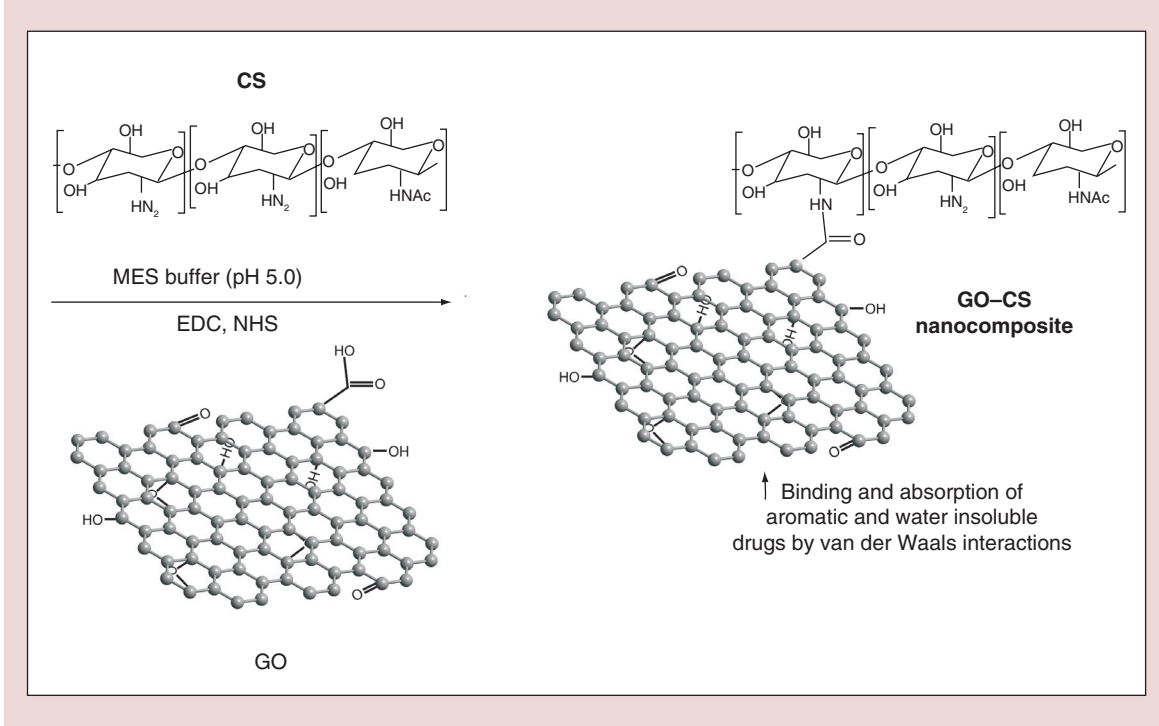


**Figure 8. Biofunctionalization of graphene by amide linkage.** The nanocarrier for drug and gene delivery, GO–CS, was prepared by the amidation of GO with CS in the presence of EDC-*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride-; NHS - *N*-hydroxysuccinimide and MES 2-(*N*-morpholino) ethanesulfonic acid. As a novel nanocarrier, GO–CS can be applied to load a water-insoluble anticancer drug, camptothecin, via π–π stacking and hydrophobic interactions.
CS: Chitosan; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; GO: Graphene oxide; MES: 2-(N-morpholino) ethanesulfonic acid; NHS: N-hydroxysuccinimide.

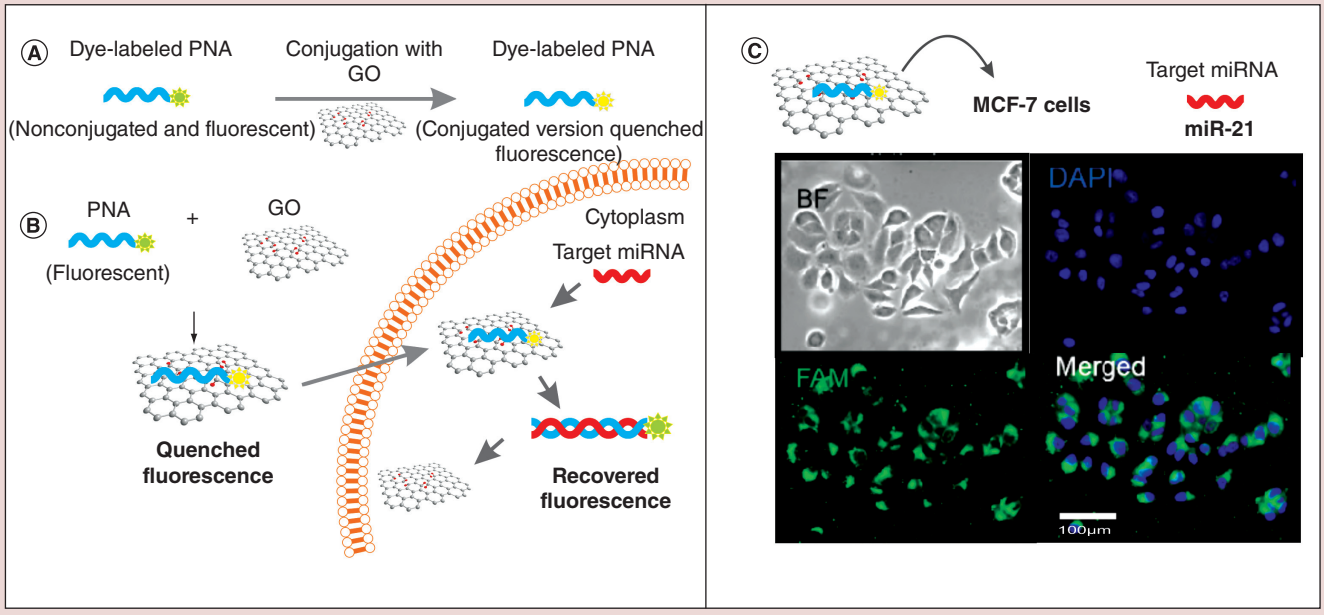


**Figure 9. Graphene-based nanotechnology to detect miRNAs. (A)** The dye-labeled PNA is fluorescent when nonconjugated; after interacting with GO, however, the fluorescence is quenched. **(B)** Graphene oxide nanoparticle-based strategy for detecting intracellular miRNAs. GO has been utilized as a fluorescence quencher of dye-labeled nucleic acid probes for multiplex miRNA detection *in vitro* and monitoring in living cells. Fluorescence of the dye-labeled PNA is quenched when interacting with GO and further hybridization of the PNA with the target miRNA led to the release of the PNA probe from the GO surface and fluorescence recovery. The ability of the PNA–GO probe to enter cells and the minimization of nonspecific fluorescence signal allows sensitive monitoring of multiple miRNA targets with low basal fluorescence even in living cells. **(C)** Fluorescent microscopy of MCF-7 cancer cells after treatment with GO-probe for miR-21 detection. MCF-7 cells exhibited high fluorescence due to high miR-21 expression.
GO: Graphene oxide; PNA: Peptide nucleic acid.
Adapted with permission from [102].

of the luciferase gene [106]. Furthermore, Feng and co-workers delivered a plasmid containing the gene coding enhanced green fluorescent protein (EGFP) to HeLa cells by using a similar nanocarrier [107]. Chen *et al.* used a polyethylenimine with higher molecular weight [108], in which the presence of GO reduced the cytotoxicity of polyethylenimine. They also delivered a plasmid containing the luciferase reporter gene to HeLa cells using this gene delivery system in presence of 10% fetal bovine serum (FBS) and demonstrated that serum proteins did not reduce transfection efficiency [108]. More recently, Zhou and co-workers delivered plasmid DNA (pEGFP) to mammalian cell lines and zebrafish embryos using polyethylenimine with grafted ultra-small GO [109]. In contrast, Xu *et al.* encapsulated inorganic nanoparticles and gold nanorods in GO nanosheets, which not only significantly decreased cytotoxicity and allowed better functionalization of polyethylenimine but also achieved high transfection efficiency and enabled higher viability of HeLa cells [110].

However, polyethylenimine is not the only substance that can be attached to graphene-based nanomaterials for gene delivery. Bao *et al.* used a chitosan-functionalized GO (a nanosystem also used by them for drug delivery, as previously mentioned) for gene delivery. A plasmid containing the luciferase gene was delivered to HeLa cells, in this experiment they showed by agarose gel electrophoresis that this complex could condense DNA for easy cellular uptake [76].

Furthermore, it is also possible to perform a dual functionalization in GO using, for example, PEG and polyethylenimine to enhance plasmid DNA transfection efficiency. This gene carrier developed by Feng *et al.* was also shown to be light-responsive [111].

Another example of a nanosystem responsive to the environment is the photothermally controlled gene delivery carrier developed by Kim *et al.*, constructed conjugating low-molecular-weight branched polyethylenimine and GO reduced via PEG. The enhanced gene transfection efficiency of this nanocarrier was attributed to accelerated endosomal escape by locally induced heat [112]. Table 2 summarizes some of the nanocarriers based on graphene, used for gene delivery.

## How is graphene internalized after *in vivo* administration or *in vitro* cell exposure?

Imagine that graphene or its derivatives are injected or administrated by a different pathway to living beings.


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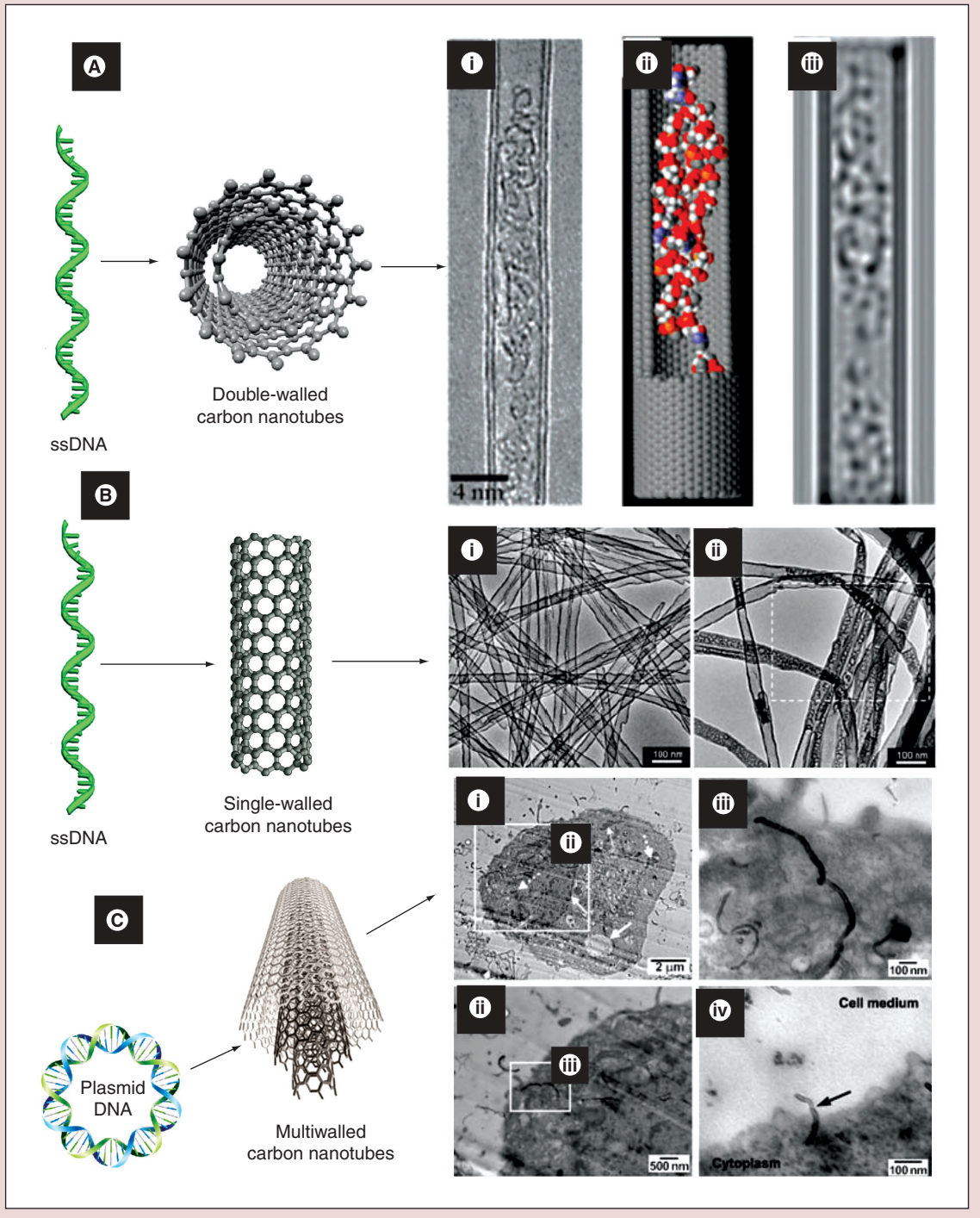


**Figure 10. DNA encapsulation and gene delivery.** **(A)** Encapsulation of ssDNA by double-walled carbon nanotubes. **(i)** Shows a transmission electron microscope (TEM) image of ssDNA molecules containing 30-base-long cytosine homopolymers (defined as C30) encapsulated double-walled carbon nanotubes. **(ii)** A structure model of the TEM image and **(iii)** a simulated TEM image. **(B)** Encapsulation of ssDNA by single-walled carbon nanotube. TEM images of ssDNA solution **(i)** without $CaCl_2$ and **(ii)** with $CaCl_2$ (DNA-$CaCl_2$) after soaking. In **(iii)** occurred internalization of ssDNA. **(C)** Encapsulation of large DNA plasmid by multiwalled carbon nanotubes. After encapsulation, HeLa cells were treated with plasmid DNA-multiwalled carbon nanotubes. **(i)** The entire cell; **(ii & iii)** are magnifications of the highlighted boxes. **(iv)** A multiwalled carbon nanotube crossing the cell membrane. Dotted white arrow: chromatin; dashed white arrow: mitochondria; thin white arrow: Golgi complex; medium white arrow: nuclear membrane; thick white arrow: vacuolum.
ssDNA: Single-stranded DNA.

To which organs do they preferentially go? How do they enter a cell? How are they eliminated from the body? These are questions that are being answered by new research involving these materials.

It is already known, for example, that dextran functionalized GO, when injected intravenously into mice, is distributed to various organs in which it can be still be found 4 h later (stomach, lung, kidney and intestine). The majority is found in the liver and spleen and persists for a longer amount of time [70].

Nanoscale graphene sheets (NGSs) functionalized with PEG and attached to $^{125}$I are similarly distributed to various organs right after injection, but mainly accumulate in the liver and spleen. These nanoparticles are also internalized in bone soon after injection (probably macrophage uptake in the bone marrow) [114].

Nanoscale GO (NGO) if conjugated to $^{125}$I and given to mice through intratracheal injection primarily remains in the lungs. After some time, NGO reaches other organs by passing through the air–blood barrier. The liver and intestines presented increase in radioactivity over the first few hours. Through this administration pathway, NGO was slowly cleared from the lungs (more than 3 months) [115].

When there is a tumor present, enhanced permeability and retention effect of graphene's nanomaterials is observed. In other words, prominent uptake of these nanomaterials is observed in the tumor area, because it possess tortuous and leaky vasculature that trap nanomaterials. For example, NGS-PEG-Cy7 complexes were almost completely internalized in the tumor after intravenous injection. Low fluorescence was present in other organs in mice that had either 4T1 breast cancer tumors, KB human epidermoid carcinoma tumors, or U87MG human glioblastoma tumors [43]. Almost negligible fluorescence was observed in the liver and spleen, which was also seen in the work of Rong *et al.* using GO conjugates to assess 4T1 tumors; the latter research group hypothesized that it can occur due to the fact that they are pigmented and light absorbing, making it difficult to be detected and measured by direct fluorescence [116].

Cell entry of graphene nanomaterials depends on the size and oxidization degree of the involved nanomaterials – two important characteristics that determinate the membrane perturbation induced by them [117]. The primary pathway for graphene QDs, in other words, is caveolae-mediated endocytosis. However, energy-dependent endocytosis and clathrin-mediated endocytosis are also involved in their cellular uptake [118]. Dextran functionalized GO enter HeLa cells through energy dependent endocytosis mechanism [107], which is also the preferential pathway used by 4T1 murine breast cancer cells to uptake PEGylated GO nano-platforms conjugated with the photosensitizer molecule 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-alpha (HPPH) [116,119]. GO and carboxyl graphene nanoplatelets can be passively internalized by the human hepatoma cell line (Hep G2) [120].

When it comes to being eliminated from the body, graphene nanomaterials use renal and fecal pathways [107,114]. Larger particles are preferentially eliminated by feces and smaller ones by urine. PEGylated GO (sheet size with 10–50 nm) and NGO [115] and NGS [43] are mainly eliminated by the renal path-

Table 2. Gene delivery systems using graphene-based nanomaterials.

| Graphene-based nanomaterial | Gene | Target cell in the study | Ref. |
|---|---|---|---|
| Graphene oxide-chitosan | Luciferase reporter gene | HeLa cells | [84] |
| Graphene oxide-low-molecular-weight branched polyethylenimine- | Luciferase reporter gene | HeLa and PC-3 cell lines | [106] |
| Graphene-polyethylenimine (1, 2 and 10 kDa) | Enhanced green fluorescent protein (EGFP) | HeLa cells | [107] |
| Graphene-polyethylenimine (25 kDa) | EGFP | HeLa cells | [108] |
| Graphene-polyethylenimine (25 kDa) | Luciferase reporter gene | HeLa cells | [108] |
| Grafted ultra small graphene oxide-polyethyleneimine | EGFP | H293T and U2Os cell lines | [109] |
| Graphene oxide-gold nanorods-polyethylenimine | EGFP and luciferase reporter gene | HeLa cells | [110] |
| Graphene oxide-gold nanoparticles-polyethylenimine | EGFP and luciferase reporter gene | HeLa cells | [110] |
| Reduced graphene oxide-PEG-polyethylenimine | EGFP | HeLa cells | [113] |
| Reduced graphene oxide-PEG-low-molecular-weight branched polyethylenimine | Luciferase reporter gene | PC-3 and NIH/3T3 cell lines | [112] |


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way, and dextran functionalized GO (50–100 nm) is mainly removed by the fecal pathway [70].

## Graphene-based nanomaterials toxicity & biocompatibility

The toxicity and biocompatibility profile of graphene-based nanomaterial is not yet completely understood. There are many useable nanomaterial and graphene derivatives and a large amount of cell types to test their effect on. Here, we summarize graphene-based nanomaterials' influence on bacterial, mammalian and plant cells.

### Bacteria

GO sheets have demonstrated antibacterial activity [121] and are currently being studied for their applicability as antibacterial material (e.g., in band-aids) [122]. Liu *et al.* compared the antibacterial activity of graphite, graphite oxide, GO and rGO using *Escherichia coli* and reported that under similar concentrations, GO had the highest toxicity, followed by rGO, especially hydrazine reduce GO [121]. They also disrupted cell membranes and caused oxidative stress [123].

Akhavan and Ghaderi observed that although Gram-negative bacteria were more resistant due to their outer membranes, Gram-positive and Gram-negative bacteria were inactivated when in direct contact with the extremely sharp edges of graphene nanosheets. In addition, Hu *et al.* also observed the loss of membrane integrity on bacterial cells (*E. coli*) in contact with GO and rGO nanosheets [124]. This proposed mechanism, loss of membrane integrity, is supported by Tu *et al.* observation that bacterial cells exposed to pristine graphene and GO nanosheets suffer degradation of their inner and outer cell membranes because these nanosheets can phospholipids [122].

Iron ion ($Fe^{2+}$) conjugates improve the graphene-based nanomaterials toxicity on bacteria. These iron ions enter the cell and are oxidized forming $Fe^{3+}$ and producing lethal hydroxyl radicals. This does not require direct physical contact and depends on the aqueous leaching of $Fe^{2+}$, its intracellular transport and the generation of the radicals inside the cells [125].

Another interesting antibacterial nanocomposite is the polyvinyl-N-carbazole-GO; it contains 3% of GO and does not show significant cytotoxicity in mammalian cells. However, this nanomaterial 'encapsulates' the bacterial cells, reducing their metabolic activity and causing cell death [70,126].

Although there are several reports describing antibacterial activity of graphene-based nanomaterials, on the other hand, there are also published studies demonstrating the opposite effect. Examples include the observation that *E. coli* could accumulate on a graphene electrode surface without growth inhibition [70]. Also, GO was not toxic to *Shewanella* species and *E. coli* species because they can reduce the graphene by microbial respiration; the nanosheets were exfoliated in anaerobic conditions in a mixed-acid fermentation environment [127]. The disparity between the antibacterial properties of graphene-based nanomaterials between groups has most likely arisen due to the use of diverse bacterial strains coupled with differences in experimental conditions. Nevertheless, further studies are required to understand the antibacterial role of carbon nanomaterials.

### Mammalian cells

The GO cytotoxicity in mammalian cells *in vitro* was verified using lung epithelial A549 cells: significant cell death was not induced. Minor toxicity was observed at doses higher than 50 μg/ml [128].Using the same cells, Hu *et al.* observed that the concentration of FBS influenced GO nanosheet toxicity. At 10% FBS, the cytotoxicity was significantly lower than that observed with 1% FBS. The cytotoxicity was diminished by protein adsorption to the nanomaterial preventing direct interactions with cell membranes [124]. The protein adsorption to the surface of nanomaterials in biological media induces the formation of a nanostructure-protein corona structure. However, this complex did not have an effect on graphene's properties due to its surface which would not allow charge transfer. So, it is possible that this complex formation presents the benefit of reducing the nanomaterial cytotoxicity without interfering with function [67].

The presence of GO less than 20 μg/ml was not toxic to human fibroblast cells. The nanomaterial was only cytotoxic at doses higher than 50 μg/ml and induced cell apoptosis [129]. GO and graphene biocompatibility were also tested in human erythrocytes and skin fibroblasts. The toxicity was dependent on the nanomaterial's degree of aggregation and mode of interaction with cells, wherein GO was shown to be more hemolytic and graphene sheets were more damaging to mammalian fibroblasts [130]. Only after long incubation times with human retinal pigment epithelium cells did GO have any influence on cell morphology, which suggests that GO is potentially biocompatible in these particular cells [131].

The HepG2 human hepatoma cells were used to compare single-wall carbon nanotubes and GO cytotoxicity. GO was more biocompatible than the nanotube, which induced reactive oxygen species production and apoptosis [132].

Graphene nanomaterials also possess the ability to penetrate alveolar spaces better than CNTs. However, pulmonary toxicity was observed for NGO intratracheally injected [115]. Graphene family nanomaterials,

in fact, the smallest particles tend to present higher lung deposition that may impair the nanomaterial clearance, and possibly lead to fibrosis [133].

Furthermore, Zhang *et al.* demonstrated that FLG sheets induced time-dependent caspase-3 activation in PC12 neuronal cells (derived from the pheochromocytoma of the rat adrenal medulla). Moreover, it also increased intracellular generation of reactive oxygen species and induced mitochondrial injury at a dose of 10 μg/ml [134]. Agarwal *et al.* also used neuroendocrine PC12 cells in addition to oligodendroglial cells and osteoblasts to test the rGO's cytotoxicity and observed that these nanocarriers were biocompatible with all these cell types, unlike CNT that inhibited the proliferation and viability of PC12 cells and the proliferation of osteoblasts [135].

Mouse neuronal cells were also exposed to graphene by Li *et al.* to explore how neurite maturation was affected using a mouse hippocampal culture model. They demonstrated that graphene was biocompatible, had no influence on cell viability, and significantly enhanced the number of neurites and their length [136].

MCF-7 epithelial breast cancer cells and U87MG human glioblastoma cells were also exposed to rGO exhibiting little toxicity, with an $IC_{50}$ value of 80.28 and 85.39 mg/l, respectively [42].

Cervical cancer HeLa cells were used to verify graphene cytotoxicity. Gollavelli *et al.* concluded that graphene can be used as a biocompatible imaging probe with an $IC_{50}$ value of 100 μg/ml [137]. Similarly, Lu *et al.*, working with the same cells, reported low cytotoxicity of GO nanosheets [138].

Ruiz *et al.* studied the effects of GO on HT-29 mammalian colorectal adenocarcinoma cells. These cells attached to the GO and their morphology remained intact. The use of this particular carbon material promoted their attachment and proliferation [139].

Furthermore, the toxicity of graphene-chitosan was tested by Fan *et al.* using the L929 cells (derived from normal subcutaneous areolar and adipose mouse tissue – a murine aneuploid fibrosarcoma cell line). These cells adhered to this biocompatible composite, seemingly biocompatible [140].

Wojtoniszak *et al.* concluded that the toxicity of graphene-based nanomaterials on L929 cells depended on the type of dispersant used and the concentration of the nanomaterial. They used PEG, polyethylene glycol-polypropylene, glycol-polyethylene glycol (Pluronic P123) and sodium deoxycholate as dispersants. GO functionalized with PEG (from 3125 to 25 μg/ml) was the most biocompatible nanomaterial in this study [141]. Pan *et al.* observed no obvious cytotoxicity in A-5RT3 cells (metastatic human squamous cell carcinoma) treated with poly(N-isopropylacrylamide)-GO [86]. Recently, Liu *et al.* tested the toxicity of a naphthalene-terminated PEG and oxidized graphene nanosystem for drug delivery. The nanosystem lacking drugs had no *in vitro* cytotoxicity on HeLa cells, but when attached to doxorubicin, led to considerable cell toxicity [95].

Sasidharan *et al.*, tested the cytotoxicity of rGO, carboxylated graphene and pristine graphene on Vero monkey kidney epithelial cells. Pristine graphene accumulated on cell membranes of Vero cells leading to apoptosis, whereas the carboxylated graphene exhibited low toxicity of the tested materials [142].

Testing the toxicity of nanomaterials is necessary because it defines their potential for *in vivo* application; cytotoxicity must be low in order to use these nanosystems for delivery or high to use them to kill unwanted cells (e.g., cancer cells). When it comes to killing cancer cells, the PDT is an important method. It was developed by Dong *et al.*, using graphene-based composite nanomaterials that increased their toxicity upon specific light irradiation. In their first study of the graphene-based PDT, they loaded a zinc phthalocyanine on the surface of rGO, obtaining significantly higher cytotoxicity against cancer cells [143]. Since then, many studies using that same technique have been described [144].

However, there is contrasting evidence regarding the cytotoxicity of graphene-based nanomaterials due to varying characteristics, such as concentration, size, shape, type of dispersants and mainly surface functionalization [104]. Functionalization can profoundly change their cytotoxicity by attenuating the hydrophobic interactions between graphene or GO with cells and tissues [104]. The complexes such as GO-PEG-Rituxan-Dox [96], GO-PEG-SN38 [91], GO-chitosan-Ibuprofen [98] and GO-chitosan-5-fluorouracil [98] showed negligible or no toxicity *in vitro* for drug delivery. These studies tested the toxicity of the aforementioned complexes in B-cell lymphoma Raji cells, the human colon cancer HCT-116 cell line, CEM a human T cell lymphoblast-like cell line and the MCF-7 breast cancer cell lines, respectively.

### In plants

Graphene-based nanomaterial cytotoxicity in plant cells has been seldomly studied. Begum *et al.* analyzed graphene's influence on root and shoot growth, biomass, shape and cell death in cabbage, tomato, red spinach and lettuce. They reported that graphene significantly inhibited plant growth and biomass levels. It also decreased the number and size of leaves in a dose-dependent manner and caused oxidative stress-induced necrosis in cabbage, tomato and red spinach seeds during development. No cytotoxicity was observed on let-


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tuce seeds during their development [145]. On the other hand, Nair *et al.* studied the effects of graphene on the germination of rice seeds. The presence of graphene decreased the rate of germination and impaired the development of root and shoot systems [146]. In plants, the toxic effect of graphene-based nanomaterials also seems to be related to the nanomaterials' concentration, the exposure time and the plant species used during the study [147].

## *In vivo* biodistribution & toxicology

The reports found in the literature generally indicate that functionalized graphene-based nanomaterials are less toxic than the nonfunctionalized ones *in vivo* [104]. However, when toxicity was tested by inhalation of graphene nanoplates (several layers of graphene sheets) by mice, they were found to be inflammogenic in lung and pleural space [148].

In order to assess nanographene biodistribution, Yang *et al.* fluorescently labeled PEG-graphene composites and injected them into mice bearing tumors. The composite accumulated near tumor sites (with lower uptake by the reticuloendothelial system after 24 h) with no short-term toxicity [43]. A year later, the PEG-graphene composite was labeled with radioactive iodine and injected in mice intravenously. It accumulated initially in the reticuloendothelial system, liver and spleen (clearance occurring after 3–15 days), but still did not induce toxicity at a dose of 20 mg/kg [114]. Two years later $^{125}$I-labeled PEGylated-GO was given to mice by oral feeding, without any obvious tissue uptake (indicating limited intestinal adsorption of these nanomaterials). However, after intraperitoneal injection, it accumulated in the reticuloendothelial system although toxicity to the treated animals was negligible [78]. In addition, intravenously injected GO was not toxic in mice at low and medium doses (0.1 and 0.25 mg). However, the high dose group (0.4 mg) developed granulomas in the lungs, liver, spleen and kidney with approximately 45% of the mice dying [129]. Zhang *et al.* did not observe toxic effects after exposing mice to 1 mg/kg of GO for 14 days, but after increasing the concentration to 10 mg/kg, they observed lung injury and inflammation with bioaccumulation and granuloma formation [70]. However, GO (when functionalized with dextran, a biocompatible polymer) labeled with $^{125}$I, was found to accumulate in reticuloendothelial system after intravenous injection of mice until being cleared within a week without noticeable toxicity. Yang *et al.* introduced GO in mice by intraperitoneal injection that did not accumulate in the reticuloendothelial system, and although it was retained in the mouse for a longer period of time, toxicity to the treated animals was significantly lower [75].

The pristine graphene, pluronic-graphene and GO were tested with injection into mouse lungs. In this experiment, GO caused severe and persistent lung injury (with inflammation and apoptosis), whereas, the pristine graphene minimized this reaction and pluronic-graphene was the least toxic [149].

Gollavelli and Ling chose zebrafish embryos (*Danio rerio*) to study the effects of microinjection of multifunction graphene attached to polyacrylic acid and fluorescein o-methacrylate. They did not observe toxicity induced by the composites, and they exhibited good biodistribution from head to tail of the embryos [137]. A year later, Liu *et al.* also used zebrafish to study the effects of graphene-based curcumin-loaded nanosystems, wherein they observed zebrafish could excrete them rapidly and the compound had nearly no influence on the zebrafish development [95].

Likewise, rabbits were used by Yan *et al.* to check if GO could cause damage to eyesight. The nanomaterial was intravitreally injected into the animals eyes causing few changes in eyeball appearance, intraocular pressure and an electroretinogram. Moreover, a histological examination suggested that there was no significant toxicity to the eyes, cell's growth and proliferation [131].

In order to avoid conjugation of fluorescent molecule to graphene-based composites to track their distribution, some research groups have been exploring the intrinsic fluorescent properties of graphene QDs. Zhu *et al.* labeled cells using graphene Qdots [74], and a year later Zhang *et al.*, developed an improved version of the Qdots with high photostability and low cytotoxicity [150]. Nurunnabi *et al.* studied the carboxylated photo luminescent graphene Qdots and observed that they accumulate in liver, spleen, lung, kidney and tumor sites after intravenous injection in mice. These materials did not cause appreciable toxicity in the treated animals and no organ damage or lesions were observed after 21 days at 5 or 10 mg/kg concentrations [151].

## Blood–brain barrier overlap by graphene nanomaterials

The brain is an organ protected by the blood–brain barrier (BBB). The BBB participates in controlling the transfer of molecules between the blood and brain parenchyma. This system of protection in the brain acts against toxic and infectious agents while maintaining the ionic and volumetric environments; however, it also creates an obstacle for effective systemic drug delivery to the brain [152]. Under the physiological conditions, circulating molecules can only gain access to the brain via diffusion (paracellular or transcellular) or specific receptor-mediated or vesicular mechanisms (adsorptive transcytosis) [66,153].

Treating neurologic diseases, such as brain tumors, inborn metabolism errors and infectious and neurodegenerative diseases is a daunting challenge due to the BBB impermeability to many drugs [154]. Several studies have shown that graphene and its derivatives can function as drug nanocarriers [153,155]. Graphene and GO, with extremely high specific surface areas, can interact with various biomolecules in drug delivery and gene transfection [104,156].

For the treatment of malignant gliomas, the most aggressive types of brain tumors, Liu *et al.* (2013) [99] developed a transferrin (Tf)-conjugated PEGylated nanoscaled GO (GO) for targeted delivery of the anticancer drug Dox (Tf-PEG-GO-Dox). In this study, the Tf-PEG-GO-Dox conjugate was administered systemically to mice, and it was found that the concentration of the drug in glioma tissue was significantly higher than in normal brain tissue compared with the other groups that used different methods for drug delivery. According to the authors, the conjugate Tf-PEG-GO-Dox penetration of the BBB may have been favored by the high vasculogenesis exhibited by gliomas. The newly formed vessels do not exhibit BBB characteristics: the gaps between neovascular endothelial cells in glioma are larger, up to 0.38 μm [71,157]. Another feature of gliomas is the secretion of soluble factors capable of degrading the tight junctions of the BBB to facilitate cell invasion of brain tissue [87]. Thereby, leaky vasculature allows a nanocarrier in the size range of 100 to 400 nm (similar to that of GO used in this study) to bypass the BBB.

Science has made significant progress through the use of graphene for the treatment of other neurodegenerative diseases such as Alzheimer's disease (AD). AD is a disorder with an insidious onset and progressive course, the prevalence increasing with age. It is characterized by neuronal degeneration and death, related to the deposition of plaques in the brain composed primarily of 39–43 amino acid amyloid-β peptides (Aβ) that are derived by enzymatic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases [29,69]. The inhibition of Aβ aggregation and destabilization of preformed Aβ fibrils are attractive therapeutic and preventive strategies for AD treatment [73].

In the study developed by Li *et al.* (2012) a conjugate was synthesized using thioflavin-S (ThS)-modified and GO (GO-ThS). The nano-GO has high ability to generate local heat when subjected to laser irradiation by near-infrared (NIR). GO was covalently linked to ThS, which can selectively attach to Aβ aggregates, forming the conjugated GO-ThS-Aβ. This conjugate can effectively dissociate amyloid deposits both in buffer and mouse cerebrospinal fluid (CSF) and protect cells from Aβ-related toxicity by NIR irradiation. Furthermore, the hyperthermic effects of GO have been considered useful in clinical treatment with reduced side-effects [75,91]. Some studies have shown that NIR optical absorbance of nano-GO can result in mild photothermal heating that increases the cell membrane permeability without significantly damaging cells. This mechanism can be an enabler for the passage of graphene nanocompounds across membranes of epithelial cells of the BBB [76,77].

In a recent report, Yang *et al.* developed an MRI-detectable nanovector based on nanoparticles of poly(amidoamine) dendrimer-grafted gadolinium-functionalized nanographene oxide (Gd-NGO) to effectively load chemotherapeutic Epirubicin (EPI) and Let7g miRNA into brain tumor cells. Using the Gd-NGO as contrast agent for MRI, the authors identified the location and extent of BBB opening, induced by focused ultrasound (FSU) and demonstrated that Gd-NGO injected into mice not only was able to delivery EPI and miRNA into brain tumor tissue but also successfully transfect the tumor cells. These results suggest that GO may be a promising nonviral vector for chemogene therapy and molecular imaging diagnosis in future clinical applications [158].

The toxicity of graphene was tested in an *in vitro* model of the BBB by measuring trans-endothelial electrical resistance (TEER). The results from this work show that TEER values after graphene exposure remained similar to the TEER values of the control model and thus, indicates that graphene does not interrupt the integrity of the *in vitro* BBB model. Additionally, the real-time effects of graphene toward BBB individual components, astrocytes and endothelial cells, was also monitored using an array-formatted whole cell based electrical impedance sensing system. The resistance values measured after graphene was incorporated with the cells showed little difference compared with the control, indicating that graphene does not interfere with cellular attachment. In conclusion, this study highlights the biocompatibility of graphene and the broad potential of using these nanomaterials for biomedical applications [159].

## Different applications of carbon-based nanoparticles: graphene versus CNTs

As mentioned above, carbon-based nanoparticles such as graphene have been shown to have some promising medical applications. However, this nanomaterial can also originate other important carbon-based nanoparticle: the CNT. Nonetheless, when they are compared, graphene presents some advantageous features over CNTs: its surface area is larger and can be easily complexed to other molecules, which ensures a large number of different possible applications [107]; it also


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presents better dispersivity, which helps, for example, its photothermal sensitivity[69;148]; PEGylated nanographene can enter and can be retained easily in tumors when compared with CNTs [107,144] and graphene's production costs are almost negligible [107].

Ryoo *et al.* reported that a favorable environment for transfection could be achieved by coating substrates with rGO, GO and/or with multiwalled carbon nanotubes (MWCNTs). The expression of the GFP gene when transfected in NIH-3T3 fibroblasts was higher using graphene nanomaterials: the substrates coated with rGO and GO induced an increase up to 250% in gene expression when compared with cells on uncoated substrate [160,161].

There are also other studies highlighting further advantages of graphene for other applications such as drug delivery. Rhodamine B, for example, was loaded easily on GO – a nanomaterial with larger loading capacity than CNT.

In cancer research, Markovic *et al.* showed that nanographene had best *in vitro* photothermal cancer cell killing efficiency than CNT. Sheets of graphene coated with polyvinylpyrrolidone generated more heat than single-wall carbon nanotubes coated with DNA or sodium dodecylbenzenesulfonate, causing, consequently, more cell death of U251 human glioma cells [144].

Graphene has been the material of choice for development of biosensors. This nanomaterial offers better reproducibility than CNT (because single graphene sheets have the same chirality and electric properties, different from single CNT). An electrochemical glucose sensor was successfully developed using the enzyme glucose oxidase and graphene or MWCNTs; however, despite being able to provide higher current signal, the CNT sensor was more vulnerable to interfering substances.

Nevertheless, the CNTs, which have a history much earlier than graphene, have also some advantages over the latter. CNTs not only have lower costs for generation of functional nanocomplexes with polymers such as PEG, but also have better photoluminescence characteristics – thanks to its properties, CNTs are more likely to be used in biomedical imaging without the need of external labels.

Though, this does not mean that it is necessary to choose between these two carbon-based nanomaterials; some recent studies used a combination of graphene and CNTs, which showed promising results. A nanocomplex made of rGO and MWCNTs was able to enhance, up to 200%, the GFP expression in HeLa cells. In NIH-3T3 and NG97 cell lines this nanocomposite revealed itself as a great gene delivery system: with high transfection efficiency and low cytotoxicity. A nanoparticle hybrid was also successfully synthetized by using grapheme nanosheets, CNT and iron oxide, to perform the delivery of anticancer drugs. 5-Fluorouracil release could be triggered by pH after internalization by HepG2 cells; these nanoparticles were also nontoxic for Chang liver cells.

## Conclusion

Scientists consider graphene as a natural wonder of the material world. That's because graphene and its derivatives can be used in a large number of fields to generate, faster computer chips to new therapeutic strategies. An increasing number of studies have been published in the recent years analyzing their potential application for gene and drug delivery and tissue engineering, which lead us to believe that these materials hold promise for solving future medical problems. Nevertheless, there are still several obstacles that must be overcome such as, rapid and highly efficient synthesis, which can guarantee the thickness of one atom to conserve material properties, for potential real-world applications.

## Future perspective

Due to a wide range of unique and fascinating properties that graphene-based materials possess, these materials have found extensive applications in the biomedical area in last years. In this review, we summarized the latest progress in graphene and its derivatives and their potential applications for drug delivery, gene delivery, biosensor and tissue engineering. Furthermore, we also describe their *in vitro and in vivo* toxicity and biocompatibility in bacterial, mammalian and plant cells. Although the potential uses for graphene seem limitless, there are still many unsolved issues and challenges that must be considered.

First, an emergent need is to develop methods of production on a scale with industrial potential that maintain the desirable properties of the material. Despite efforts to develop a scalable production method, it is still difficult to produce single-layer samples of graphene. Moreover, large scalable methods are prone to produce defective graphene, which can significantly affect the physicochemical properties of graphene. As an attempt to overcome this issue, Kim and coworkers developed a scalable production method for depositing rGO flakes by supersonic atomization and spraying that resulted in less graphene flakes aggregation and therefore, improving the quality of the graphene layer produced. Thus, unlike other methods, it is possible to deposite rGO on any substrate, such polymeric and flexible ones with no post-treatment required [162]. In 2014, McNerny and coworkers demonstrated a direct production of graphene using CVD. Graphene is

grown at the interface between a nickel (Ni) film and the silicon dioxide ($SiO_2$) substrate. By this approach, authors showed that graphene grown at the $Ni/SiO_2$ interface can be directly retained on a clean silicon dioxide substrate by mechanical removal of the nickel or by *in situ* delamination of the nickel at high temperature. Importantly, this process eliminates the need for an additional step to transfer graphene from the metal catalyst film to the substrate and resulted in greater than 90% coverage across centimeter-scale dimensions [163]. To achieve large, high-quality single crystals of graphene, Hao and coworkers used the CVD method to grown graphene on cooper (Cu) by a controlled oxygen (O) surface. They discovered that graphene nucleation density can be decreased through depositing O on the Cu surface enabling repeatable growth of single-crystal graphene domains. Another major finding by the team was that graphene film's electrical properties seems to be of the same quality compared with mechanically exfoliated graphene, even its growth was in the presence of O [164].

The second challenge is regarding graphene-based biosensing. Over the last few years, graphene-based biosensors have brought great perspectives on the use of this material for real-time sensing. Its ultrahigh surface area (2630 $m^2/g$) can serve as a good support for biomolecules. However, the major limitation for the current generation of graphene-based detectors is concerned of their lack of specificity and also to its poorly sensitivity. In fact, most of biomolecules can adsorb on graphene, which limits selectivity. Moreover, graphene sensitivity can range from hundreds to thousands times lower than what a commercial device would require. Graphene field-effect transistor (FET) biosensor performance has low sensitivity which is limited by the lack of zero-band gap of graphene. This lead to increased leakage current, leading to reduced sensitivity. However, reducing graphene sheets to nanoscale dimensions or adding dopants modify the gate voltage or open the zero-band gap can overcome this limitation. Zhang and coworkers reported the use of a graphene-nanoparticle hydrid FET for real-time detection of hydridization of ssDNA with high sensitivity (2.4 nm). This hybrid structure was used as a channel material and had the PtNPs directly syntetized onto the rGO sheets by photochemical reduction [150]. Recently, Ren and coworkers reported an ultra-sensitive and selective acetylcholinesterase (AChE) biosensor for organophosphorus pesticides detection based on a direct electrodeposition of electrochemical reduced graphene oxide (ERGO)-Au nanoparticles (AuNPs)-β-cyclodextrin (β-CD) and Prussian blue-chitosan (PB-CS) on glass carbon electrode. The authors described an efficiently electron transfer between the analyte and the electrode surface, as well as an improved performance of the biosensor due to the electrocatalytic synergy effect between them. Moreover, ERGO and AuNPs were found to increase the surface loading of AChE and therefore, provided a suitable microenvironment for the immobilization of AChE. As the authors described, the resulting ERGO-based enzyme biosensor exhibited excellent sensitivity, good stability, fast electrochemical response and good reproducibility and therefore can provide an advantageous and high-performance platform for sensing applications [165].

Although recently advances on graphene-based materials for drug delivery seems encouraging, there are still many challenges remain toward clinical applications. In order to explore the truly potential of graphene materials as delivery system, efforts must be address to better understanding the mechanisms of clearance and long-term toxicity, tissue distribution and cellular-uptake mechanism, intracellular and *in vivo* metabolic pathway, as well to overcome problems such as solubility or stability in order to enhance drug delivery. A promising strategy for enhancement of cancer treatment using a graphene-based codelivery nanosystem of a cell-membrane-targeted anticancer protein and a chemotherapeutic agent for combination cancer treatment has been reported. This designed nanocarrier composed of GO, PEG linker and a furin-cleavable peptide attached to two drugs – TNF-related apoptosis-inducing ligand and Dox was found to preferentially accumulate at the tumor site by means of the enhanced permeability and retention effect after intravenous administration, specially due to the ability of the membrane-associated cytokine TNF-related apoptosis-inducing ligand to directly bind to the surface of cancer cells, and therefore efficiently release its cargoes to their distinct sites of action separately, in a site-specific manner [166]. Recently, it has been reported the development of a multifunctional nanocarrier based on a paramagnetic graphene QDs (GQDs), folate and Dox that was efficiently taken up by the cancer cells overexpressing folate receptors and then promoting an inhibitory effect on HeLa cells, as confirmed by an *in vitro* cytotoxicity assay. The multifunctional nanocarrier not only exhibit excellent biocompatibility but also showed a longitudinal relaxivity $r_1$ of 11.49 $mM^{-1}s^{-1}$, enhancing the brightness of the $T_1$-weighted MRI, showing that their possible use as positive contrast agents for MRI [167].

The last ultimate challenge which we discuss is related to the application of graphene on tissue engineering. Handle graphene is not so easy and turn it as a plan thin layer gets so hard works as it easily crumples. To control the process of crumpling and unfolding of large-area graphene Zhao's group linked graphene with prestretched rubber film [168]. This turns this


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## Executive summary

**A brief history of graphene**

- Although since 1859, there were reports of the basics of obtaining graphene (formerly known as graphon), only in 2004 was it isolated by simple mechanical exfoliation.
- Chemical vapor deposition is a commonly used method to synthetize graphene, but this is not the only method available. There is also graphitization of silicon carbide surfaces, intercalation/exfoliation methods and other reductive chemistry reactions.
- Graphene and its derivatives' unique advantageous characteristics made it useful in a wide range of fields. These carbon-based materials have been shown to be innovative and significant in optics, electronics, biotechnology and medicine.
- The 21st century has become the Carbon Age, in which efforts have been made to characterize graphene and carbon-based materials to determine the range of potential applications.

**Important graphene derivatives**

- Like graphene synthesis, the synthesis of graphene derivatives can also be performed by applying various methodologies.
- We review the structures and synthesis of ultrathin graphite, graphite oxide, graphene oxide, reduced graphene oxide and nano-graphene oxide.

**Graphene as an advantageous carbon-based material for biological & medical use**

- Nanotechnology is a field of interdisciplinary research in which graphene-based materials receive special attention for being suitable for biological and medical applications.
- Graphene-based materials possess outstanding mechanical, electronic, optical, thermal and chemical properties and are also biocompatible materials.

**Tissue engineering with graphene nanomaterials**

- By using graphene-based materials, it is possible to synthesize scaffolds with desirable properties that facilitate use in tissue engineering applications with different cell types. These features include biodegradability, high porosity, the possibility of surface chemical modification, mechanical characteristics that support cells adhesion and proliferation, biocompatibility and pliability.
- Even induced pluripotent stem cells are able to attach, proliferate and differentiate in graphene and graphene oxide-coated biomaterials.

**Drug delivery with graphene nanomaterials**

- The surface properties of graphene nanomaterials make them suitable as drug nanocarriers with a relatively low cost for preparation.
- The nanocarriers can also be developed to improve drug delivery by performing modifications that allow pH-dependent release or release in a specific biophysical environment.

**Gene delivery with graphene nanomaterials**

- DNA can interact with pure graphene-based materials by hydrophobic and $\pi$–$\pi$ stacking interactions by overcoming the electrostatic repulsion of phosphate groups.
- It is also possible to functionalize graphene materials to introduce positive charge on their surface to interact with DNA by electrostatic interactions.
- Gene delivery using these materials has already been tested in a variety of cells, through different graphene-based materials functionalization. It is also important to highlight that these materials optimise the delivery by protecting the DNA before releasing it.

**How is graphene internalized after *in vivo* administration or *in vitro* cell exposure?**

- After *in vivo* application, graphene is initially distributed to a variety of organs, but eventually accumulates in the liver and spleen. In tumors, there is an enhanced permeability and retention effect which is vital to targeted drug delivery.
- It is possible to improve the targeting of these materials by modifying their pathway of administration or functionalizing them.
- Cell entry of graphene nanomaterials depends on the membrane perturbation induced by them which is mainly dependent on their size and oxidization degree.
- Graphene nanomaterials are eliminated via renal and fecal pathways.

**Graphene nanomaterials toxicity & biocompatibility**

- In bacteria, graphene nanomaterials can be toxic to some species and innocuous to others. When toxicity is observed, the material presents antibacterial activity, inducing oxidative stress and disrupting the cell membrane. This toxic effect may be influenced by iron ($Fe^{2+}$) conjugation.
- In mammalian cells, it is also not possible to establish as a general rule that graphene nanomaterials are either cytotoxic or biocompatible. It seems to depend on concentration and functionalization, the cell in question and the medium components (mainly FBS) in which cells are immersed.

## Executive summary (cont.)

**Graphene nanomaterials toxicity & biocompatibility (cont.)**

- In plants, the toxic effect of graphene-based nanomaterials seems to be related to the nanomaterial concentration, the exposure time and the plant species used during the study. The cytotoxicity in plant cells has been seldomly studied.

***In vivo* biodistribution & toxicology**

- Graphene-based nanomaterials toxic effects can be reduced by functionalization, which can also control distribuition inside the body.
- The observed toxicity is also related to the materials' concentration and dose and when a tumor is present the distribution tends to favor accumulation near tumor sites.

**Blood–brain barrier overlap by graphene nanomaterials**

- Although the blood–brain barrier is very selective, functionalized graphene-based materials can permeate, without interrupting its integrity, which is important to the development of new strategies to treat brain tumors and Alzheimer's disease.

system an attached-detached pattern in nanometers scale which enables one end of the graphene be linked on the rubber and the other end detached from the rubber. In this way when the rubber was relaxed, the end detached graphene maybe compressed to crumple [168]. And in the other way, when the rubber film was stretched back, the linked graphene end can be pulled on the crumpled graphene to unfold it. So, in this way, stretching and relaxing a large-area atomic-thick graphene can be controlled by the crumpling and unfolding of a rubber film. This approach has opened possible to turn graphene from being transparent to opaque through crumpling it or to turn it back to transparent by unfolding it. Also it is possible to use this crumple and unfolding graphene as contracting and relaxed muscles when graphene is layered with different polymer films. Using graphene's conductivity properties, in the same way which muscles work, electricity would crumple graphene film or contracting the artificial muscle; when the electricity is cut off, it relaxes. It is also possible to control the degree of contraction and relaxation by controlling the voltage [168]. A variety of technologies can be enabled through these artificial muscles as energy harvesting and storage to robotic and drug delivery.

In summary, graphene-based materials emerge as a novel nanomaterial platform for biomedical applications; however, there are still many challenges that are critical and inevitable and must be study in details.

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