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Prospects and Challenges of Graphene in Biomedical Applications

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Dedicated to Professor Maurizio Prato on the occasion of his 60th birthday.

Graphene materials have entered a phase of maturity in their development that is characterized by their explorative utilization in various types of applications and fields from electronics to biomedicine. Herein, we describe the recent advances made with graphene-related materials in the biomedical field and the challenges facing these exciting new tools both in terms of biological activity and toxicological profiling in vitro and in vivo. Graphene materials today have mainly been explored as components of biosensors and for construction of matrices in tissue engineering. Their antimicrobial activity and their capacity to act as drug delivery platforms have also been reported, however, not as coherently. This report will attempt to offer some perspective as to which areas of biomedical applications can expect graphene-related materials to constitute a tool offering improved functionality and previously unavailable options.

1. Introduction

Variations in covalent bonding between carbon atoms leads to naturally occurring different materials called carbon allotropes. Each of them has distinctive physical and chemical properties owing to the unique spatial arrangement that carbon atoms adopt. Allotropes of carbon include graphite, diamond and carbon nanotubes, among others. The atomic structure of graphite is characterized by the multiple stacking of one-atom thick sheets formed by carbon atoms arranged in a hexagonal lattice. The isolated two-dimensional crystal structures made of single atomic layers of graphite are called "graphene".

The existence of single graphene sheets had been discussed in theory more than 50 years ago.[1] Yet, the existence of two-dimensional, atomically thin crystal materials was considered physically impossible.[2] In 2004, a single sheet of graphene was isolated and characterized by Novoselov and Geim.[3] Since then, research on graphene has been increasing almost exponentially, attracting the interest of various scientific fields. Graphene's optical, electronic and thermal properties are proving to be extraordinary and placed it in the spotlight of material scientists, physicists and chemists alike.[4]

Interest in monoatomic graphene sheets also drew attention to other carbon-based materials that are now examined under a different light. The most notable of them is graphene oxide - graphene sheets derivatized with oxygen-containing functional groups. By analogy to graphite and graphene, graphene oxide is the "building block" of graphite oxide. More particularly, it is the result of graphite's oxidation under acidic conditions, first described by Brodie et al. more than 150 years ago.[5] Today, the most popular method for the production of graphene oxide is based on the principle first introduced by Hummers and Offeman (commonly referred to as the *Hummers method*) that involves the oxidation of graphite by potassium permanganate in concentrated sulphuric acid.[6]

Graphene is composed almost entirely of sp^2 hybridized carbon atoms and their electrons participate in aromatic conjugated domains. In graphite, van der Waals forces are those that keep graphene sheets tightly together. Even in the case of single graphene sheets synthesized de novo (for example, through chemical vapor deposition of carbohydrates on a metal catalyst) the apolar nature of the carbonaceous material makes it highly hydrophobic.[7,8] On the other hand, the oxidation of graphite to graphite oxide loosens its firmly packed graphene sheets. This occurs due to the random introduction of carbonyls, hydroxyls and epoxides on the planar surfaces and edges of the carbon sheets (**Figure 1**). Graphene oxide sheets can then be exfoliated from graphite oxide particles through ultra-sonication. Due

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to their derivatization, graphene oxide sheets are more hydrophilic and the hydrogen bonds between their polar functional groups and water molecules offer reasonable colloidal dispersibility under appropriate pH conditions.[9]

The great advantage of using graphene oxide (GO) over other carbon-based materials is the considerably more reliable aqueous dispersibility and colloidal stability of both single- or few-layered GO that are earning the material great popularity in the biomedical field. The physicochemical characteristics of GO materials render them also chemically versatile templates of high surface-to-volume ratio, which can be adjusted to the needs of a variety of biomedical applications, ranging from the detection of biomarkers to imaging and cancer therapy. The most popular biomedical applications of graphene oxide are schematically represented in **Figure 2**. Apart from the hydrophilic GO, graphene and reduced GO are also explored in biomedical applications due to their interesting optical and electrical properties.

2. Graphene as Sensors of Biomolecules

The nanotechnology field is in constant search for new materials that can be engineered for biosensing purposes, i.e., the accurate, sensitive and selective detection of biomarkers. Recently, the graphene family of materials has shown great potential and the proposed applications for graphene-based biosensors have shown great diversity. Graphene biosensors for thrombin[10] and caspase-3[11]can provide tools for the effective diagnosis and monitoring of chronic and acute pathological conditions alike. Also, single nucleotide polymorphisms can be traced for early identification of genetic disorders such as Alzheimer's or cystic fibrosis.[12,13]

The use of graphene nanomaterials for biosensing applications involves two alternative technologies. One that uses a probe molecule on the graphene sheet that interacts with the analyte and another that is label-free and is based on measurement of the changes in electrical properties of the graphene platform on interaction with an analyte. Some of the most important examples that have reported use of graphene materials for biosensing purposes are summarized in **Table 1**. In a recent study, graphene oxide (GO) was coated with dye-labeled single stranded oligonucleotide molecules (aptamers) via π–π stacking.[14] The fluorescence resonance energy transfer (FRET) effect between the GO and the dye led to quenching of its fluorescent signal. On interaction with a target molecule complementary to the aptamer, strong complexation between the two occurs and as the newly formed complex desorbs from the GO surface, the FRET effect is cancelled and fluorescence can be restored to detectable levels. This strategy of fluorescence quenching and recovery has been utilized to measure disease-related enzyme activities such as viral helicase, nuclease, and methyltransferase.[15–17] Alternatively, Feng et al. reported a GO-based electrochemical sensor for label-free detection of entire cancer cells.[18] A 26-mer DNA aptamer with high affinity towards nucleolin, a membrane protein over-expressed in various cancer cells was covalently bound to GO sheets. The anchoring of the cells to the aptamer molecules altered the electrical impedance and cyclic voltammetry readings of

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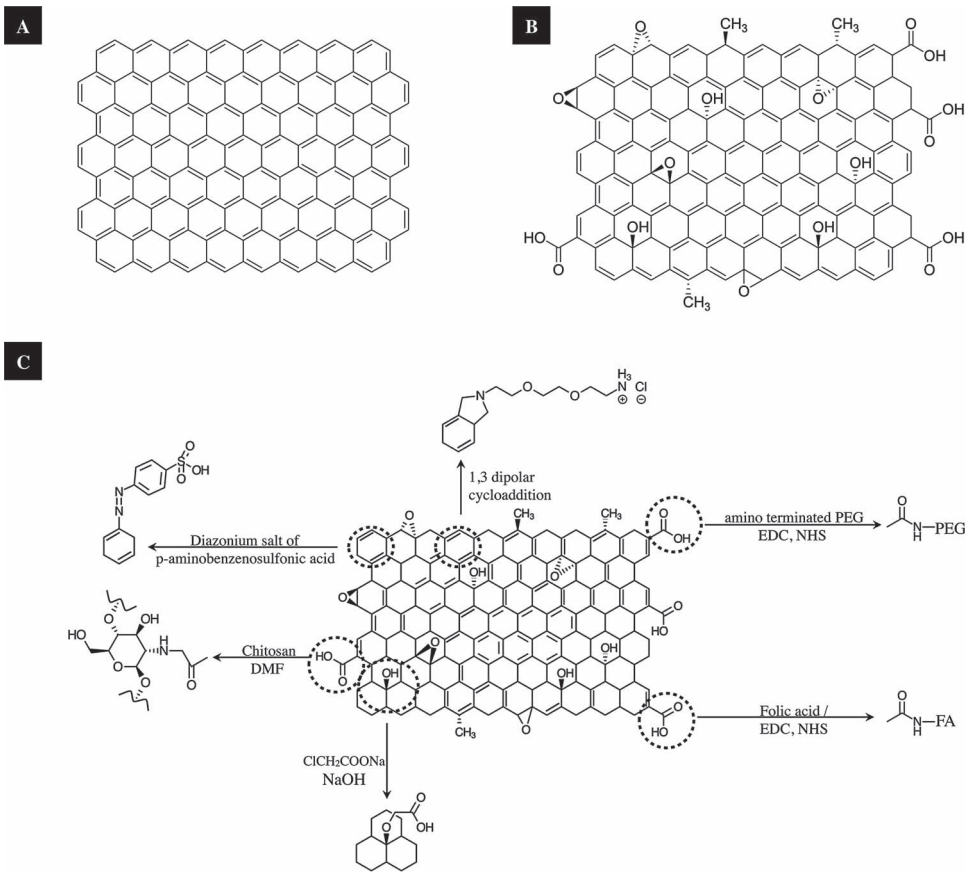

Figure 1. Graphical representations of graphene and its derivatives. A) A graphene sheet consisting of carbon atoms of sp^2 hybridization. B) Graphene oxide sheet consisting of either sp^2 or sp^3 hybridization, due to their derivatization with carbonyls, epoxides and tertiary alcohol functional groups. C) Chemical functionalisation strategies of graphene and graphene oxide.

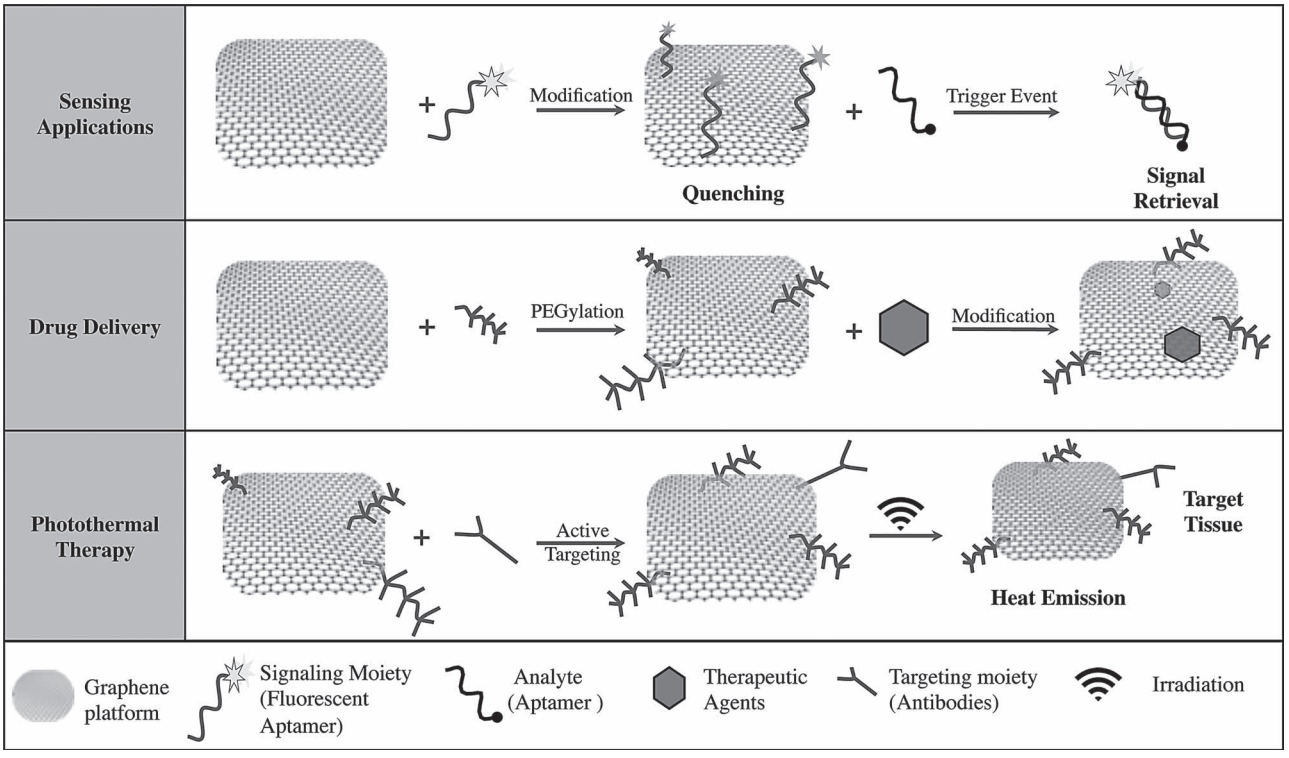

Figure 2. Most common biomedical applications of graphene related materials including sensing applications, drug delivery and photothermal therapy.

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Table 1. Graphene Materials in Biosensing Applications.

Material	Fluorescent Label	Analyte	Probe	References
GO	Fluorescein	Thrombin	Human Thrombin Aptamer	[19]
GO	-	Glucose oxidase	-	[20]
GO	Quantum Dots	ssDNA	ssDNA (complementary)	[21]
Graphene	Fluorescein	Thrombin	Human Thrombin Aptamer	[14]
GO	Fluorescein	Bleomycin	ssDNA	[22]
Graphene	-	Single Nucleotide Polymorphism	hpDNA	[13]
GO	Fluorescein	Caspase-3	Lysine	[11]
Glassy carbon/ Chitosan/ GO electrode	-	Thrombin	Human Thrombin Aptamer	[10]
Glassy carbon/ GO/ Nafion/ Au electrode	-	Thrombin	Human Thrombin Aptamer	[23]
GO/ Fe_3O_4/ Au electrode	Ferrocene	ATP	Aptamer	[24]
GO	Fluorescein	Flavonoids	ssDNA	[25]
GO	Fluorescein	Exonuclease	hpDNA	[17]
Graphene Electrode	-	Media Nutrients	-	[26]
GO	-	Fe^{3+}	-	[27]
Graphene/ Chitosan/ Myoglobin Electrode	-	Trichloroacetic acid	-	[28]
GO/ Nafion/ Myoglobin electrode	-	H_2O_2, $NaNO_2$, O_2	-	[29]
Graphene/ Glassy Carbon Electrode	-	Epinephrine	-	[30]
Graphene/ PTCA Electrode	-	Nucleolin	AS1411 Aptamer	[18]
GO	Fluorescein	dsDNA	ssDNA	[31]
GO	Fluorescein	Exonuclease/Methyltransferease	DNA	[16]
GO	Fluorescein	Helicase	ssDNA	[15]
GO	-	Phospholipase	Phospholipid	[32]

the electrode thus detecting the presence of the cancer cells. Some types of modifications proposed pristine graphene and reduced graphene oxide in view of their utility in biosensing applications are shown in **Figure 3**b and d.

The quenching efficiency of fluorescence in graphene when in close proximity to fluorophores is even better than carbon nanotubes.[14] This is probably due to the planar conformation of graphene sheets that allows for a better interaction between the probes and the analytes.[21] Their flat, two-dimensional shape also accounts for the lower detection limit that can be achieved, as in the case of thrombin detection. Reported studies suggest that graphene could detect orders of magnitude lower concentration thrombin analytes compared to what could be detected with carbon nanotubes. Graphene sheets have also been implemented in DNA quantification, single nucleotide polymorphism (SNP) detection and G-quadruplex ligand formation.[25] Such detection processes require laborious, complex, time-consuming and usually costly techniques, including among others gel electrophoresis and Southern blot analysis.[13] Engineered graphene platforms promise simpler, faster, cheaper, reproducible and more versatile alternatives to all above techniques. The properties of graphene that are utilized in these cases can be a combination of: a) strong, preferential binding of single-stranded DNA (ssDNA) (compared to double-stranded DNA); b) very sensitive FRET effect, as mentioned above; c) detection of minute changes in electrical properties that provide accurate signals with minimal noise.

The incorporation of graphene materials has also been proposed in the design of electrochemical sensors, with pristine graphene and reduced graphene oxide increasingly explored as electrode components. Their properties can be summarized in excellent electrocatalytic activity, broad electrochemical field for detection purposes, low-detection limit, high charge transfer ability and high stability[10] Equally important is the fact that the use of graphene-based electrosensors is usually label-free which means that the only properties that affect the readings are those of the electrode alone.[26]

3. Graphene for Drug Delivery and Imaging

Graphene materials have been proposed to offer high therapeutic molecule loading capacity due to their high available surface area and have been explored as potential drug delivery systems. **Table 2** summarizes most of the molecules that have been loaded onto graphene materials. As can be seen, the majority of drugs that have been complexed with graphene surfaces feature planar aromatic domains, such as doxorubicin (DOX). The main reason behind this is that stable π–π stacking between their aromatic rings and the graphene carbon surface leads to stable complex formation, avoiding chemical conjugation.[33] Alternatively, the overall negative charge of graphene oxide has also been utilized to establish electrostatic interactions with positively charged (highly hydrophilic) polymers

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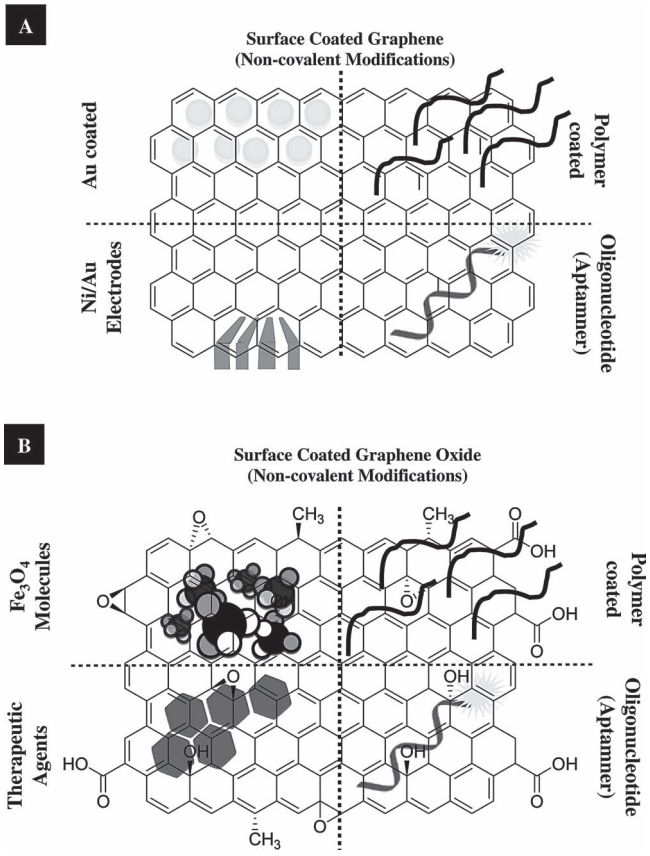

Figure 3. Surface coating of graphene and graphene oxide for use in biomedical applications. Different moieties could be used for the non-covalent coating of graphene (A) and graphene oxide (B). These range from polymers, single stranded oligonucleotides to gold (Au), ferric oxide (Fe_3O_4) and therapeutic agents (e.g. Doxorubicin).

such as polyethylenimine (PEI). PEI was also used to impart a positive charge to the graphene platform and to subsequently complex with negatively charged RNA or DNA molecules[34] for gene transfer purposes. The PEI-graphene-nucleic acid complex has shown to offer better protection from enzymes (e.g., nucleases). Alternatively, graphene oxide offers a variety of functional groups on its surface that also allow the possibility of versatile surface biconjugation. Polyethylene glycol (PEG) and chitosan molecules have been covalently linked to GO in an attempt to alter its blood circulation profile and impart better biocompatibility.[35] Finally, folic acid (FA) molecules have been covalently bound to graphene oxide along with anti-neoplastic drugs in an attempt to actively target the graphene platform to FA-receptor presenting cancer cells.[36]

GO has also been used as a multi-modal platform that not only serves drug delivery purposes but can also act as a tool for imaging purposes. One of the first examples on how this material could be used in imaging was made by Wang et al. in a study that displayed the ability of GO to provide real-time, in situ monitoring of living cells using apatmer–carboxyfluorescein GO nanocomplexes.[49] According to another study, Hong et al. reported a graphene oxide-based platform that was covalently conjugated to amine-terminated PEG molecules which were in turn ligated to Gallium 66 (^{66}Ga) labeled triacetic acid. An antibody with an affinity for tumor neo-vasculature was also conjugated onto GO (via the PEG chains) and positron emission tomography (PET) imaging was used. 3-D imaging of the tumor was acquired, although there was significant uptake of the radioactive construct by the liver and spleen.[50] Graphene materials were also explored for imaging purposes which involved the non-covalent conjugation of DOX and iron oxide particles on covalently PEGylated GO sheets.[51] More particularly, this work illustrated how GO could be used for theranostic applications, in which the graphene platform acted simultaneously as a carrier for DOX, while the iron oxide particles were used as T_2 contrast agent for magnetic resonance imaging (MRI). Most of the work reported today, however, is only at the proof-of-concept stage in terms of clinical development.

4. Graphene for Photodynamic Therapy

GO has also been investigated as a therapeutic modality due to its ability to absorb energy at the near-infrared (NIR) spectrum (700–1100 nm) and subsequently emit heat leading to thermal ablation of tissues. The mechanism of photothermal ablation of tissues involves the production of reactive oxygen species (ROS), caspase activation, the mitochondrial membrane depolarization and DNA fragmentation.[52] These factors combined are potent enough to kill cells that are within a specific range of the heat-emitting graphene platform. Carbon nanotubes offer similar photothermal capabilities, however graphene is considered an alternative material that may offer improved efficacy at lower concentrations.[53] Reported studies have shown that GO can absorb low power radiation, thus saving healthy tissues from unnecessary heating and without significant changes in its absorbance capacity over time.

In another example, polyethylene glycol was covalently linked to GO.[54] Conjugation was increased upon reduction and led to a significantly enhanced absorbance in the near-infrared spectrum. Upon irradiation, the reduced GO sheets could heat up their immediate environment and reach a temperature range that could lead to photothermal ablation of tissues. Furthermore, fluorescence spectroscopy suggested cellular uptake and cytotoxicity assays revealed an effective decrease (20%) in the viability of treated cells upon irradiation.

5. Graphene as Antimicrobials

There have been a few attempts to study the toxicity of graphene nanomaterials and their effect on bacteria, resulting in contradictory results regarding their possible antibacterial activity. Akhavan et al.[55] described how graphene "nanowalls" caused effective membrane damage in gram-positive bacteria, even though no membrane damage was observed using gram-negative bacteria. It was also reported that reduced graphene oxide 'nanowalls' were more toxic to bacteria compared to their non-reduced counterpart. In contrast, other studies proposed graphene "paper" showing no effects on bacteria,[56,57] while photo-inactivation of *Escherichia coli* bacteria was reported on the surface of graphene/titanium oxide (TiO2) composite

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Table 2. Therapeutic Molecules Loaded onto Graphene Materials.

Drug	Type of Interaction	Functionalization	Tumour Type	References
Doxorubicin	π–π stacking/ hydrophobic interactions	-	-	[33]
Doxorubicin	π–π stacking/ hydrophobic interactions	PEG (covalent)	B-cell lymphoma	[37]
Camptothecin analogue (SN38)	π–π stacking/ hydrophobic interactions	PEG (covalent)	HTC-116 colon cancer, MCF-7 breast cancer, U87MG glioma	[38]
Heparin	π–π stacking	-	Red blood cells	[39]
Doxorubicin & Camptothecin	π–π stacking/ hydrophobic interactions	FA (covalent)	MCF-7 breast cancer, A549 human lung carcinoma	[40]
		SO_3H (covalent)		
plasmid DNA	electrostatic interactions	PEI (electrostatic)	HeLa cervical carcinoma	[34]
Hairpin-shaped DNA (MB)	π–π stacking	-	HeLa cervical carcinoma	[41]
Doxorubicin	hydrophobic interactions	F127 (hydrophobic interactions)	MCF-7 breast cancer	[42]
Photosensitizer molecule (Chlorin e6)	π–π stacking	PEG (covalent)	KB nasopharyngeal carcinoma	[43]
Doxorubicin	π–π stacking/ Hydrophobic Interactions	PEG (covalent)	EMT6 murine tumor (*in vivo*)	[44]
Ibuprofen & 5-fluorouracil	π–π stacking	Chitosan (covalent)	CEM human lymphoblastic leukemia	[45]
Doxorubicin	π–π stacking/Hydrophobic Interactions	FA (covalent)	HeLa cervical carcinoma (*in vivo*)	[46]
siRNA	Electrostatic Interactions	PEG (covalent)	HeLa cervical carcinoma	[47]
		FA (covalent)		
		1-pyrenemethylamine (π–π stacking)		
Doxorubicin	π–π stacking	-	CNE1 nasopharyngeal carcinoma	[48]

films.[58] The effects of different types of graphene materials, namely graphite, graphite oxide, graphene oxide and reduced graphene oxide (rGO) on a bacterial model (*Escherichia coli*) have also been assessed.[59] The highest antibacterial activity was observed with GO followed by rGO, and was thought to reflect the severity of membrane damage and consequent oxidation stress caused. Along the same lines, Bao et al.[60] described the bacteriocidal effects of GO:silver nanoparticle films on gram positive and negative bacteria. In contrast to these studies, Ruiz et al.[61] showed that bacteria grew faster in the presence of GO and showed a better adherence and attachment to films containing GO compared to those without GO, suggesting that GO is neither bacteriocidal nor bacteriostatic. Similarly, Das et al.[62] showed that GO placed in a nutrient media plate previously inoculated with bacteria, did not inhibit bacterial growth in comparison to silver-containing GO which showed bacterial inhibition. Even though that was in agreement with Bao et al., it is important to note that silver is known for it is antimicrobial activity[63] and the effect seen cannot be directly attributed to GO.

6. Graphene for Tissue Engineering

Graphene-related materials have also shown promise in the area of tissue engineering. Kim et al.[64] synthesized a GO/$CaCO_3$ construct which showed good compatibility with osteoblast cells and increased bone bioactivity in vitro. In addition, an enhancement of hydroxyapatite formation was also observed in simulated body fluid. The engineering of graphene hydrogels as scaffolds for cellular growth has also been reported[65] using engineered graphene oxide nanosheets to build scaffolds for the proliferation of MG63 cells. Field emission scanning electron microscopy showed clear adherence of cells onto the hydrogel scaffold. Moreover, chitosan-graphene oxide scaffolds were synthesized by covalent linkage of chitosan on graphene oxide sheets.[66] These scaffolds showed enhanced cellular attachment and proliferation of MC3T3-E1 mouse pre-osteoblast cells while decreasing the degradation rate of chitosan, an important factor in the design of scaffolds for bone tissue engineering. Interestingly, the cells infiltrated the pores of the scaffold while increasing cell to cell interactions. The combination of graphene oxide with chitosan was also investigated by others,[67] and it is thought to enhance the wettability of chitosan due to the hydrophilic nature of graphene oxide. In an alternative design, chitosan-PVA nanofibers containing graphene were also investigated for their wound healing effects[68] to show that the chitosan-PVA membrane containing graphene resulted in complete skin wound healing after 10 days of application, that was not observed with control groups. This was thought to be due to the ability of graphene-containing membranes to affect the division and proliferation of cells.

Another area of interest closely related to tissue engineering is the effect of graphene materials on stem cell growth and differentiation. Lee et al.[69] reported that cellular proliferation of mesenchymal stem cells (MSCs) on graphene and graphene oxide substrates was higher than that on PDMS substrates, however it was found that the extent of mineralization of MSCs cultured on graphene was greater than that cultured on GO. Therefore, graphene was found to be more osteogenic and deposited

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more minerals compared to other substrates, due to the highest levels of dexamethasone deposition on its surface. Interestingly, the inverse was observed when MSCs were chemically induced to differentiate into adipocytes on graphene and GO, which was due to the higher affinity of insulin responsible for fatty acid synthesis, compared to its denaturation on graphene substrate. In addition, Nayak et al.[70] found that graphene-coated Si/SiO2 substrates provide a biocompatible scaffold for the proliferation of human mesenchymal stem cells (hMSCs) while accelerating their specific osteogenic differentiation, comparable to that in the presence of growth factors.

The surface properties of graphene and GO in maintaining induced pluripotent stem cells (iPSCs) have also been studied.[71] GO allowed faster adherence and proliferation of mouse iPSCs in comparison with the glass surface or a graphene substrate. Also, GO enhanced differentiation of iPSCs into the 3 different germ lines, while graphene suppressed their endodermal differentiation. Along similar lines, Park et al.[72] showed enhanced differentiation of human neural stem cells (hNSCs) on a graphene substrate, showing that graphene acted as a strong cell adhesion layer and induced the differentiation of hNSCs towards neurons rather than glial cells. Li et al.[73] also described the biocompatibility of primary cultures of mouse hippocampal neurons on graphene substrates and how the latter was capable of promoting neurite sprouting and outgrowth in the early developmental stage. This latter study proposed graphene-containing substrates as either implantable materials or neural chips to be investigated in neurodegenerative diseases where a loss of neurons and damage to neurites is observed.

In a related application, graphene-based materials have been incorporated in the design of electrodes used for neural applications. Heo et al.[74] developed graphene/polyethylene terephthalate (PET) electrodes using non-contact electric field stimulation to enhance human neuroblastoma cell-to-cell interactions. Since neuronal function is shaped to a large extent by synaptic and electrical coupling, the latter approach using graphene-containing electrodes may also offer opportunities for cell transplantation therapies in the central nervous system.

7. Toxicity and Biocompatibility of Graphene Materials

The increased use of graphene in a variety of applications and industries, will require stringent toxicological assessment in vitro and in vivo. Although a limited number ofstudies today have looked into the toxicity profile of graphene-related materials, we have attempted to offer an summary of what have we learnt regarding the cytotoxic profile of graphene.

Initially, the ability of mammalian cells to adhere into graphene substrates was studied in vitro using rat pheochromocytoma cells (PC12). Although cells could proliferate on the graphene substrates, 40% cell death was noticed using the MTT assay.[75] This cytotoxicity assay, although popular, has been proven problematic in studies using carbon nanotubes[76] and great caution should be exercised also in studies with graphene-related materials. The cytotoxicity of hydrophobic graphene has been assessed after dispersion in cell culture medium. Although very limited information regarding the quality of the dispersion was offered, Zhang et al. showed that above 10 μg/ml of graphene, the results suggested a gradual increase in cytotoxicity and a time- and concentration-dependent decrease in metabolic activity.[40] The effect of graphene on human red blood cells (RBC) has also been investigated[77] indicating that small, individual graphene oxide sheets showed higher haemolytic activity compared to aggregated sheets. However, improving the dispersion of GO using chitosan almost eliminated the haemolytic activity. In another study, Singh et al. studied the effect of GO on platelets in vitro. Even though no increased levels of LDH (lactate dehydroxylase; an enzyme used as an indicator of the existence and severity of acute or chronic tissue damage) were released from exposure to large (0.2–5 μm) 2–3 layered GO sheets, the production of reactive oxygen species was increased in a concentration-dependent manner.[78] The cytotoxicity of GO on human lung carcinoma (A549) cells was studied using GO sheets of different sizes (160, 430 and 780 nm). While, no cellular uptake of GO in A549 cells was observed, dose-dependent oxidative stress led to decreased viability at higher concentrations.[79] In addition, the effect of fetal bovine serum (FBS) and the formation of a protein corona on the cytotoxic effect of GO on A549 cells was investigated by Hu et al.[80] It was found that the presence of 10% FBS in cell media reduced the cytotoxicity of GO. This study suggested that the cytotoxic activity of GO was due to the direct physical damage caused by GO on the plasma membrane. **Table 3** provides an overview of the published reports investigating the effects from exposure of cell-cultures to graphene-related materials.

Even fewer toxicological studies using in vivo models have been performed today, which itself indicates the early stage in the development of graphene technologies and their widespread applications. Zhang et al. intravenously administered single-layered GO sheets of 10–800 nm in lateral size to Kunming mice[81] either at a dose of 1 mg/kg or 10 mg/kg. No pathological alterations were observed with the low dose after 14 days post-injection. However, the increased accumulation of the 10 mg/kg dose in the lungs and its slow clearance caused granulomatous lesions, pulmonary oedema, inflammation as well as fibrosis. Yang et al. looked into the pharmacokinetics of PEGylated graphene sheets in tumour-bearing mouse models.[82] They described a high uptake within tumour tissue with low accumulation in the reticuloendothelial system organs (liver and spleen). The same group subsequently studied the long-term biodistribution and toxicology of iodine-125 labelled PEGylated graphene sheets of about 10–30 nm,[83] reporting their accumulation in reticuloendothelial system (RES) organs, including liver and spleen, after intravenous administration that was reportedly cleared via both the renal and fecal excretion pathways. No changes in blood biochemistry, haematological analysis and histology of organs were observed even after 90 days post-injection. Moreover, looking into the biocompatibility of GO after intravenous administration in Kunming mice, Wang et al.[85] injected low, medium and high doses of GO, reporting that low and medium exposure of animals did not show any signs of toxicity. The highest dose exhibited chronic toxicity with severe side-effects triggered by a dose-dependent inflammatory response in the lung and the

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Table 3. Toxicity studies with graphene materials using in vitro models.

Material	Characterization	Properties	Cell lines	Concentration	Cytotoxicity assays	Conclusions	References
Graphene	TEM, SEM, AFM, ζ potential	3–5 nm thick	PC12	0.01–100 μg/mL	MTT, LDH, ROS, Caspase 3/7 assays	Time- and concentration- dependent decrease in metabolic activity.	[84]
		3–5 layers				Increase in cytotoxicity and in ROS levels.	
		−35.6 mV ζ-potential					
GO	XRD, AFM, DLS, XPS	0.94 nm inter-plane distance	RBC	3.125–200 μg/mL	Hemolysis, MTT, WST-8,Trypan blue, ROS	Time-dependent hemolytic activity.	[77]
	ζ-potential		Human fibroblasts			Extent of exfoliation and particle size affect toxicity.	
	Optical microscopy					Chitosan coating eliminates toxicity.	
GO	TEM, AFM, FTIR	0.9 nm thick	A549	10–200 μg/mL	CCK-8, Trypan blue, LDH assay, ROS, Cell adhesion & morphology	Dose- and size- dependent toxicity (CCK-8).	[79]
	Raman, XPS, Size distribution, ζ-potential	single layers				Small GO sheets induce oxidative stress.	
		90–780 nm lateral size				Morphology and adhesion properties unaltered.	
		33–37% oxygen content				No cellular uptake of GO.	
Graphene	SEM, AFM, XPS, ART-IR	Flat sheets	PC12	rGO substrates	MTT assay, Proliferation & differentiation studies	No significant toxicity on PC12 cells. Cellular proliferation on rGO.	[75]
		1.6 nm roughness (wrinkles)	Human oligodendroglia				
			Human fetal osteoblast cells				
GO	HR-TEM, FFT, FTIR	1.5 nm thick	Human platelets	2–20 μg/mL	Platelet activation	Platelet aggregation in a concentration dependent manner.	[78]
		2–3 layers			LDH assay, ROS	No significant LDH leakage, Concentration dependent increase in ROS.	
		0.5–5 μm lateral size			Flow cytometry		
GO	AFM	1 nm thick, 4–18 nm	A549	20–100 μg/mL	MTT assay	Dose-dependent toxicity, 50% loss of cell viability at 100 μg/mL after 24 h.	[80]

formation of granulomas and lesions. Moreover, Schinwald et al.[86] found that the graphene "*nanoplatelets*" with projected area dimensions of 25 μm and thickness of 0.1 μm induced an inflammatory response and granuloma formation in the lung and pleural space. It should be noted that the commercially available graphene '*nanoplatelet*' materials were not chemically or surface modified, therefore presumably hydrophobic and not very well dispersed. Some of us have recently reported that pure and highly dispersable GO with 1–2 nm in thickness and below 500 nm in lateral dimensions show no inflammation or granuloma formation at the mesothelial membrane after intraperitoneal injections.[87] The most important in vivo studies investigating the toxicological side effects of graphene using in vivo models are shown in **Table 4**.

Even with the scarce number of in vivo studies available, it is becoming clear that different graphene materials will have a different toxicological profile. Adverse reactions on exposure will depend on numerous factors that will need to be carefully monitored and systematically investigated before conclusions could be reached. Some of the critical design parameters to be studied include graphene sheet lateral size and surface modifications of the carbonaceous sheet surface.

8. Conclusion

Graphene materials have an undeniable potential for multiple applications in a wide range of industries including biomedical.[88] Although they are closely related to carbon nanotubes that have a longer history of development in various applications, few comparative studies have been conducted today (mainly for biosensing applications[89]), however much more work is needed and will certainly appear in the next few years. Similar to nanotubes, graphene materials can vary widely in terms of their physical and chemical characteristics such as dimensions and surface functional groups. The size distribution of graphene sheets can span from a few nanometers to the micrometer scale. Moreover, they can be linked to various

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Table 4. Toxicity studies with graphene materials using in vivo models.

Material	Characterization	Properties	Animal Model	Dose, route and duration	Conclusions	References
GO	FTIR, AFM, TEM	Flat 1 nm thick sheets	Kunming mice	0.1 mg (low), 0.25 mg (medium), 0.4 mg (high) doses. (1, 7 and 30 days)	No obvious toxicity signs with low and medium doses	[85]
			Female	*Intravenous injection*	Dose dependent lung inflammatory response (high dose)	
					Accumulation in lungs, spleen and liver.	
^{188}Re-GO	Raman, AFM	Single layered	Kunming mice	^{188}Re-GO: 20 μCi (up to 48 hours)	Accumulation in lungs, spleen and liver.	[81]
GO	ζ-potential	1 nm thick, 10–800 nm	Male	1–10 mg/kg (14 days)	Blood half-life of 4–6.5 h	
				Intravenous injection	Granulomatous lesions, pulmonary edema, inflammation and fibrosis.	
PEG-GO-Cy7	NIR, AFM, FTIR	Single & double layered, 10–50 nm thick	Balb/c mice	PEG-GO-Cy7 (12 and 48 hours)	Blood half-life of 1.5 h, Limited passive uptake by tumor, high kidneys accumulation	[82]
PEG-GO				PEG-GO (40 days and 3 months)	All biomarkers at normal levels, no noticeable toxicity.	
				Intravenous injection		
^{125}I-PEG-GO	FTIR, AFM	Single & double layered, PEGylated	Balb/c mice	^{125}I-PEG-GO: 20 μCi (1 hour to 30 days)	Two-compartmental pharmacokinetic model, accumulation in liver and spleen high renal and fecal clearance.	[83]
2. PEG-GO		30 nm lateral size		PEG-GO: 20 mg/kg (3–90 days)	No obvious toxicity of PEG-GO	
				Intravenous injection		
Graphene	SEM	1–10 layered, 5.64 ± 4.56 μm	C57BL6 mice	50 μg per mouse (24 hrs & 7 days)	Inflammatory reaction and granuloma formation with both types of injections.	[86]
				Pharyngeal aspiration		
				Intrapleural injection		
GO	TEM, AFM, FTIR, UV	Single layered (1 nm), <500 nm	C57BL6 mice	50 μg per mouse (24 hrs & 7 days)	Highly pure, readily dispersible and highly and stable GO smaller than 500nm don't cause inflammation or granuloma formation	[87]
				Intraperitoneal injection		

molecules by electrostatic, hydrophobic or covalent attachment. The type of starting material and the modifications undertaken during the oxidation reaction to produce GO, commonly result in materials with different levels of impurities and dispersibility. Therefore, it is imperative that some uniformity is agreed among the physicochemical properties of graphene derivatives after rigorous characterization to minimize heterogeneity in graphene samples. Correlation between physicochemical properties, graphene materials, and their biological function will instruct the opportunities and limitations offered for each biomedical application. Clarity in the nomenclature of the graphene materials developed in each laboratory is needed and the specific characterization protocols used to avoid generalizations about the capabilities and limitations of graphene materials that commonly lead to either false expectations or unnecessary safety concerns. As this Progress Report illustrates, it is still very early days for the development of all graphene-related materials in biomedicine that have the potential to offer very powerful new tools for the treatment and diagnosis of disease.

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