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This review article has summarized the latest progress on research regarding bioapplication of graphene oxide derivatives and expert opinion for overcoming the challenges.
Bioapplication of graphene oxide derivatives: drug/gene delivery, imaging, polymeric modification, toxicity, therapeutics and challenges

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Due to wide range and various applications of graphene in multidisciplinary fields such as electronic, solar cell, biomedical, bioengineering, drug delivery, gene delivery and semiconductor, graphene and its’ derivatives have attracted most significant interest of diverse group of scientists by the last decades. Besides numerous applications in electrical and mechanical fields, their noninvasive biomedical imaging property made them widely occupied for biological applications. Optical imaging probes plays a pivotal role in early cancer detection, image based surgery, disease diagnosis and cellular imaging. Graphene has been widely studied in drug delivery system due to their unique features and comparative less/non-toxic properties in biological system thereby launching graphene quantum dots as a potential organic optical imaging agent to substitute the toxic cadmium or tellurium quantum dots, etc. Many groups have also focused on different polymeric modification strategies to enhance the biocompatibility as well as application perspectives of graphene. In this review we have summarized recent advancements in graphene based application, and focused on relation between chemical structure and polymeric modification in relevance to the bio-safety issues. The lack of adequate biosafety studies as well as understanding the interaction between graphene derivatives and biomolecules has hindered their progress in biomedical and biological application. To proceed with biological application of graphene derivatives such development of graphene based therapeutics and drug delivery system, research community must need to understand how the graphene derivatives interact with cells lines and how they accumulate into the cells. We also have a need to learn the fate of graphene derivative in vivo once it invasive entered into biological system.

1. Introduction

Nowadays, carbon based materials especially graphene and graphene derivatives such as graphene oxide (GO), reduced graphene oxide (rGO) and graphene quantum dots (GQDs) have attracted considerable interests for various interdisciplinary sciences that spans a variety of disciplines including chemistry, physics, material sciences and nanotechnology.1 Moreover, graphene and its derivatives are expected to revolutionize the technological advances in electronics, ultrafast computing, solar energy harvesting etc. Recently, graphene has also been proposed for biomedical applications such as drug delivery, bio-medical imaging and anticancer therapy.2 However, the actual application of any nanomaterials in biology and medicine is decided critically by its biocompatibility. Although, many graphene derivatives have been widely considering in various electronic devices, very few of them have been considered for biological application. Though graphene based derivatives have been considered for various biological applications such as tissue engineering, bioengineering, drug delivery, gene delivery, optical imaging and therapeutics, to the best of our knowledge, none of the graphene derivatives have considered for clinical trials yet.3 Issues related to toxicity and bio-safety became pertinent as soon as graphene based derivatives were used for biological applications.1

Solely carbon consisting graphene materials known to be non-toxic though it’s a matter of serious concern to know how carbon derivatives like graphene decompose in biological system and how long it takes to excrete from the biological system.2 However, graphene or sources of graphene usually undergo several chemical treatment processes to be fabricated for functionalization, doping other metals, oxidation, introducing functional group and also reduction.4 It indicates that some of the graphene derivatives considered for bio-application contains other metals and/or impurities except carbon, for example graphene quantum dots contains around 10-40% of oxygen where as 60% carbon. The presence of excess oxygen is one of the principle reasons to enhance their solubility and impart optical properties. Moreover, different graphene derivatives have different chemical properties with different functionality and application thus they exert different toxicity.5
Though very limited studies have recently been conducted to draw the mechanism of toxicology that exert from graphene derivatives, especially by GO due to oxidative stress, no identical mechanism is established yet.6,7 In this review we have summarized application of graphene derivatives, polymeric modification and toxicological investigation based on recent reports. We have also pointed out the perspective and challenges of graphene derivatives for biological application and proposed ways to overcome these limitations.

1.2. Graphene Quantum Dot

GQDs is a nano form of GO which has smaller size, zigzag shape and quantum confinement properties thus show band gap mediated and size tunable optical properties. Synthesis of GQDs has gained preeminence due to their strong quantum confinements, size dependent and edge sensitive photoluminescence properties. Different synthesis routes have been employed to attain their size and photoluminescence properties.21 Syntheses of GQDs are broadly classified based on tuning the size of GQDs to atomic precision such as (i) top-down approach, and (ii) bottom-up approach.

Top-down approach is primarily based on defect arbitrated fragmentation where different carbon precursors are exfoliated and decomposed under strenuous experimental conditions (Concentrated acid treatment, strong oxidizing agents and elevated temperatures). However these methods often suffer from defined control over the size and properties of the material.22 On the other hand, bottom up approach exploits the use of polycyclic aromatic compounds to achieve an exquisite control over the size, shape and precisely regulate the physicochemical properties of the material. Bacon et al. have briefly summarized different routes to synthesize GQDs. Hydrothermal cutting is the simplest, efficient and prevalent method used to synthesize GQDs and often expanded for large scale batch production. Moreover GQDs synthesized by this method have a plethora of oxygenated groups that assists them in aqueous dispersion and surface modifications. Pan et al. have expounded the mechanism of GQDs synthesis from graphene sheets under hydrothermal treatment. They postulated that epoxy and carboxyl groups in the oxidized graphene sheets are friable and easily targeted under hydrothermal conditions for cutting. They have also observed pH dependent photo luminescent properties of GQDs. Luo et al. studied the effect of different oxidizing groups on photo luminescent properties of GQDs. Their results showed a 2 fold increase in the quantum yield compared to precursors. Feng et al. synthesized reduced graphene quantum dots (rGQD) by hydrazine reduced solvothermal method. They have shown that reduction of GQDs prevented non radiative electron-hole recombination and formation of pyrazole rings at the edges enriched their PL properties over pristine GQDs. Zhang et al. used electrochemical exfoliation method to synthesize GQDs for stem cell labelling. Production of O and OH radicals during the anodic oxidation channelled as a targeting site for electrochemical trimming of carbon nanocrystals. Ananthanarayanan et al. have also used this method to synthesize GQDs for Fe3+ ions detection. Moreover magnetic properties of Fe3+ ions resulted in GQD aggregates which could be used as contrast agents for MRI imaging. Luk et al. used microwave aided synthesis of PANI-GQDs for photonic devices. Functionalizing the GQD surface created emission traps and charge trapping sites at their surface states that could enhance the electrical and optical properties of the films. Other methods like nanolithography, ultrasonication and plasma assisted GQD synthesis have also been used.

Bottom-up fabrication strategies fostered the size controlled synthesis of GQDs with a molecular level precision with fine-tuned
physicochemical properties. However these methods are impeded due to their complex synthesis phases and a small scale production. Yan et al. synthesized step by step organic synthesis of water soluble precursors stabilized by 2', 4', 6'-trialkyl phenyl groups.\(^\text{32}\) Lu et al. synthesized ruthenium catalyzed GQDs from C60s. Ruthenium not only functioned as a catalyst but also acted as a template for the ring opened C-60 clusters. Thermally activated diffusion led to the formation of GQDs from coalesced clusters on Ruthenium with shear precision.\(^\text{32}\)

### 2. Bioapplication of Graphene Derivatives

#### 2.1. Current Limitation in Biomedical Diagnosis and Prospects of Graphene Derivatives

Early diagnosis techniques play a vital role for treating disease with minimal cost and improving treatment outcomes.\(^\text{1}\) A feasible, cost effective and reliable early detection and diagnosis technology could enhance and extend patient’s lifetime.\(^\text{35}\) However, existing in vivo diagnostic technologies such as MRI, computed tomography (CT) scan etc. are expensive and inaccessible to majority of patients. Also existing in vitro diagnostic technologies such as biosensors are not yet ready to be used in the clinic. The development of cost effective and ideal contrast agents could overcome these barriers and would accelerate the development of molecular imaging systems to be used in biomedical diagnosis.\(^\text{36}\)

Recent advances in nanomaterial based strategies for therapy and diagnosis have been very promising for the early stage diagnosis and treatment of many diseases and infections.\(^\text{37}\) Nanoparticle based therapies are able to overcome many of the existing barriers for cancer therapy as it enables the early detection and targeting of specific cells whilst minimising toxicity and being cost effective.\(^\text{38}\) Many studies have revealed that early cancer detection either in vitro or in vivo could minimise treatment costs by 50% and the risk of death could be reduced by 60%.\(^\text{39}\) In vitro analysis techniques are not yet an optimised or reliable way to detect biomarkers from the blood stream. Therefore, noninvasive imaging technology such as optical imaging, magnetic resonance imaging (MRI), positron emission tomography (PET) and X-ray computed tomography (CT scan) play vital roles in the early stage diagnosis from deep tissue and organs.\(^\text{40}\) However, the cost of current analytical tools is highly expensive due to the cost of contrast agents and imaging equipment. Previously, our group as well as other international groups have reported that semiconductor QDs are more appropriate for optical imaging compared to organic dyes (Rhodamine, Cyanine etc) due to their unique properties (ultra nano-

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Table 1. Reported synthesis process of graphene quantum dots.
size, photo quenching stability, sharp emission and size variable excitation spectrum.\textsuperscript{41, 42} However, the toxicity issue of heavy metals such as cadmium (Cd), tellurium (Te) and selenium (Se) is a major concern for their biological application. Due to serious toxicity matters, QDot has not been approved for clinical investigation, despite being studied for over a decade.\textsuperscript{54} Recently upconversion nanomaterials emerged as a new alternative to address the issues related with the impaired tissue penetration depths of the light sources. Their unique property of emitting high energy photons upon low energy NIR excitations and easy surface modifications amplified their \textit{in vivo} \textit{in vitro} imaging and PDT applications \textsuperscript{45, 46}. However, some studies report their low quantum yields and inaccurate surface modifications which resulted in uptake by reticuloendothelial system and rapid clearance from the body. Moreover their biological fate, bio distribution and toxicity evaluations are required to ensure their treatment in clinical applications \textsuperscript{47}.

Current noninvasive imaging technology and process is an expensive way for detecting diseases due to the cost of contrast agents and imaging equipment which limits its widespread application.\textsuperscript{48} As it is easy to fabricate the functional derivatives of Graphene based materials, they are known to be cost effective, nontoxic and stable imaging contrast agents used for \textit{in vitro} and \textit{in vivo} molecular imaging and biomedical diagnosis. The development of a cost effective, bio compatible, target specific, nontoxic and water dispersable graphene based nanoparticle could solve many current problems in biomedical diagnosis and molecular imaging over using of toxic quantum dots and less table organic dyes.\textsuperscript{49} The newly developed multifunctional and biocompatible graphene nanoparticle can be widely used as optical imaging contrast agents as well as photo therapy for treating cancer. The photoluminescent GQDs could also be used to develop photoluminesce based biosensor for biomarker detection through surface Plasmon resonance strategies.\textsuperscript{49} Moreover fabrication of photo-tunable different color graphene nanoparticles from carbon fiber is comparatively easier. Some research groups have also focused on \textit{in vitro} and \textit{in vivo} imaging feasibilities of GQDs in different cell lines and small animals respectively, as well as observed primary toxicity behavior of GQDs.\textsuperscript{50, 51} The observation showed that the uncoated cationic GQDs aggregated in aqueous medium thus showing significant toxicity \textit{in vitro} and \textit{in vivo}. Therefore, to prevent the aggregation coating and surface modification with polymers have been done. One of the strategies is coating of GQDs with polydopamine that greatly prevent aggregation and be used for delivery drug and gene through catechol medicated linkage.\textsuperscript{52} Photosensitivity is another mentionable limitation of GQDs that generate single oxygen upon irradiation with visible or UV light thus cells and tissues surrounding the GQDs become affected by the toxic singlet oxygen. However, this photosensitivity or photodynamic properties of GQDs are turned to positive approach as therapeutic for treating diseases and wound healing.

Chemical modifications of GQDs with biocompatible polymers enhanced the solubility of GQDs and made them feasible for both photo-cancer therapy and real time imaging for detecting the cancer cells/tumor. We have modified the surface of GQDs with polydopamine and Hyaluronic acid to impart the hydrophilicity and explore the feasibility of GQDs as multifunctional nanomaterials for cancer therapy and gene therapy.\textsuperscript{52}

### 2.2. Graphene for Drug Delivery

Since their discovery as a bio-safe material, graphene has been perceived as a carrier molecule in drug delivery research.\textsuperscript{1, 2} The large specific surface area of graphene enhances multi drug delivery opportunity to the target site from site of administration. Polymeric modification and conjugation strategies also enhance biocompatibility and circulation times \textit{in vivo}.\textsuperscript{53} Several studies have been conducted on delivery of anticancer drugs, genes and peptide through graphene derivatives by last couple of years.\textsuperscript{54-60} Simple physisorption via \(\pi\)-stacking can be used for loading many hydrophobic drugs such as doxorubicin, docetaxel with antibody for selective killing of cancer cells. Owing to its small size, intrinsic optical properties, large specific surface area, low cost and useful non-covalent interactions with aromatic drug molecules, graphene is a promising new material for drug delivery through nano-carrier approach. Large specific surface area, \(\pi\)-\(\pi\) stacking and electrostatic or hydrophobic interactions of graphene can assist in high drug loading of poorly soluble drugs without compromising potency or efficiency.

Joo and his group reported that PEGylated GO loaded Doxorubicin via \(\pi\)-\(\pi\) interactions shows promising real-time release of DOX from PEGylated GO at the specific loci after an external triggering by GSH.\textsuperscript{55} Another research group reported that GO loaded with Doxorubicin exhibits higher drug release at pH 5.3, due to the reduced interaction between DOX and drug carrier.\textsuperscript{56} GO loaded with DOX shows enhanced cellular toxicity and promising tumor growth inhibition, almost 66-91\% cell death.\textsuperscript{57-59} Another Chemotherapy drugs Paclitaxel and Methotrexate loaded on graphene oxide via \(\pi\)-\(\pi\) stacking and amide bonds had amazing cancerous effect on lung cancer and breast cancer resulting about 66 to 90\% tumour growth inhibition.\textsuperscript{60, 61} Buprofen which is used as NSAIDs drugs conjugated with chitosan functionalized GO via amide linkages, suggest that GO exhibit higher (20\%) biocompatibility than GO sheets for CEM & MCF-7 cell lines.\textsuperscript{62} GO loaded with second generation photosensitizer chlorin e6 (C6e) resulted in its higher accumulation in tumor cells leading to higher photodynamic efficacy upon irradiation.\textsuperscript{63, 64} Nano GO is another important materials for drug delivery area. Nanographene oxide (NGO) is opted as a novel and efficient nanocarrier for delivery of water insoluble aromatic anticancer drugs into cells. In their approach, Nano GO was first conjugated PEG–Nano GO. The non-covalent \(\pi\)-\(\pi\) stacking was used to load doxorubicin (DOX) and camptothecin (CPT) analog, SN38 onto PEG–NGO conjugate and \textit{in vitro} pH-dependent drug release studies were reported. These complexes also showed high cytotoxicity in HCT-116 cells and was 1000 times more potent than CPT.\textsuperscript{65, 66} Kim et al. reported that near infrared (NIR), acidic pH and high intracellular concentration of GSH favored intracellular cytosolic delivery of DOX. Cells treated with PEG and branched polyethyleneimine (BPEI)-functionalized GFO (PEG–BPEI–GFO) nano-carriers unveiled to near-IR irradiation encouraged endosomal disruption and consequent DOX release which triggered the cellular toxicity.\textsuperscript{57} Graphene derivatives have been conjugated with a biopolymer like gelatin and polyethylene glycol as functionalizing agents for drug delivery applications. Gelatin and polyethylene glycol not only favoured the reduction of graphene but also functionalized GNS assisted in loading DOX onto GNS. The GNS–DOX complex also exhibited high toxicity towards U251, 1800 and A-SRT3 cells through endocytosis.\textsuperscript{68, 69} Poly NIPAM and other polymers have been used with graphene for loading various drugs like camptothecin,\textsuperscript{70} Methotexate,\textsuperscript{71} and 5-fluorouracil.\textsuperscript{72} In recent past researchers have begun to synthesize smaller graphene derivatives, often referred as graphene quantum dots (GQDs). These GQDs exhibit intrinsic fluorescence, and also used for theranostic purposes. Synthesized GQDs with different color emission loaded with anticancer drugs doxorubicin were reported for image guided higher therapeutic efficacy about 55 to 90 cell growth inhibition.\textsuperscript{73-78}

<table>
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<th>Graphene derivatives</th>
<th>Drug</th>
<th>Application</th>
<th>Ref</th>
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Table 2. The table shows application of different graphene derivatives for drug delivery.
2.3. Graphene for Tissue Engineering

Functional carbon-based nanomaterials have become important due to their unique combination of chemical and physical properties. In tissue engineering research, selection of a scaffold plays a vital role for designing and developing a hydrogel with optimized properties such as conductivity, mechanical properties and elasticity. Selection of biocompatible and biomimetic scaffold also plays vital role for minimizing toxicity that happens through auto-immune system. Zhang et al. incorporated GO into poly (vinyl alcohol) hydrogels to improve mechanical strength of the hydrogel. More recently, researchers have turned their attention to utilize the multifunctional nature of carbon derivatives in engineering tissue scaffolds. Most notably, carbon materials have been incorporated to fabricate electrically conductive scaffolds. Most of the biomaterials used for tissue engineering applications are electrically insulating, as they are made from nonconductive polymers. Another study demonstrated the process of self-assembled graphene hydrogel via a convenient one-step hydrothermal method. The self-assembled graphene hydrogel with inherent biocompatibility of carbon materials is attractive in the fields of biotechnology and electrochemistry such as tissue scaffolds and bionic nanocomposites. Graphene derivatives are found to be a promising agent to be considered as a composite material in tissue engineering areas due to non-significant toxicity, natural source, and excellent thermal and electrical conductivity.

2.4. Graphene as Photomedicine

Non-uniform coverage of oxygen functional groups in GO sheet results in ordered small sp$^2$ clusters which are isolated within sp$^3$ C-O matrix. The presence of finite molecular sp$^2$ clusters within a sp$^3$ matrix can lead to confinement of π-electrons in GO. Radiative combination of electron-hole pairs in such sp$^2$ clusters can give rise to fluorescence. The size of the local sp$^2$ cluster determines the local energy gap and therefore the wavelength of the emitted fluorescence. Emission from UV-visible region can occur from sp$^2$ clusters with size less than 1 nm. On the other hand, sp$^3$ domains larger than 2 nm possess smaller gaps and may account for red to near-IR emission. The strong optical absorbance of GO in the near-IR region has been applied to in vivo photothermal therapy. Many research groups reported on the application of graphene and its derivatives for cancer therapy. For example, polyethylene glycol functionalized GO, enhanced the therapeutic efficacy and showed high cellular uptake. A promising therapeutic outcomes were observed from PEG-GO conjugates though many studies attributed that the reduced GO has better photoablation properties over the non-reduce graphene derivatives. To get a synergistic and enhanced therapeutic efficacy for cancer treatment a combinational therapeutic multifunctional nanoparticle was designed. The PEG-GO nanoparticle conjugated with doxorubicine shows a combination of photothermal and chemical therapy for cancer treatment. Recent results show that GQDs have the highest singlet oxygen generation capacity over other conventional photosensitzers (PS) due to their multistate sensitization. In comparison to the conventional PS, GQDs exhibited enhanced 1$^2$O$_2$ quantum yields. This enhancement was envisaged based on the generation of singlet oxygen species during their transition from excited state to the triplet state in the visible region (below 636 nm) which is not seen in conventional PS.

2.5. Theranostics: A Combination of Diagnostics and Therapy

Since graphene and GO, especially graphene quantum dot has wide range of excitation and emission properties as investigated previously, many research groups mainly aimed to introduce a novel and new graphene based optical imaging agent that can be considered as a unique contrast agent for deep tissue and cell imaging, and cancer therapy as well. Previous studies on photoluminescent graphene demonstrated several methods to produce different tunable colours from nanosize GO. Acid exfoliation, tunable laser irradiation, electronic beam irradiation, autoclaving and many other methods have been reported to fabricate photoluminescent graphene in the last couple of years. However, the combinatorial application of graphene for therapeutic and imaging has yet to be reported. To acquire an efficient theranostic effect, the material should facilitate multimodal imaging modalities with suitable surface area that flaunts them as a...
plinth to perch the drugs and afford sufficient surface modifications to assist in site specific targeting. Though different theranostic systems have been designed, they often lack some of the above mentioned qualities. Since cancer cells have a tendency to develop an intrinsic multiple drug resistance (MDR) profile, there is a need to design multimodal therapeutic systems with effective targeting. To overcome these impairments, scientists came up with synthesizing composite systems that satisfies all the above mentioned complications. As GO has unique properties and easy for functionalization, these materials were chosen as an optimal material to design theranostic nanocomposites. Several graphene based nanocomposite formulations have been synthesized and been envisaged as an ideal theranostic and multifunctional nanomedicine. Recently peptide and magnetic GO functionalized mesoporous silica nanomaterials have been synthesized to selectively target glioma cells. GO enhanced the drug loading capacities of the system and featured a pH responsive drug release assisted photothermal treatment. Moreover these materials gained dual receptor mediated and magnetic guided drug transport with MRI imaging. Another interesting property of GO nanosheets based theranostic materials is their wavelength dependent photoluminescence. This exhilarating feature could be either due to the wavelength dependent fluorescence from OH groups in the GO or the solvent relaxation times due to the excited GO that could be compared to the fluorescent times. Tagging a photosensitizer with an aptamer to the magnetic GO nanosheets enables multi-luminescent label free cell imaging with photothermal and photodynamic MRI image guided therapy. Graphene based nanomaterials have also been used as a surface enhanced Raman scattering material to selectively deliver and monitor the drug release from the carrier system. Since the pipazine ring has a stronger affinity for the [100] planes of gold, Graphene can be

Fig. 2. (a) Schematic illustration of the $^{1}\text{O}_2$ generation mechanisms by conventional PDT agents (left) and GQDs (right). (b) Fluorescence intensity of GQDs at 680 nm versus the $\text{O}_2$ concentration in solution. (c) The dependence of the $^{1}\text{O}_2$ quantum yield ($Q_\Delta$) on the fluorescence intensity ratio at 680 nm ($F/F_0$). Adapted with permission from ref 80. Copyright © 2013 Nature publishing group. Simultaneous photothermal, photodynamic and optical imaging properties of GQDs (d), adapted with permission from ref 38. Copyright © 2014 American Chemical Society.

Fig. 3. (a) Schematic representation showing EpCAM antibody and A9-aptamer-attached theranostic GO for the separation and capturing of CTC from infected blood. (b) Schematic representation showing label-free multicolor luminescence imaging of CTC using EpCAM antibody and A9-aptamer-attached theranostic GO. (c) Schematic representation showing that the theranostic GO can be used for combined synergistic treatment. Adapted with permission from 84. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
preferred as an optimal material to seed and grow the noble metals on their surface thereby creating an interface with the non-thiolated molecules and generate adequate SERS signals. Since the drug affinity to the GO can be reduced in acidic pH, these SERS signals can easily assist in monitoring the drug release from the carrier. Moreover presence of GO can assist in photothermal therapy to treat skin cancer.

2.6. Graphene for Gene Delivery

Graphene mediated gene delivery is another emerging area of research which has been considered by the research groups who are focusing non-viral gene delivery. Majority of the scientific works reported that the graphene derivative could be used as a promising carrier with high gene packing density due to large surface area. Some reports also demonstrated that the graphene derivatives could overcome many barriers and increase gene accumulation through targeting the specific cells resulting in increased gene transfection. However, graphene derivatives are required to be modified by polymers to make the surface cationic and interact with the anionic genes. Mostly polyethyleneimine (PEI), PEG and poly(sodium 4-styrenesulfonates) (PSS) were considered for gene delivery through graphene derivatives. Zhi et al. has reported that layer-by-layer assembled GO carried miR-21 targeted siRNA and adrimycin simultaneously that overcome multidrug resistance. The polymer associated with GO significantly enhanced cellular uptake in MCF-7 cells. Another inciting study was reported by Khademhosseini group where GO based injectable hydrogel were used to host angiogenic genes and demonstrated them as a potential cardiac implant for vasculogenesis. This invention can be widely used in tissue engineering research for generating blood vessel to circulate blood and nutrients for the cells located inside the hydrogel. The thermogenesis properties of reduced rGO have been properly implicated by Kim et al. where they showed that the light sensitive rGO can be used to escape or overcome the barriers of current gene therapeutic strategies. Translocation of a gene from cellular membrane to nucleus is a hurdle due to endosomal barrier between the routes. Though many strategies have been taken into consideration to overcome this barrier, progress is far away from the expectation. This report has attributed that heat generated through irradiation of the rGO helps to overcome the endosomal escape thus enhancing gene expression.

3. Polymeric Modification of Graphene Derivatives

Polymer nanocomposites based on graphene and graphene derivatives have found their eminence in various biomedical applications viz. tissue engineering, drug delivery and biosensors. GO based nanomaterials have recently gained much attention in 2D carbon family for multifarious applications. The surface and the edges of GO possess hydroxyl and carboxyl groups that expedite easy functionalization and impart a dynamic change in the physicochemical properties of the composite materials. Moreover higher surface area and structural defects can foster interactions with the polymeric materials. Recent studies shows that 2D graphene based carbon nanomaterials reinforced polymer composites displayed enhanced mechanical properties over 1D carbon nanomaterials. Having a higher surface area with low aspect ratio and higher crosslinking densities with the polymer, 2D graphene based materials could be uniformly dispersed in the polymer matrix and promote efficient load transfer from polymer matrix to the nanomaterials. As Graphene based materials stood to be an excellent reinforcement for polymer materials by improving the mechanical properties and enhancing their load bearing abilities, they have been directed for tissue engineering applications. Li et al. synthesized flexible and fluorescent crosslinked chitosan scaffold reinforced with GO for Tissue engineering applications. Their results demonstrate that swelling and degradation of the scaffold was entirely contingent on percentage of the GO loaded. Yang et al. used GO to fabricate 3D porous scaffold and showed that addition of GO resulted in uniform pore structures with higher por density. Apart from enhancing the mechanical properties of the scaffold, higher surface area of GO provided higher intermolecular interactions with the cell culture media and enriched the growth, differentiation and proliferation of the cells on the scaffold.

Fig. 4. Schematic of the polyethylenimine (PEI)/poly (sodium 4-styrenesulfonates) (PSS)/graphene oxide fabrication and multidrug resistant reversion (a) adopted with permission from ref 91. The scheme represents preparation of injectable hydrogel incorporating with PDNA and GO for acute myocardial infarction therapy (b). Adapted with permission from ref 92, Copyright © 2013 American Chemical Society. Synthesis scheme of PEG-BPEI-GO nanocomposite. BPEI-rGO was synthesized from BPEI-GO. To enhance colloidal stability of BPEI-GO and BPEI-rGO, polyeleyn glycol monomethyl ether (mPEG) was conjugated by 1,1’-carbonyldimidazole coupling (c). Adapted with permission from ref 93, Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Recently GO loaded Poly L-Lysine (PLL) thin films served as an adhesive layers to stack multiple layers of cardiac cells. Surface charges play an important role in growth and differentiation of the cells adsorbed on the surface of the scaffold. Tu et al. affected the effect of surface charge on growth and branching of neuronal cells. Their results demonstrate that selective coating on the GO surfaces varied the neurite length and branching of the neuronal cells. An et al. designed a Poly Lactic acid/Poly Urethane polymer matrix loaded with GO for antimicrobial applications. Their results showed 100%
reduction in bacterial cell growth with just 5wt. % of GO in the matrix but no effect on normal human cell lines.\textsuperscript{100}

Owing to their exceptional optical properties at near-IR region, higher surface area and high drug loading capabilities, graphene based nanomaterials were opted as an excellent nanocarrier system for drug delivery applications. Most of the efficient drugs for cancer therapy suffer from reduced therapeutic efficacy due to their hydrophobicity and easy elimination from the host before reaching the targeted site. Graphene based nanomaterials emerged as an excellent solution to this problem. Most of the hydrophobic drugs like doxorubicin (Dox), Paclitaxel, Dexamethasone were attached onto the surface of the graphene surface by either hydrophobic interactions or π-π stacking with the graphene surface. Zhou et al. synthesized an efficient pH responsive drug delivery system where the drug, Dox was released from the carrier system based on charge reversal poly electrolyte under acidic conditions.\textsuperscript{101} Chowdhury et al. synthesized ligand free targeting of graphene nanoribbons loaded Dox for cancer therapy.\textsuperscript{102} Their results suggest that Gnrbs were preferentially taken up by the cells that express epidermal growth factor receptors and regulated by papillomavirus – E5 protein.\textsuperscript{103} Having exceptional electrical conductivity, Graphene based nanomaterials could tune the drug release. Weaver et al. synthesized polypyrrole coated GO nanosheets to channelize the drug release, dexamethasone from the carrier. Their results showed 100 % ON/OFF voltage gated drug release with no passive diffusion of the drug from the polymer matrix.\textsuperscript{103} Though graphene based composites were opted as a suitable carrier, their π-π interactions with the neighboring molecules stacks one over other and often suffer from aggregation and instability. Swain et al. explored the effect of polymer coating on surface of GO and stability. Upon surface functionalization with polyvinylpyrrolidone (PVP) crosslinked poly (vinyl alcohol) (PVA), GO displayed a stability for over 27 months.\textsuperscript{104} Swain and Chen et al. compared the efficiency of rGO systems over GO for drug delivery applications. Their results show a two fold increase in the drug loading in rGO compared to GO due to the conservation of aromatic structure that improved the surface area for the drug molecules to be loaded on the surface by π-π interactions.\textsuperscript{105} Another interesting study was based on using rGO-chitosan nanocomposite as microneedle based transdermal drug delivery applications. Justin et al. demonstrated that effect of drug loading and release was based on the amount of rGO loaded in the composite. Moreover their results demonstrate that the synthesized material were susceptible to withhold the tissue insertion and can deliver drugs to epidermis.\textsuperscript{106}

Current advancements in technology and science aided graphene based materials for photothermal therapy at near-IR region. Graphene similar to gold nanoparticles and CNTs absorb the near-IR radiation and efficaciously convert them to thermal vibrations by which they can thermally ablate the targeted tissue. Since human body lacks chromophores that can absorb at near-IR region, near-IR radiation can access tumor site with deeper penetration (low scattering) compared to other high energy radiations. Markovic et al. compared the photo thermal efficiency between GO sheets and carbon nanotubes.\textsuperscript{107} One fold increase in the photo thermal properties of graphene sheets was due to their ultra-small size and uniform dispersion over CNT which tend to aggregate when loaded in the polymer matrix. Sirivivyanun et al. explored the use of GO as a Photosensitizer for photodynamic therapy. Their results proved a two photon fluorescence imaging of the cancer cells with a photocytotoxicity at a wavelength of 780nm.\textsuperscript{108} Li et al. loaded iron oxide nanoparticles in poly lactic acid matrix, surface coated with GO and used as hyperthermic and image contrast agent for ultrasound, Photo acoustic and MRI imaging.\textsuperscript{109} Nguyen et al. also explored the use of near-IR absorption properties of GO as a two photon and photo acoustic imaging agents.\textsuperscript{110}

Recently, much focus is shed over the use of graphene and graphene based materials for biosensor applications. With high electron transport mobility, unprecedented mechanical strength, excellent thermal and electrical conductivity made this an appropriate material for biosensors. Ease of surface functionalization renders high sensitivity, selectivity and stimuli responsive characteristics to the biosensors. Graphene based electrodes increased the surface area that headed towards increased detection limit with a dynamic linear range. Recently, Ouyang et al. fabricated G-PEDOT biosensor for simultaneous detection of both purines and pyrimidines. G-PEDOT complex enhanced the surface area that led to amplified electrocatalytic oxidation of DNA bases.\textsuperscript{111} Another interesting study was based on using G to lower the pKa value of the substrate. Zhou et al. fabricated Photoluminescent glucose biosensor that could preferentially shrink and expand to glucose. Graphene enhanced the pKa value of the substrate and charged the PBA that led to the formation of bisborate complex with glucose and fold inward.\textsuperscript{112} Zhu et al. investigated the enhancement in photoluminescence property of GO under acidic pH to monitor the growth and proliferation of cancer cells. Positively charged GO-PEG enabled π-π HUMO-LUMO electronic transition at lower pH compared to π-π* assisted electronic transitions at neutral pH which augmented the fluorescence intensity.\textsuperscript{113} Tian et al. explored the use of GO-PEDOT composite as an excellent material for tissue electrode interface. Doping of GO affords low impedance, high charge storage capacity, high charge injection limit to perform electrical simulation and biocompatibility.\textsuperscript{114} Another interesting application of GO was to use them as a bioenergy storage device. Byun et al. synthesized graphene-polyprrole hybrid nanostructured bio energy storage device to gate the release of ATP and control the activity and mobility of actomyosin.\textsuperscript{115}

Over the past years great focus is shed over edifying the biological applications of graphene based materials. Owing to their exceptional mechanical, optical, electrical and thermal properties, graphene based materials were chosen to improve the characteristics of the targeting material. Though Graphene based materials are biocompatible and nontoxic they often suffer from long term stability. Polymer coatings over the graphene based materials surmounted this issue by enhancing their stability and improving their biocompatibility. Apart from alleviating the stability of graphene based materials, apt selection of polymer materials over the graphene surface could facilitate for diverse biomedical applications.

4. Toxicology and Biosafety of Graphene Derivative

4.1. Recent Studies on Potential Toxicity of Graphene Derivatives

It is very important to investigate the physicochemical interaction of the nanoparticles with the \textit{in vitro} and \textit{in vivo} organelles before applying or considering for biological application. Since graphene was primarily been considered as an electronic material, latter on many studies have established graphene for bioapplication such as tissue engineering, drug delivery, stem cell research etc., but it requires extensive observation on both \textit{in vitro} and \textit{in vivo} interaction with cell and biomolecules. Graphene is composed of only carbon atoms, however, GO and graphene quantum dot contains oxygen due to oxidation. Though very few \textit{in vitro} and \textit{in vivo} toxicity studies have been reported previously, not much focused on biochemistry and histological impact.\textsuperscript{138,133} Therefore, our group has conducted an extensive toxicity evaluation experiment to conduct deep investigation based on
Table 3. Polymeric modification of graphene and graphene derivatives and their application.

<table>
<thead>
<tr>
<th>Graphene/Graphene Derivatives</th>
<th>Polymer</th>
<th>Application</th>
<th>Key results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO</td>
<td>Poly L-Lysine (PLL)</td>
<td>3D Tissue Engineering</td>
<td>GO-PLL thin films were used as an adhesion layer between stacked multilayered cardiac cells. Low external electric field equipped the stacked tissue with frequency dependent actuation (open/close) and strong spontaneous tissue beating.</td>
<td>98</td>
</tr>
<tr>
<td>Amine functionalized graphene</td>
<td>poly pyrrole</td>
<td>Bio energy storage devices</td>
<td>Graphene facilitated control and gated release of ATP by electrical stimuli. Graphene functionalization led to an increase in adhesion and mobility of actomyosin with no loss of the actin function upon repeated electrical stimuli.</td>
<td>115</td>
</tr>
<tr>
<td>Graphene</td>
<td>poly(4-phenylenediamine) (PoPD)</td>
<td>Biosensor</td>
<td>Adenosine triphosphate (ATP) Biosensor with a detection limit of 0.3nM and dynamic range of 10 nM–2 nM. Presence of analyte disturbed the interactions between the aptamer and the graphene surface and decreased the current response.</td>
<td>116</td>
</tr>
<tr>
<td>Graphene</td>
<td>PEDOT</td>
<td>Biosensor</td>
<td>Biosensors that can simultaneously detect both Purines and pyrimidines. Surface area and the conductivity of the film were enhanced after the reduction of GO / Graphene improved the electrochemical properties, flexibility and Specific capacitance of the composite material. Compared to GO-PANI, G-PANI exhibited improved biocompatibility and enhanced cell survival rate.</td>
<td>117</td>
</tr>
<tr>
<td>Graphene</td>
<td>poly(4-vinylphenylboronic acid) (PBA) /N,N'-methylenebis(acrylamide)</td>
<td>Biosensor</td>
<td>Selective and sensitive photoluminescent glucose biosensor that could preferentially fold/unfold to Glucose. Graphene lowered the pKa value of the PBA that charged PBA groups on the electrode surface and can preferentially form bisborate complexes with glucose and resulted in shrinking of the micro gels.</td>
<td>112</td>
</tr>
<tr>
<td>GO/Graphene</td>
<td>Polyamine (PANI)</td>
<td>Biosensors</td>
<td>Addition of GO / Graphene improved the electrochemical properties, flexibility and Specific capacitance of the composite material. Compared to GO-PANI, G-PANI exhibited improved biocompatibility and enhanced cell survival rate.</td>
<td>118</td>
</tr>
<tr>
<td>GO</td>
<td>Poly Ethylene oxide (PEG)</td>
<td>Biosensors</td>
<td>Fluorescent and positively charged GO-PEG was synthesized. π-π* facilitated HUMO-LUMO electronic transition enhanced the fluorescence intensity at lower pH compared to n-π* assisted electronic transitions at neutral pH. Monitored the growth and metabolism of the cancer cells based on the subtle changes in the local pH.</td>
<td>113</td>
</tr>
<tr>
<td>Graphene</td>
<td>PEDOT</td>
<td>Biosensors</td>
<td>Graphene and PEDOT hastened the electron transfer between the H2O2 and Hemin. Efficient, stable and selective Hydrogen peroxide biosensor with a detection limit of 0.08 nM ranging from 10 7 to 10 5 M. Graphene Biosensors were synthesized with a detection limit of 64 µM. Loading Nitrogen doped graphene into the matrix increased the capacitive current and decreased the charge transfer resistance of the electrode Electrochemiluminescence alcohol dehydrogenase biosensor was fabricated with a detection limit of 2.5µM.</td>
<td>118</td>
</tr>
<tr>
<td>nitrogen-doped graphene</td>
<td>chitosan-poly(styrene sulfonate)</td>
<td>Biosensors</td>
<td>Glucose Biosensors were synthesized with a detection limit of 64 µM. Loading Nitrogen doped graphene into the matrix increased the capacitive current and decreased the charge transfer resistance of the electrode Electrochemiluminescence alcohol dehydrogenase biosensor was fabricated with a detection limit of 2.5µM.</td>
<td>119</td>
</tr>
<tr>
<td>Graphene</td>
<td>poly-(3,4-ethylene dioxythiophene) and polystyrene sulfonate</td>
<td>Biosensors</td>
<td>Electrochemiluminescence alcohol dehydrogenase biosensor was fabricated with a detection limit of 2.5µM.</td>
<td>120</td>
</tr>
<tr>
<td>Reduced graphene oxide</td>
<td>polyPoly (anilineboronic acid) (PABA)</td>
<td>Biosensors</td>
<td>Sialic acid biosensor where boric acid of PABA preferentially reacts with diols of Sialic acid resulting in the ester formation after the reaction. Graphene layer dramatically improved the dynamic range and limit of detection of the sensor to 2 µM–1.83mM and 0.8 µM respectively Covalent linking of graphene to PCL resulted in homogeneous dispersion of graphene in the polymer matrix with enhanced tensile strength and plasticity. 14 times increase in electrical conductivity of the composite with 10% graphene content.</td>
<td>121</td>
</tr>
<tr>
<td>Graphene</td>
<td>PCL</td>
<td>Biosensors/ Tissue Engineering</td>
<td>Covalent linking of graphene to PCL resulted in homogeneous dispersion of graphene in the polymer matrix with enhanced tensile strength and plasticity. 14 times increase in electrical conductivity of the composite with 10% graphene content.</td>
<td>122</td>
</tr>
<tr>
<td>GO</td>
<td>poly(3,4-ethylenedioxythiophene) (PEDOT)</td>
<td>biosensors/ Bio implants</td>
<td>Doping of graphene not only enhanced the mechanical properties but also enlarged the active surface area of the electrode. With low impedance, high charge storage capacity, high charge injection limit to perform electrical simulation and biocompatibility, this could be an excellent material at electrode-tissue interface.</td>
<td>114</td>
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<tr>
<td>Graphene</td>
<td>58s Bioactive glass</td>
<td>Bone tissue Engineering</td>
<td>Used as a reinforcement for 58s Bioactive glass which improved the compressive strength and fracture toughness of the scaffold with favourable biocompatibility for bone tissue engineering</td>
<td>123</td>
</tr>
<tr>
<td>single- and multi-walled GO nanoribbons (SWGONRs, MWGONRs); GO nanoplatelets; Single and Multi walled Carbon Nanotubes</td>
<td>polypropylene fumarate</td>
<td>Bone tissue Engineering</td>
<td>Enhancement in the mechanical properties of the composite compared to pristine polymer. 2D graphene material (SWGONR, MWGONR, and GONP) showed an increase in the mechanical properties compared to 1D graphene materials (SWCNT, MWCNT). Reinforcement primarily based on the structure (surface area, aspect ratio and crosslinking density) of the nanomaterial.</td>
<td>94</td>
</tr>
<tr>
<td>Graphene Nanoribbons</td>
<td>PEG-DSPE (1,2-distearyl-sn-glycero-</td>
<td>Drug delivery</td>
<td>Oxidized graphene nanoribbons provided a higher surface to load Doxorubicin on their surface by π -π stacking. Further coating with</td>
<td>102</td>
</tr>
</tbody>
</table>
GO

Poly(N-vinyl caprolactam) (PVCL)

GO nanosheets

poly(pyrrole)

poly(acrylic acid)/gelatin

GO nanoparticles

Poly Ethylene oxide (PEG)-Alginate

Graphene nanoparticles (GQDs)

polyvinylpyrrolidone

Poly Ethylene oxide (PEG)

Poly Ethylene oxide (PEG)

Graphene Quantum Dots

Polydopamine

GO/ rGO

polyvinylpyrrolidone (PVP)

GO

Poly L-Lysine

GO

Chitosan

GO

Poly Lactic acid/ Poly Urethane

GO nanosheets

poly(acrylic acid)/gelatin

GO

Chitosan/ Hydroxyapatite

GO

Poly (propylene carbonate) (PPC)

3-phosphoethanolamine

Drug delivery

Energy driven endocytosis mediated GO-PVCL nanocarrier delivered camptothecin to cancer cells.

Drug delivery

Electrically activated controlled ON/OFF delivery of Dexamethasone was achieved. Physical properties of GONS led to customize the physiochemical properties and drug loading parameters of the composite film.

Drug delivery

Apart from enhancing the mechanical properties of the composite film, GO assisted in drug loading and assisted in transdermal therapy. Micro-needles developed were able to withstand insertion and able to penetrate till epidermis and deliver the drug.

Drug delivery

Thermo and Photo responsive hydrogel composite microspheres were synthesized. Heat liberated during Photo activation of GO assisted in phase transition of pNIPAAm and enabled drug release

Drug delivery

Multifunctional Iron Oxide loaded Poly Lactic acid microcapsules decorated with GO were synthesized for image guided photo thermal theranostic agent. These conglomerate systems served as contrast agents for Ultrasound, MRI and photo acoustic imaging.

Drug delivery

3D GONPs were synthesized and functionalized with biocompatible alginite PEG to deliver Doxorubicin via glutathione mediated drug release

Drug delivery

Bionocompatible nanocarrier system loaded with paclitaxel to target Human lung cancer A549 and human breast cancer MCF-7 cells.

Drug delivery

Smaller sized and highly dispersed Graphene nanoparticles outperformed the Single walled carbon nanotubes in Photothermal therapy. A two fold increase in the heat generated by Graphene nanoparticles created an oxidative stress and depolarized mitochondrial membrane and lead to apoptosis and necrosis mediated cancer cell death.

Drug delivery

rGO showed 3-4 fold enhancement of optical absorption in NIR region compared to GO. Ultra small size and appropriate surface coating enhanced the blood circulation time of rGO-PEG over GO-PEG. Efficient tumour ablation with ultra-low power of 0.15W/cm2

Drug delivery

Higher NIR absorption property of GO facilitated Photo acoustic imaging and Two photon absorption cross section for the Two photon imaging sensitive dye.

Drug delivery

pH responsive charge reversal electrolyte on GO enabled controlled release of Dox from the carrier.

Drug delivery

Hybrid Two photon Photodynamic therapeutics were synthesized that can generate reactive oxygen species under preferential NIR absorption can generate reactive oxygen species under preferential NIR absorption

Drug delivery

Surface functionalized, stable and nontoxic polydopamine coated Graphene Quantum dots were synthesized that could be facilitate single cell imaging and used as an optical contrast agent and drug carrier

Drug delivery

Effect of surface functionalization over the stability of GO is studied. Coating of GO with PVP-PVA provided electrostatic type stabilization for over 27 months

Drug delivery/ Tissue Engineering

GO grafted Poly ethylene imide as a potential vector for gene delivery enhanced the gene transfection and localization of DNA in the nucleus. Layer by layer assembly of GO and Poly L Lysine showed a significant increase in the growth, differentiation and proliferation of Mesenchymal stem cells. Larger surface area and higher intermolecular interactions between the osteogenic media and the GO, favoured the osteogenic differentiation of mesenchymal stem cells.

Gene transfection

Regenerative medicine

GO

Addition of GO not only increased the mechanical properties and pore formation but also enhanced the cell proliferation and bioactivity of the chitosan 3D porous scaffold

Tissue Engineering

Antibacterial property with minimal intrinsic toxicity and no effect on normal cell proliferation and differentiation.

Tissue Engineering

Graphene reinforced the poly (acrylic acid)/gelatin hydrogel matrix by improving the mechanical properties (Improved tensile strength and elongation at break by 71% and 26% respectively).

Tissue Engineering

Enhanced bio mineralization by GO-Chitosan improved the cell adherence, proliferation and elevated the osteoblast function

Tissue Engineering

1 wt. % of GO enhanced the thermo-mechanical properties of PPC. Super critical foaming technology was used to prepare 3D porous scaffolds for Tissue engineering applications. Addition of GO to the matrix resulted in uniform pore structure with high pore density and small pore size.
biochemical and histological observation in GQDs treated animals. Our observation does not reveal any significant toxicity exert from GQDs in vitro and in vivo. GO has several advantages over graphite or graphene such as their dispersion in aqueous media, which is essential for biological application. GO contains hydrophilic functional groups that enable chemical modification and functionalization. In vivo studies of GO is based on appraisal of bioaccumulation and excretion. Route of administration is also one of the important parameters to be considered in case of toxicity of nanomaterials. Due to the increasing importance of GO, there is a need for more detailed and accurate in vitro and in vivo studies regarding the toxicity of the GO. Wang et al. reported that GO could induce dose- and time dependent cytotoxicity and also cell apoptosis can cause cell floating and apoptosis.\textsuperscript{134-136} Another group reported that GO showed less toxicity in fibroblast Hela cells over other carbon materials like multiwall carbon nanotube and nano-diamond.\textsuperscript{137} In addition to GO, GO based polymer nanocomposites also found to show toxicity on bacterial cells.\textsuperscript{138} Size-dependent toxicity of graphene nanoflakes were investigated using a cell-based electrochemical impedance sensor which depends on interdigitated ITO electrode. Their results showed that increased toxicity with smaller graphene nanoflakes can be used for electrochemical impedance sensing, optical imaging of cells, and also bioassays.\textsuperscript{139} Another graphene derivative, graphene nano-walls posed greater toxicity upon their contact with the bacterial cell membrane leading to the efflux of RNA from the cells. GO and some of their derivatives like oxygenated and carboxylated GO nanomaterials showed toxicity in human cancer cells by MTT assay.\textsuperscript{9,141} Zebrafish is considered as the most used animal model to evaluate the in vivo toxicity of graphene-related materials. One of the research reported that MWCNTs, GO, and reduced GO did not show high toxicity to zebrafish embryos, but had some sub-lethal effects on the heart rate, hatching rate, and the length of larva.\textsuperscript{142} Nano-size GO and reduced GO showed lower toxicity in biomedical areas with higher photothermal effects.\textsuperscript{143}

### 4.2. Biological Effect of Graphene Derivatives

Though not much, but important studies have been conducted earlier to understand the mechanism of interaction between graphene and biomolecules especially intracellular organelles. The study reported by Li et al. shows that the commercially available pristine graphene increased reactive oxygen species (ROS) in vitro.

#### Table 4. Toxicity of graphene derivatives in vitro and in vivo.

<table>
<thead>
<tr>
<th>Graphene derivatives</th>
<th>Study</th>
<th>Model (Cell line/ animal)</th>
<th>Observation</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO Quantum dots</td>
<td>In vitro, In vivo</td>
<td>Hela cells/ female BALB/c mice</td>
<td>No apparent in vitro and in vivo toxicity of GQD, resulting from its small size and high oxygen content compared with that of the widely used GO-PEG</td>
<td>132</td>
</tr>
<tr>
<td>GO Quantum dots</td>
<td>In vitro, In vivo</td>
<td>KB, MDA-MB231, and A549 cells / BALB/c nude mice</td>
<td>No acute toxicity or morphological changes of Carboxylated GQDs were noted in either system at the tested exposure levels. GO may induce severe cytotoxicity and lung diseases. Higher dose of GO showed</td>
<td>38</td>
</tr>
</tbody>
</table>

Fig. 5. (a) Signalling pathway of Cell apoptosis involved in pristine-graphene. This scheme shows that cell apoptosis causes through ROS-activated MAPKs and TGF-beta pathways. (b) Signalling pathway of macrophage activation stimulated by graphene nanosheets. Graphene may recognize by certain types of TLRs this activating kinase cascades by MyD88-dependent mechanism. Figure adapted from ref 141, 142, respectively. Copyright © (2012), with permission from Elsevier.

| GO | Poly(m-aminobenzene sulfonic acid)/ polyoxymethylenebis(a mine) (NH2-PEG-NH2), poly(ethylene glycol) monomethyl ether | Tissue Engineering | Effect of surface functional charges on the growth and branching of neuronal cells were investigated. Compared to neutral, zwitterionic and negatively charged surfaces, positively charged GO surfaces exhibited enhanced maximum neurite length and branching |
| GO | Genipin cross-linked Chitosan | Tissue Engineering/ Drug delivery | Fluorescent and flexible Genipin cross-linked chitosan reinforced with GO improved the tensile strength of the material. Swelling behaviour and degree of degradation were governed by the concentrations of GO. Excellent biocompatibility with no systemic toxicity. |
| Multi walled Carbon Nanotubes; Graphene | Poly (L-Lactic Acid) | Tissue Engineering | Protonated GO showed higher toxicity to zebrafish larvae, but had some sub-lethal effects on the heart rate, hatching rate, and the length of larva. Nano size GO and reduced GO showed lower toxicity in biomedical areas with higher photothermal effects. |
GO \textit{In vitro} A549 cells toxicity in mice organ than lower doses. The effect of GO on A549 cells is dose and size related.  

GO \textit{In vitro} Hela cells GO toxic in Hela cells.  

GO composite \textit{In vitro} Escherichia coli, Bacillus subtilis, Rhodococcus opacus, Cupriavidus metallidurans CH4 and NIH 3T3 fibroblast cells Nano composite shows lower toxic in bacterial and mammalian cells.  

Graphene \textit{In vitro} HeLa cells Evaluate Size-dependent toxicity of graphene nanoflakes.  

Graphene Oxide Nanowalls Bacterial activity \textit{E. coli} and \textit{S. aureus} Cell membrane of the bacteria was effectively damaged by direct contact of the bacteria.  

Oxidized graphite nanoribbons \textit{In vitro} HeLa, MCF7, SKBR3 and NIH3T3 cells Oxidized graphene nanoribbons showed cytotoxic effects than GO.  

GO and carboxyl graphene nanoplatelets \textit{In vitro} Hep G2 cells GO and carboxyl graphene nanoplatelets - treated cells demonstrated toxic in cancer cells.  

Reduced GO \textit{In vitro} Wild-type zebrafish Toxicity to zebrafish embryos and sublethal effects on the heart rate, hatching rate, and the length of larvae.  

Nanoco-GO and Nano-Reduced GO \textit{In vitro} U87MG human glioblastoma cells Nano-GO and nano-rGO appeared to show similar levels of toxicity on Breast Cancer cells.  

Fig. 6. (a-c) Small GQD (15 nm; 50 mg/kg i.v.) were compared with large GQD (40 nm; 50 mg/kg i.v.) and the conventional immunosuppressant dexamethasone (0.5 mg/kg i.v.) for their ability to reduce serum transaminase (a) and IFN-\gamma levels (b), as well as liver infiltration of IFN-\gamma-producing CD4+ and CD8+ T cells (c). EEM contour maps (after removing Rayleigh scattering) of GQD (blue lines) and the urine of mice treated with GQD (red lines). The shaded region highlights the overlap of GQD and urine emission (d). Figure was adapted with permission from ref 14. Copyright © 2014 American Chemical Society.

5. **Size and Dose Dependant Therapeutic Effect of Graphene**
The size of the nanoparticles (crystal, semi-crystal, semiconductors or metals etc.) lower than 5 nm mostly and equally accumulate in liver and kidney and finally excrete through kidney, whereas previous studies proved that the larger particles accumulate in the liver. Unfortunately, no in depth investigating have been done to understand the effect of a larger nanoparticles and their effect in liver and/or other organs. A very recent and advanced study reported by Volarevic et al. demonstrated that the larger GQD of 40 nm in size highly accumulated in liver can alleviate immune-mediated liver damage. In addition the rate of liver accumulation is higher for high dose (50 mg/kg) compare to lower dose. Though many previous studies reported earlier showed that graphene derivatives especially GQDs with smaller size in diameter (5-10 nm) interfere with regular cellular mechanism through interacting with intracellular pathways and induce apoptosis, and reduce immunity but this study on GQDs with larger diameter shows complementary results. Nevertheless, both in vitro and in vivo study shows that the GQDs with the size of 40 nm in diameters play a critical role as a therapeutic agent to be used to treat liver inflammation/ hepatitis.

6. Conclusion, Challenge and Prospects

The as developed and well characterized GQDs can be used not only for biomedical imaging but also nanocarrier mediated drug delivery, gene delivery, tissue engineering, stem cell research, photothermal cancer therapy and also molecular imaging. The GQDs also have promising prospects for applying in PL based biosensor development. However, more basic and broad research is required to optimize the reaction condition with proper analytical methods to get the unified structure of GQDs with higher properties. Waste management and utilization of the byproducts is the biggest challenges regarding large scale production the GQDs by chemical exfoliation methods. Since the synthesis process conducted in highly acidic medium, the acids such as nitric acid and sulfuric acid are required to be neutralized by adding excess amount of salts. Therefore, it produce huge amount of byproducts that is one of the major concern for scaling up the production system. Though we have found no significant toxicity of GQDs in biological systems however, further elaborate toxicity studies are required to observe bio-degradation of GQDs in biological system after administration. Studies also required to see if the graphene components interact with genetic molecules such as DNA. GQDs required further long term biosafety studies before considering them as biomaterials for drug delivery, gene delivery even for therapeutic applications. Graphene has several unique and promising properties that facilitates them to be considered for various biomedical applications such as drug delivery, gene delivery, tissue engineering and as well as photomedicine that can be considered as therapeutic agent. Large surface area, ease of functionalization extends their opportunity as a drug delivery carrier. It is widely accepted as a scaffold in tissue engineering as they divulge unique mechanical properties. Optical properties of graphene derivatives facilitate in vitro and in vivo imaging which is an emerging research field in biomedical and molecular imaging. A promising discovery of graphene derivatives, their photosensitizing property unveiled a window to use graphene derivatives as a photomedicine to treat diseases like cancer and wound healing. Very recent findings include the application of large graphene nanoparticle as therapeutic agent for treating hepatitis, oxidative stress, apoptosis and autophagy. However, considering the safety issues and hurdles of clinical trials to get an approval in clinical application of graphene derivatives is time consuming and it hardly unlikely that graphene based materials will be available in market for biological application before 2030. Therefore, many must to do research is required to understand its pharmacokinetics, biodegradability, biocompatibility and acute/chronic toxicity studies before graphene can be considered as a promising materials for biomedical application.

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Notes

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