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Functionalized Graphene Oxide as a Vehicle for Targeted Drug Delivery and Bioimaging Applications

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Abstract

Graphene Oxide (GO) has attracted tremendous attention as a most promising nanomaterial among the carbon family since itsemerged as a polynomial functional tool bearing rational application in diverse fields such as biomedical engineering, electrocatalysis, biosensing, energy conversion, storage devices and others. Despite having certain limitations due to their irreversible aggregation performance owing largely to the strong vander Waals interactions; efforts have been made to smartly engineer its surface chemistry for multimodal realistic applications. The use of such GO based engineered devices has galloped rapidly in last few years principally due to its excellent properties such as huge surface area, honeycomb like structure allowing vacant interstitial space to accommodate compounds, sp^2 hybridized carbon, improved biocompatibility and cell surface penetration due to electronic interactions. Amongst multifaceted GO dynamics, in this review, attempts have been made to discuss the advanced applications of GO or graphene based materials (GBNs) in biomedical field involving drug or therapeutic gene delivery, dual drug or drug-gene concoction targeting, special delivery of drug cocktail to brain, stimuli responsive release of molecular payloads, Janus structured smart applications for polar-nonpolar combination drug loading followed by targeting together with smart bioimaging approaches. In addition, the advantages of duel drug delivery systems have been discussed in details. We have also discussed various electronic mechanisms, detailed surface engineering to meet microcosmic criteria for its utilizations, various novel implementations of engineered GO as mentioned above together with discussions of its inevitable toxicity or disadvantages. We hope that target audience, belonging to biomedical engineering, pharmaceutical or material science field, may acquire relevant information from this review which may further help them design future studies in this field.

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Keywords: Bioimaging; controlled release; duel drugs; drug delivery; Janus structure.

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Abreviations:

AuNPs, Gold Nanoparticles; APS, 3-aminopropyl triethoxysilane;bcPLu, block copolymer pluronic; BSA, Bovine Serum Albumin; β-CD, Betacyclodextrin; CT,Computated Tomography; CPT, Camptothecin; CAs, Contrast Agents;CEF, Cephalexin; CXCR4, C-X-C chemokine receptor type 4;CuS, Coper Sulphide; CEA, CarcinoembryonicantigenCNTs, Carbon Nanotubes; CS, chitosan; Ce6, chlorine6; Cis-Pt, Cis-Platin; DDSs, Drug Delivery Systems; DFT, Density Functional Theory; DDMAT, 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid; DOX, Doxorubicin; 2D, FA, Folic Acid; 5-FU, 5-fluorouracil; Two Dimensional; DNA, Deoxyribo Nucleic Acid; E. Coli, Escherichia coli, EPR, Enhanced Permeation and Retention;FRET, Fluorescence Resonance Energy Transfer;FI, Fluorescence Imaging;GEM, Gemcitabine hydrochloride; GMA, glycidyl methacrylate; GBNs, graphene based nanomaterials; GO, Graphene Oxide; Glu, glucosamine; GEF, Gefitinib GQDs, GSH, glutathione; GNRs, Graphene Nanoribbons;Graphene Quantum Dots; HA, hypocrellin A;IONP, Iron Oxide Nano particle; ICG, Indocyanine Green; KGM/SA,Konjac glucomannan/sodium alginate; Lf, Lactoferrin;Me-LOGr, Microwave-enabled Low-Oxygen Graphene;MNs, Magnetic Nanoparticles; MBs, Molecular Beacons; MRI, Magnetic Resonance Imaging;MTX, Methotrexate;MI, Multimodal Imaging; MeB, Methylene Blue; NIAcAcAl, N-isopropyl acrylamide-coacrylamide co-allylamine; NmPDT, Nanomaterial-mediated Photodynamic Therapy;NmPTT, Nanomaterial-mediated Photothermal Therapy; NGS, Nanographene Sheets;NIR, Near Infrared Regions;PEI, poly-ethyleneimide; PAI, Photoacoustic Imaging; PVP, polyvinylpyrolidone; PNIPAAm,poly(N-isopropylacrylamide);PEG,poly-ethyleneglycol; PVA, poly(vinyl alcohol); PAMAM, polyamidoamine; PCT, Paclitaxel; PET, Positron Emission Tomography; PNPs, polymers nanoparticles; PS, Photosensitizers; PLL, poly (L-lactide); PDT, Photodynamic Therapy; PTT,P-gp,P-glycoprotein; Photothermal Therapy; PCL, poly-caproyllactone;PAA, Polyallylamine; PSA, polysebacic anhydride; PMMA, Poly(methyl methacrylate); QSR,Quercetin; RB, Rose Bengal; RAI, Radionuclide Imaging; RI, Raman Imaging, rGO, reduced Graphene Oxide; RAFT, Reversible terminated Addition Fragmentation chain Transfer; SPECT, Single-Photon Emission Computed Tomography;SERS, Surface-Enhanced Raman spectroscopy; TPFI, Two-Photon Fluorescence Imaging; Tf, Transferrin; UV, Ultra-Violet.

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1. Introduction

At present, various malignant diseases have substantially affected and compromised the human life and become the cause of threat all over the world [1]. Therefore, exponentially emergent call for advances of the efficient treatmentand diagnosis of various malignant diseases has encouraged for anextensivearray of interdisciplinary field to modernize an effective and nontoxic drug delivery systems (DDSs). With the advancement of science and technology, various routes have been emerged so far for dealing againstsuch threat [2,3].To enhance the persistence rate of patients suffering from such diseases, the convenience of novel technologies for early diagnosis and monitoringplay a vital role. With current developments in nanotechnology field, the potential application of nanosized materials for special types of cell target therapy such as efficient delivery of biological entities to the targeted site and competent detection of diseases are being paid enormous attention so far [4–6]. Since now, numerous nanostructured materials have been envisioned and discovered for such highly focused biomedical applications.

Among these nanostructured materials, graphene based nanomaterials (GBNs) e.g. graphene oxides (GO), reduced graphene oxide (rGO), graphene quantum dots (GQDs) etc., are extensively explored for various drug targeting strategies, gene therapy, bioimaging application with the possibility of highly engineered and efficient multi-function diagnostics and therapeutics agents as they acquire exceptionally excellent physicochemical properties along witha number of incomparable characteristics such as extreme small sizes, high specific surface areas and exclusive arrangement of carbon atoms. At 2004, Geim and Novoselov isolated graphene by single layer exfoliation technique in University of Manchester and characterized to establish it as a novel 2D carbon naomaterial with single atom layer, however, endowed with flat sp^2 hybridized structure, long π-π stacking aromatic chain and polar functional group on both the surfaces [7]. In 2008, 2D graphene oxide (GO), which had been synthesized by classical Hummers' method [8], was first exploited in biomedical field as novel, improved drug carrier to load water insoluble anticancer drugs such as Doxorubicin and SN-38 [9-10]. Interstingly, in one case pristine GO was used as nanocarrier of drug [9] where in the other case [10], PEGylated GO acted as superior cargo-boat to deliver SN-38 with better efficacy than Irinotecan, one FDA approved anticancer prodrug for colon cancer. The superior activity of GO or functionalized GO has been attributed to their 2D structures as they are reported to acquire exceptionally excellent physicochemical properties along witha number of incomparable characteristics such as extreme small sizes, high specific surface

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areas and exclusive arrangement of carbon atoms [11]. GO, an oxygenated derivative of the graphene, based on its specific honeycomb lattice structures and biocompatibility, provides such sites to integrate and fabricate with various types of biomolecules, such as drugs, antibodies, DNA, peptide, protein, enzyme etc. In addition, graphene and its derivatives exhibit excellent optical properties, thus they consider to be promising and attractive candidate for bioimaging, generally for cells and tissues; GO and its derivatives are extensively applied in fluorescence bioimaging, surface enhanced Raman scattering (SERS) imaging and magnetic resonance imaging (MRI) [12-14] and offered the extended applications of GO based DDSs in materials science [15-17]. Such biomolecules and hydrophobic drugs possess limited clinical utility as they show poor solubility in the physiological environment.

It is well known that carbon nanomaterials aggregate in buffers solutions due to screening effect of charge. Therefore, surface modification is the key to render the solubility and the biocompatibility of carbon nanomaterials for biological systems. It is the physicochemical characteristics of GO which make it physically and chemically versatile candidate and differentiate it with other carbon nanomaterials. Hence, the principal advantage of GO over other carbon-based nanomaterials is its aqueous and colloidal stability and controlled release for sustainable drug release [18,19]. Owing a high surface-to-volume ratio GO enables to load more than one drug simultaneously in a single nanocarrier [20]. Recently a report of a dual DDSs with cocktailing two anticancer drugs Doxorubicin (DOX) and Cisplatin (Cis-Pt) has been described and found that cancer cell apoptosis and necrosis rate increased by two times after the combining the drugs, suggesting this dual DDS has great potential for clinical applications [21- 23].

Furthermore, due to its unique size and structure, Liu *et al.* investigated thatin passive targeting graphene appears more efficient than that of the carbon nanotubes by providing a favorable environment for superior permeability and retention effect [24].

Interestingly, GO exhibits superior quenching abilities to the other carbon nanomaterials in quenching efficiency and its kinetics. Fan *et al*. introduced a comparative study between GO and CNTs based fluorescent sensor for the detection of DNA (Deoxyribo Nucleic Acid) where the former resulted in detection and quantitation of lower amount of DNA than that of the latter [25]. Moreover, the 2D graphene sheets may be easily complexed to various other functional nano-particles for potential multimodality imaging and therapy applications, while the nanoparticle modification on individual nanotubes has been relatively more complicated.

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To overcome from these complications and to explore the prospective of GO based DDSs, these bio-molecules are functionalized on the surface of GBNsby means of various surface coating strategies. These surface functionalization strategies are applied through non-covalent and covalent bonding resulting in improved biocompatibility and regulation of their properties inside the biological systems [26- 28]. To regulate the terminologies used in GO and validate the toxicological consequence for the comparable results, the toxicological index of GO-based formulations approach plays promising role.

Further to explore the optimum dosage that maintains a balance between the therapeutic effects and nanotoxicity of GO-based formulations, the proper knowledge of the biocompatibility of GO-based formulations with relevant pre-clinical *in vitro* and *in vivo* models are crucial, so that the results obtained can be easily interpreted for the further clinical applications.Therefore, GO-based nanostructured systems can encourage the development of ideological approach for the expansion of novel technologies which can help to overcome against the detection limits for early diagnosis and provide improved targeting approaches [29].

On the other hand, recently GO-based nanomaterials emerged as new alternative to address the issues related with the impaired tissue penetration depths of the light sources, owing to intrinsic optical (absorption in the Near Infrared Regions/NIR or Ultra-Violet/UV regions) and thermal properties of these surface engineered GO which further can be utilized for selective therapies through hyperthermia, recognized as one of the other promising ways to treat some malignant diseases through thermal ablation [30, 31].

This critical review aims to update all the possible avenues related to GO or GO based materials pertaining to our scope, that have been or being undertaken by various scientists across the globe. In addition, we have undertaken a special note on GO based dual drug delivery with or without targeting because such multimodal drug delivery based on a single carrier may take the height of drug delivery application to a different level by augmenting their release pattern or improving their bioactivity by synergistic mechanism. In pursuit, molecular modelling and simulation approaches have been perturbed in this review to elicit role of chemistry of both carrier and guests together with their loading mechanisms for achieving such polynomial drug cocktail. Furthermore, application of novel Janus structured materials based on GO is being coined nowadays to facilitate dual or targeted drug delivery, which has been another prime target area of this critical review. Smart bioimaging, which may ease down the therapeutic decision by medical

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practitioners through apt diagnosis, is in galore with GO based material which have been summarized in this review for future benefits of the scientists and professional who are working in this field. Moreover, not only we presented some perspectives on the challenges or constraints counting the advanced techniques and facile methods to improve the drug loading and dispersing as carriers; but innovative ideas and opportunities in this promising research field are also proposed and their solutions suggested. Finally, the review also highlights the future domains and avenues of implementing GO based materials in relevant biomedical applications. Thus, this review provides an overview of the state of the understanding and challenges in this field and would be highly beneficial not only to experienced scientist but also to graduate and undergraduate students in the areas of biomedical and nanomaterials science and engineering.

2. Functionalization of GO

The functionalization of graphene sheet is an effective way which helps them to better disperse and stabilize within a polymer matrix. There are two chief approaches for the functionalization of graphene. (Table 1.)

2.1. Noncovalent Functionalization

Noncovalent modifications require moieties which show extremely high hydrophobicity and usually involve Van der Waals forces, π-π interactions [32], hydrogen bonding [33], electrostatic interactions [34], and coordination bonds [35] with GO. As graphene sheets also exist of Van der Waals forces and π-π stacking which make their surface modification significant with such moities. In general, such noncovalent functionalizationon GO surface can be attained either wrapping of polymers and biomacromolecule, or via absorption of such molecules on the surface of GO [36]. In this regard, Liu and coworkers [37] synthesized a composite material with graphene and PNIPAAm (poly N-isopropylacrylamide) by reversible terminated addition fragmentation chain transfer (RAFT) of PNIPAAm with graphene. They found pyrene functionalized polymers have the property to attached both sides ofthe graphene sheet to form a sandwich-like structure via π-π stacking which further helpful to stack higher amount of drug than the non functionalized graphene. Zhi *et al.* [38] enhanced aqueous solubility of GO via reducing its excessive oxidative moities due to electrostatic noncovalent interfaces of GO with L-tryptophan (an amino acid). Their study clearly explained that the increase of π-π interactions between the GO and L-tryptophan

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molecules increaded the dispersibility of GO in aqueous media. Hu *et al.* prepared a graphene derivative by non-covalently functionalizing GO sheets with pluronic F127, an amphiphilictriblock copolymer with excellent biocompatibility [39]. In this study, in order to increase the π-conjugation before coated by F127, GO was reduced to rGO, then poly (propylene oxide) (PPO) segments of F127 were used to bound to the surface of reduced GO through hydrophobic interactions and the poly(ethyleneoxide) (PEO) segments of F127/rGO nanohybrid results excellent solubility and stability in both of aqueous solutions and physiological environment. However, the adsorption of polymers onto GO surface via a noncovalent route is not as strong as the covalent linkage and susceptible to the inconsistant external environment, which makes the DDSs not that much stable with biological systems *in vitro* or *in vivo*. Along with this, non-covalently functionalized GO may load less quantity of aromatic drugs as compared to covalently functionalized GO, because most of the conjugated sites of the GO sheets are partially engaged by coated polymers.

2.2. Covalent Functionalization

Covalent functionalization follows the chemical bonding with surface moities present on the surface of GO with the help of strong acid-based treatment. The harsh acidic conditions might also be introduced structural defects, resulting advancement in physicochemical properties of GO [40]. DDSs based on covalently functionalized GO with suitable surface functional groups are emerged as potential tools and widely explored for systemic targeting platforms. Xu *et al.* [41] used a covalent conjunction strategy for PCT loaded on GO derivatives, whereby PCT was connected with biocompatible six-armed PEG by covalent functionalization onto the GO surface. The modified GO-PEG-PCT system had a high loading ratio of along with superior stability under physiological conditions. The covalent functionalization for GO sheets can also be realized by introducing small molecules onto the GO sheets. For example, Zhang *et al.* functionalized GO sheets with sulfonic acid groups (SO_3H), followed by a covalent grafting of folic acid (FA) biomolecules to the GO sheets [42]. The FA-conjugated GO i.e. FA-GO were able to well dispersed and maintained stability in a physiological solution for a long time. The combination of GO and FA provides a novel molecular recognition strategy to specifically carry anticancer drugs into folate-receptor-positive malignant cells, which covered the way for the development of smart DDSs [43]. Recently, GO was covalently functionalized with D-mannose using mannosylated

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ethylenediamine. The mannosylation of GO drastically reduced its toxicity and improved its biocompatibility in red blood cells [44]. Thus, covalent functionalization of GO contributes to future biomedical applications with active biomolecules. With the help of stimuli specific polymer functionalization the effective drug release rate on the tumor site can be released rapidly when the modified DDSs reach at the target cells and resulted to a more effective therapy. For example, Wen *et al.* [45] conjugated PEG with GO via cleavable disulfide bond (GO-SS-PEG), which exhibited great biocompatibility, considerable degradability and the targeting ability of delivering drugs to specific tumor cells with high intracellular glutathione (GSH) concentrations via redox reaction. Similarly, Kim and collaborators developed a photothermally triggered DDSs by functionalizing GO covalently with branched polyethylenimine (bPEI) and PEG successively [46]. The GO-bPEI-PEG nanocomposite exhibited high water stability along with high DOX loading efficiency as compared with the GO alone. Chen *et al.,* developed PEGylated GO to build a highly efficient drug loading and photothermally triggered DDSs [47]. The GO-PEG system shows better water stability and high NIR absorbance. Conjugation of CS on GO is another example of covalent functionalization which results in better biocompatibility as well as drug and gene delivery. CS is used as a linker to combine FA with functional GO and also provide encapsulation, better stability, biocompatibility, and controlled release of active molecules [40]. Various reports on the encapsulation of DOX onto GO via charged folate conjugated CS explain the superiority of the system over GO, resulting in pH responsive drug release [41]. Further, Yan *et al.* used polyethylenimine (PEI) to functionalize GO covalently for an efficient nanocarrier which shows high stability in both water and physiological solutions, and further combined with biomolecules and markers to enhance their drug loading and delivery capacity [48]. Besides single functionalization, the dual covalent functionalization of GO exhibits the attachment of distinct molecules through different mechanisms. Recently, Shi and his group [49] established a scheme for chemoselective dual functionalization of GO using benzoquinone. The two functional groups were covalently functionalized onto GO through an epoxide ring opening reaction and the second moiety with amine group was covalently attached through a Michael addition. Thus, the morphology of the GO sheets was preserved and the functionalization did not cause any further reduction of GO. Hence temperature and pH responsive functional groups efficiently functionalized on surface of GO followed by chemical reactions and extend the application of GO via preserving its structure and properties. This strategy is particularly suitable for the conjugation

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of biomolecules and widely subjugated for modifying GO with proteins, drugs, aptamers, or peptides to obtain multifunctional GO for applications in therapy, biosensing, and bioimaging [50, 51].

3. Advantages and disadvantages of GO

3.1. Advantages of GO

GO play a significant role in sorting out the drawbacks occurs in biomedical field.Drug release can also be tuned or stimulated by the intracellular environment. In fact, drug release in a cell is due to the change of the environmental condition (i.e. pH, temperature etc.) between the extracellular matrix and cytoplasm. In this section, we will discuss how the characteristic properties of GO provide a room for significant and effective DDSs.

3.1.1. pH Responsive GO for Controlled Release

As compared to the healthy cells, infected cells are usually sensitive and possess unique physicochemical properties, microstructural features and unique micro environments which can be targeted accordingly by GO. Since GO has both sp^2 and sp^3 domains within it, not only it provides the π-π interaction for therapeutic molecules at surface, but also it helps to develop targeted DDSs exploiting strategic ligands attached onto it [52–54]. Moreover, GO surface chemistry is unique due to presence of both –OH and –COOH groups onto it. The surface is highly anionic due to negative charges present on the surface arising from ionized $–O^-$ and $–COO^-$ functional groups. This undergoes intensive protonation in low pH where degree of protonation depends on lowering of pH. The protonation of surface moieties leaves it to be non-ionic thus hydrophobic. Thus, at low pH, inside aqueous solution, GO form aggregates with GO-water-GO sandwitch strucuture. On the contrary, at higher pH, the anionic surface groups remain charged, thus rendering it to be hydrophilic where degree of hydrophilicity depends on elevation of medium pH. This ultimately leads in dissolution of GO, altering its surface dynamics within itself as well as with water. This phase transition of GO with variation of medium pH results in different wettability, water penetration into GO sheets, hydrolytic cleavage of GO-guest chemical bonds subsequently releasing the guest (e.g. therapeutic molecules) from GO sheets. In addition, the GO response has been also dependent on layer by layer (LBL) structure of GO nanosheets which

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regulate the entry of ions (dependent on pH) inside the same, its swelling, interfacial dynamics as well as release of molecules from inside the GO nanosheet [52-54].

It has been reported earlier that GO has high drug loading efficiency and the release behavior of the loaded drug is adjusted by varying the pH of the DDSs [55].

As an environmental pollutant, antibiotics increasingly affect the health issues in past few years. Antibiotics overdose results in antibiotic resistant genes, which significantly cause health hazards. In this context, Bytesnikova *et al*. in 2018 applied GO as a remediation of the environment as it has characteristic properties to binding nucleic acids and catalyzing their decomposition. They discussed the factors influencing the binding of nucleic acids and the response of antibiotic resistant genes to GO, together with the presence of salts in the water pH. Finally they conclude that by modifying the water conditions with the adjustment of pH and temperature one can increase the efficiency of GO [56].

Considering the excellent dispersion of GO in water, GO was initially presumed to be hydrophilic due to the presence of the hydroxyl and epoxy groups present in the GO sheet basal plane [57]. Later on experiments involving pH changes and salt addition suggest that it was the peripheral carboxyl groups which are actually accountable in determining the solution behavior of GO [58]. Shih and his group explained the pH-dependent behavior of GO in aqueous solutions. They investigated the mechanisms behind the aggregation and the surface activity of GO at different pH values and found that at acidic pH, the carboxyl groups are easily protonated resulting the GO sheets less hydrophilic and tends to aggregates. However, at basic medium i.e. high pH, the carboxyl groups are deprotonatedand thus GO shows hydrophilic character and dissolved like a salt in aqueous medium [59].

In fact the colloidal stability of GO solutions is due to the electrostatic repulsions between ionized carboxyl groups. Kim *et al.* further suggested that GO behaves like a surfactant, as it has ability to adsorb at a water air interface, by lowering the surface tension of water [60].In addition, GO has been used to stabilize Pickering emulsions of organic solvents in water [61]. The basal plane of GO is much more hydrophobic than the carboxyl-decorated edges, and the large differences in both the hydrophilicity and structural dimensions make GO behave like an amphiphile. Thus GO can perform as hydrophobic as well as hydrophilic agent according to the requirement of our goal.

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In order to that, Bai and co-workers demonstrated the pH induced sol gel transition property of GO– PVA hydrogel to conclude that the hydrogel thus formed is used to selectively deliver the drug to the intestine (pH 6.8-7.4) without releasing it in acidic gastric juice (pH 1-2) which generally cause the stomach discomfort, therefore GO can be utilized for loading and release of drug in physiological medium selectively[62].Through proper tuning of this unique property, GO can be formulated into a smart DDSs having controlled release property in various specific microenvironments depending on their characteristic pH that differentiate cancer cell from the normal cell e.g., Yang *et al*. reported the release behavior of water soluble anticancer drug Doxorubicin and found that at acidic medium (pH-2) the release of the drug was more than 70% after the time period of 30 hours which was 4 times more than the medium of pH 7 and 10, hence this drug with GO give a higher drug release at acidic pH compared other pH [63].

The zeta potentials of GO suspensions can alsorender with pH as it is highly sensitive to it. Chen *et al.* prepared a multilayer film of GO and branched PEI on a terephthalate substrate and founda stable suspension of GO at all pH mediums [64].

Thus the reported pH-dependent behavior of GO originates from the degree of deprotonation of the carboxyl groups present at the edges of GO sheets. The electrostatic repulsions between the ionized carboxyl groups of GO are the major driving force for pH dependence.

Also, it is found that a novel magnetic GO, grafted with brush polymer *via* surface-initiated (SI) RAFT polymerization,can be applied as a nanocarrier for magnetically induced and pH-triggered delivery of doxorubicin anticancer drug.In this SI-RAFT technique, first a RAFT reagent called 2-(Dodecylthiocarbonothioylthio)-2-methylpropionic acid (DDMAT) was incorporated onto magnetically functionalized GO nanosheet and later polymerized with glycidyl methacrylate (GMA) using DDMAT. Subsequently the epoxy ring of the latter was opened with hydrazine (N_2H_4) which helped to load anticancer drug DOX by SI-RAFT technique. The imine interaction and π-π stacking was the major driving force for DOX coupling onto the polymer. The imine bond (-N=C_l-) is cleavable under weakly acidic condition (~ pH 6.0) inside body such as cancer tumor microenvironment and can release DOX which is linked with Polymerized GMA via the imine chain. This technique has been applied successfully to render pH responsive release of DOX from such GO nanocomposite. The resulting drug-nano composite has been reported with better bioavailability, lower toxicity and improved therapeutic activity when administered *in vivo* [65].

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Recently, scientists have focused to engineered multifunctional nanomaterials with controlled release of drug bysustaining a constant drug dosage in a regulated rate for a specific period of time. The most intriguing properties of GO and its derivatives are their remarkable solubility and stability in physiological media and biocompatibility which make them promising biomaterial substrate for controlled drug delivery.

In this context, an efficient approach was developed by Zhao *et al.* by integrating the GO with biocompatible polymer PEG and folic acid (FA) to form a nanovehicle GO-PEG-FA as an efficient and targeted DDS. The release kinetics of DOX from the carrier in different medium of pH was also investigated. Cumulative release of DOX at pH 7.4, 6.5 and 5 was 6.43%, 8.01% and 15.74%, respectively, which reveal that the drug has the favorable release in the acidic medium due to the higher solubility of DOX in acidic medium, hence it can be concluded that the DOX release should be regulated simultaneously by the solubility of the given drug and the designed GO based carrier supports in pH dependent controlled release characteristics [85].

Further in order to describe novel composite materials for the controlled release Wang and coworkers examined the release behavior of 5-fluorouracil (5-FU) with pH sensitive Konjac glucomannan/sodium alginate (KGM/SA) and KGM/SA/GO hydrogels were prepared, where GO is drug-binding agent for anticancer drug loading and release. The release amount of 5-fluorouracil (5-FU) incorporated into KGM/SA/GO hydrogels was about 38.02% at pH 1.2 and 84.19% at pH 6.8 after 6 h and 12 h, respectively. Therefore, the release rate of 5-FU from the KGM/SA/GO hydrogelscould be efficiently controlled with GO.The results showed that GO has a great potential for drug-binding as well as controlling the release rate of drugs from an efficient nanocarrier for the site-specific drug delivery [66].

3.1.2. Temperature Sensitive GO

Temperature is a typical example of triggers at the diseased site that could be exploited with the nanocarriers [66]. In modern drug delivery approach, the status of thermosensitive nanocarrirers are not only applied as traditional DDSs but also for the enhanced stability, solubility and reduces immunogenicity, toxicity of the targeted drugs. GO as thermosensitive nanocarriers attract enormous attention for controlled and targeted drug delivery.On the basis of this advantage, Bardajee and his team synthesized a temperature sensitive nanohydrogel ofNIPAAm with GO and

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the resulting nanocomposite showed potential drug loading capacity and relative drug release behaviour with increase in temperature [67].

Another GO based hydrogel(GO-PVA/PNIPAAm hydrogel) in which GO is the crosslinker between the two biocompatile polymers i.e. PVA and PNIPAAm-GO was preparedand temperature responsive behaviour of hydrogel was examined.The results demonstrated that the mechanical strength of the hydrogen has beenimproved with increasing composition of temperature sensitive GO. Furthermore, the PVA/PNIPAAm hydrogel exhibited a phase volume transition temperature at around 34.9 ^{0}C, which was reduced by 1 ^{0}C when conjugated with GO. This specific advantagerepresented that GO based hydrogel could be a potentialchoice in drug delivery field[68].

Wang and coworkers demonstrated the comparative study about pure polymers nanoparticles (PNPs) and their thermoresponsivehybrid with GO nanosheets for drug delivery application.The loading efficiency of drug molecules (Adriamycin) with GO–PNP (~87%) has been close to that withGO (~91%), but significantly higher than that with PNPs (~46%). The release efficiency of GO–PNPhybrids with the highest surface coverage of PNPs (~85 PNPs / mm^2) has been about 22%, which was very comparableto that of PNPs (~25%) and significantly higher than that of GO (~11%). The thermo-sensitive GO–PNP hybrid consisted of considerable better drug loading and release performance than both PNPs and GO and thus it can be applied as a novel nanocarrier fortemperature-controllable drug release. The unique superiority of this drug carrier system also lies in the fact that the drug loading and release are controllable by adjusting temperature and PNP covering on GO surface [69].

3.1.3. Near Infrared (NIR) or Laser sensitive photodynamic therapy (PDT)

Compared with other light irradiation techniques, near infrared NIR (700-1000 nm) light is considered as the most advantageous region in biological applications owing to its high ability ofpenetrate tissues [70-71].

Sahu *et al.*non-covalently functionalized nano GO sheet (NGO) with block copolymer pluronic and further conjugated the system with positively charged photosensitizer organic hydrophilic dye*i.e.* methylene blue (MeB), through electrostatic attraction for mutual photodynamic-photothermal therapy (PDT-PTT). Polymer functionalized NGO exhibitedrelatively higher stability than non functionalized NGO in physiological medium. Also

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the complexNGO displayed dual character of being a photothermal material as well as an efficientphotosensitizervehicle. The release behavior of the photosensitizer from NGO surface has been pH-responsive and acidic environment enhanced the release behavior of organic dye considerably. This nanohybrid complex system explains the enhanced uptake of the targeted molecules by cancer cells than non infected cells and in the absence of light, it displayed no major toxicity towards the cells. On the other hand, when irradiated with selective NIR laser lights, it induced significant cell death. Intravenous injection of the complex into tumor bearing mice showed high tumor accumulation, and when the tumors were exposed to NIR lights, it caused total ablation of tumor tissue through the combined action of photodynamic and photothermal effects. This work shows the potential of NGO for synergistic complexion of both phototherapy of malignant area [72].

In 2016, Kulluru *et al.*first investigatedthat NGO exhibits single-photon excitation wavelength dependent photoluminescence in the visible and short NIR region, suitable for *in vivo* multi-color fluorescence imaging. They demonstrated both *in vitro* and *in vivo* experiments to explain that NGO is highly sensitive towards the singlet oxygen formation andhence it can be applied for combined nanomaterial-mediated photodynamic therapeutic (NmPDT) and photothermal therapy (NmPTT). Both NmPDT and NmPTT effectively result the destruction of B16F0 melanoma tumors in mice using ultra-low intense NIR light. The average half-life time of the mice examined by the GO-PEG-folate-mediated NmPDT has been beyond 30 days, which is approximately 2 times longer than that of the mice treated with doxorubicin (17 days). Overall, the experiment highlighted effectiveapplication of NIR using GO-PEG-folate nanocomposite as a theranostic nanomedicine to exert simultaneously *in vivo* fluorescent imaging as well as combined NmPDT and NmPTT effects for clinical cancer treatments [73].

PDT is considered as a promising therapy for cancer, because it is a non-invasive therapy which has many significant advantages such as remote controllability, spatiotemporal selectivity, and repeatability without cumulative toxicity [74].Together PDT and GO represent selective therapy via hyperthermic process toward cancer cells [75].In recent years, GO-based nanomaterials as photothermal sensitizers have attracted attention of researchers due to their wide absorption spectrum of wavelengths from UV to NIR and the ability of converting absorbed light into localized heat by surface plasmon resonance [76]. Furthermore, GO with better biocompatibility and lower cost is beneficial to this application [77].

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PDT mainly involves three components: PS, light source and oxygen. When exposed to the light of specific wavelength, PS is transformed from a ground state (singlet state) into an excited singlet state, then crosses to an excited triplet state. However, most of these PSs cannot satisfy all the characteristics of the ideal PSs due to their low solubility, poor tumor selectivity, restricted absorption wavelength, long treatment period and fast photo bleaching [78-79].In order to overcome these issues, GO has been developed as an ideal carrier of PSs mostly benefiting from its large specific surface area and various surface functional groups. These characteristics enable it to be functionalized with hydrophilic macromolecules and targeting ligands or active agents to improve aqueous solubility and control drugs delivery toward specific types of cancer cells [80].

It is a promising approach to enhance PDT efficacy through sensitizing strategies. Ding *et al.*loaded photosensitizer hypocrellin A (HA) and sensitizer TiO_2 onto GO to increase the ability of producing ROS through mutual sensitization mechanism. *In vitro* cell experiments showed that HA-TiO_2-GO exhibited significantly lower cell survival percent (about 30%) than HA-TiO_2(about 50%) and TiO_2-GO (about 55%), suggesting the potential of HA-TiO_2-GO for improving the efficacy of PDT[81].In addition, in order to enhance the target selectivity of PSs to provide accurate PDT, PSs loaded GO can also be used for activate PDT. For example, Cho*et al.* conjugated photosensitizer chlorine6 (Ce6) on nano-sized GO via a redox-responsive cleavable disulfide bond (GO-SS-Ce6) which was used as an active therapeutic agent for PDT. According to the analysis of the UV/Vis and fluorescence spectroscopy, the fluorescence of Ce6 conjugated onto GO was strongly quenched without reducing agent such as GSH though exposed to the light, which avoided off-target effect caused by non-specific activation and poor target selectivity of PS. They observed that cells treated with GO-SS-Ce6 exhibited strong fluorescence while very slight fluorescence appeared in cells treated with free Ce6, which showed that Ce6 conjugated GO had a better uptake ability than free Ce6 in cancer cells [82]. Particularly, in cancer treatment, GO-based multifunctional nanomaterials have been discovered to integrate imaging and therapeutic in one single platform to realize good therapeutic efficiency with minimized side effects [50, 51].

3.1.4. Janus structured GOs for multivariant (differentially polar dual drugs) Release

Janus structured nano-material is asymmetrically functionalized nano-material where two surfaces of the material are functionalized with two polymers of differential polarity. It is named on Greek God Janus with two faces. One surface of the polymer is grafted with hydrophobic

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polymeric chain whereas the second surface is polymerized with hydrophilic one. In modern drug delivery approaches, this is advantageous when cocktailed drug, loaded onto the GOs with differential polarity alterations. This is particularly exploitable in case of GO because due to its unique structure, GO provides the scope to convert it into anisotropic Janus structure. Due to the polar functional groups such as –OH and –COOH groups, polar polymeric tails can be impregnated on one surface of it. *Vis a vis*, GO has hydrophobic sp^2 and sp^3 carbon atoms too it its structures. Exploiting this, on the other surface of GO, hydrophobic polymer can be attached. This engineering explores the opportunity for attachment of differentially polar drug on opposite surfaces of GO.For example, Khoee *et al.* in 2018 reported that GO has been converted into Janus nanostructure by cross linking one surface with poly caproyl lactone (PCL) as hydrophilic polymer whereas other surface being cross linked with N-isopropyl acrylamide-coacrylamide co-allylamine terpolymer as hydrophobic one [83]. The author could subsequently loaded quercetin (QCR, hydrophobic) and 5-Flurouracil (5-FU, hydrophilic) drug duo onto this Janus structured GO and successfully delivered this against cancer. The second advantage of this nanostructure was that the polymers being temperature sensitive, could efficiently deliver drugs based on the temperature of tumor microenvironment.

The Janus based GO nanomaterials are reported to produce stimuli responsive properties such as pH, Near Infrared Radiation, light or combination of them. For example, Li *et al*. designed Janus chorded mesoporous silica nanoparticles (UCNP-SiO_2-mSiO_2-PMO) containing hydrophilic domain of UCNP-SiO_2-mSiO_2 in contrast of hydrophobic domain of PMO (Figure 1).UCNP is upconversion nanoparticle (UCNP, upconversion nanoparticle = NaGdF4:Yb,Tm@NaGdF4, mSiO_2 = mesoporous silica shell, PMO = periodic mesoporous organosilica). UCNP has been reported for its towering ability to convert near IR to high energy emission such as heat energy thus offering promising opportunity for the scientists to catalyze thermoresponsive release of molecules bound to this. Now, SiO_2 and m$SiCO_2$ provides the Janus structure cage dual compartments to its hydrophilic surface thus aid in accommodation of multiple hydrophilic molecules in a single asymmetric Janus surface.Furthermore, when this kind of Janus nucleus is co-bonded with GO, it provides GO enough storage space of molecules of opposite polarity. This also endows GO efficiently to catapult near IR or heat mediated release of its guest molecules.Exploiting this, efficient co-loading of hydrophobic paclitaxel (PCT) and hydrophilic DOX have been furnished on UCNP-SiO_2-mSiO_2-PMO and subsequently targeted against

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malignant cells. Furthermore, the authors engineered janus nanostructured surface with thermoresponsive 1-tetradecanol and photosensitive azobenzene in order to convert normal drug release to smart release with the aforementioned stimuli. Interestingly, the drugs from the combination revealed more tumoricidal efficiency (~50%) compared to that of their individual formulation (~25%) [84].

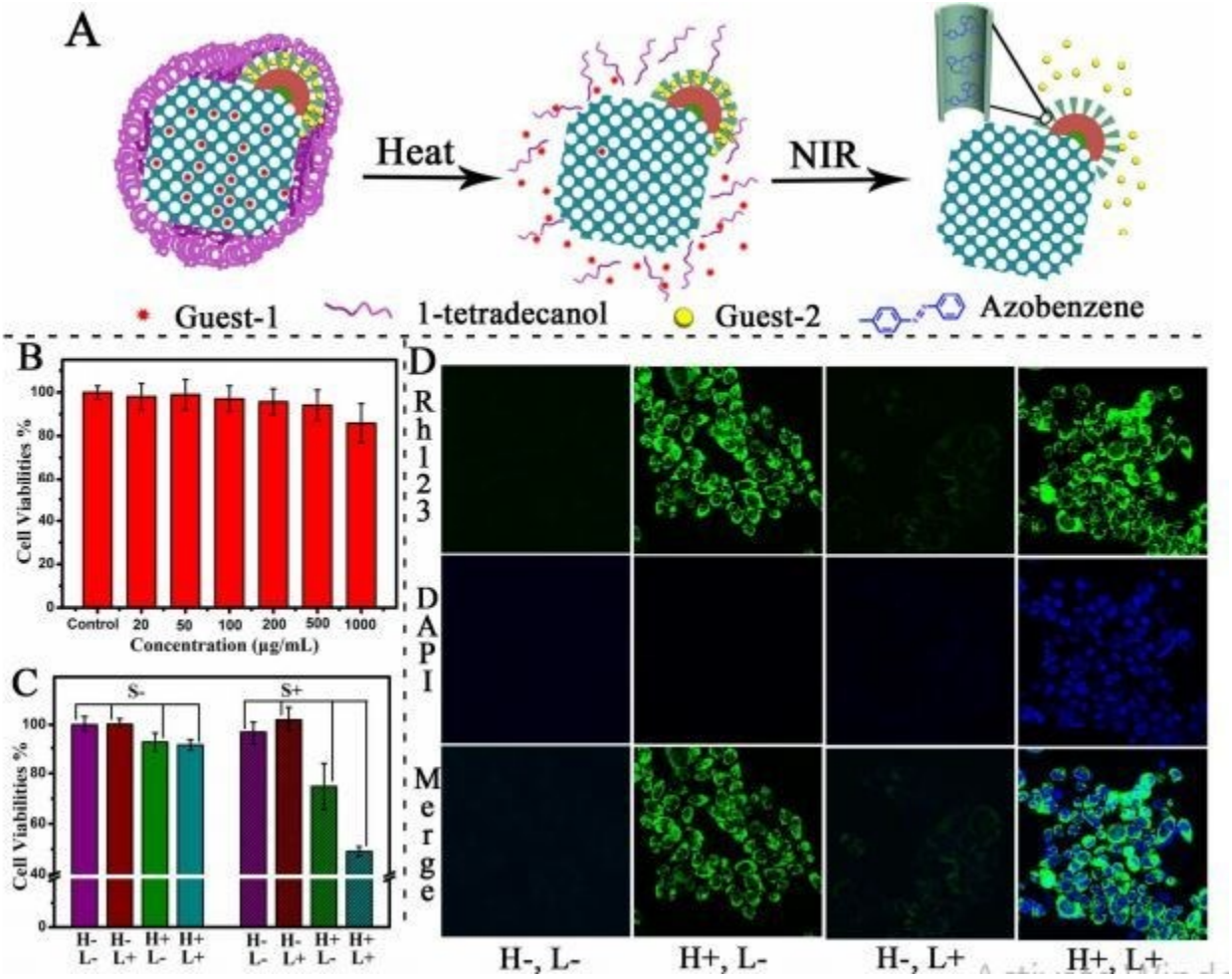

Figure 1. Schematic presentation for dual-control drug release systems by using the dual-compartment mesoporous Janus nanocomposites. (B) MTT cell viability assay of Janus UCNP@SiO2@mSiO2&PMO (C) Cell viabilities of paclitaxel and DOX co-loaded UCNP@SiO2@mSiO2-Azo&PMO-PCM Janus nanocomposites under the heat (H) and NIR light (L) treatment (S means sample). (D) Confocal laser scanning microscopy (CLSM) observations of the HeLa cells after incubation with the Rh123 (green) and DAPI (blue) co-loaded mesoporous Janus nanocomposites with or without heat (H) and NIR light (L) stimuli.Reprinted from [84], Copyright 2014, with permission from American Chemical Society.

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3.2. Disadvantages of GO

Although nanostructured GO-based DDSs due to various therapeutics potential have achieved significant advances to improve the therapeutic efficacy and minimize the adverse side effects of drugs, at the same time the clinical use of drug delivery systems often requires the association of therapeutics and diagnostics to realize personalized patient treatments.

3.2.1. Aggregation in biological media

As an excellent candidate for solution processing, the colloidal stability of GO plays decisiverole for controlling the excellence and performance of the proposed DDSs. Increasing the ionic strength or decreasing the pH of aqueous dispersions of GO results in the coagulation of GO particles and thus affect the colloidal stability [59, 87-88].

Colloidal stability of GO has been extensively studied in aqueous and organic media and it is accomplished that both magnitude and scaling laws for the van der Waals forces are affected considerably by the 2D lattice structure of GO.Also GO exfoliates and shows stable dispersions in polar organic solvents. However, introducinga nonpolar solvents cause colloidal instability at a critical volume fraction. Analyzing the aggregation of GO in mixtures of different nonpolar solvents and N-methyl-2-pyrrolidone, Gudarzi *et al.*proposed that the solvents with dielectric constants less than 24 may not be able to favor stable colloids of GO resulting in aggregation of GO [89]. However, dispersions of GO in polar solvents establishsurprisingly high stability at high concentration of acids and salts. An exciting fact of this study was that aggregation of GO is highly sensitive to pH as it shows abnormal behavior in the presence of acid and base. This evidence can have advance impact on GO storage as GO, self-generates proton during interaction with water [90]. Therefore, slightly basic dispersion of GO can become slightly acidic over time and becomes much more sensitive to ionic impurities.

Meng and his group formulated a multi-step ultracentrifugation-based technique to isolatethe conical arrangement of GO sheets. GO sheets act as large aggregated particles than the expected individual sheet which have a tendency togenerate irreversible coagulation when excessively high polar saltssuch as NaCl and $MgCl_2$are introduced. On the other side, by introducing amphoteric salts such as $AlCl_3$, the GO dispersion remains stable which attributed to the inversion of surface charges of GO sheets. Although there is disadvantage of GO regarding the colloidal stability in different medium due to its aggregation phenomenon, but using the different inorganic salts according to the demand we can overcome by this threat [91].

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3.2.2. Irregular size

GBNs are nothomogeneous, and they vary in number, lateral dimension, surface chemistry, defect density or quality of the individualgraphene sheets and composition or purity and the size of the graphene sheet produced in bulk amount cannot be controlled [92]. To overcome these obstacles, development of a facile method of synthesizing GO is required to potentially control the size and quality for the targeting drug delivery approach. McAllister et al. explained that the lateral size of GO obtained by complete oxidation of graphite particles was independent of the size of the graphite particle, demonstrating that the controlling factor is not the size of graphite particles. However, Zhao *et al.* showed that the controlled oxidation of graphite particles from Hummer's method had a significant effect on the size of GO. High surface area GO sheets were obtained by sonicating GO with controlled oxidation. Further, Li *etal.* recognized that the formation of epoxy groups on graphene sheets could weaken the interaction between the sheets. They explained that the size of the sheets might be reduced with increased oxygen content therein due to the higher density of carbon-oxygen bonds, allowing cracks to form over hydroxy and epoxy coated sites on the graphene sheet during oxidation. Hence previous study demonstrated that the size of the GO could be controlled not only by a balance of edge-to center penetration versus crack propagation rates but also by the degree of graphite oxidation.

3.2.3. Toxicity study of GO

GO is a promising candidate for targeted DDSs and its*in vivo* toxicity, cytotoxicity and uniform genotoxicity attract researchers to considered GO in either biological applications. The toxicity analysis of GO has not developed anynonconflict evidence in current research interest [93]. However, many studies show that GO could cause cell apoptosis, lung granuloma formation, pulmonary edema and platelets aggregation [94]. Furthermore, hemocompatibility is also an importanttoxicity assessment of GO [95]. The hemolytic properties of GO are caused by the strong electrostatic interaction between the GO surface and the lipid bi-layer of the erythrocyte membrane [96]. Many detection methods have suggested that the material properties of GO such as reactive oxygen species, high surface area and charge, unique particle size and functional groups on its surface and edges can affect its toxicity in organism [97].In addition, *in vivo* and *invitro* experiments have shown that GO displayedobservable dose-dependent toxicity [98]. Surface modification is a suitable and effective method to reduce toxicity and improvebiocompatibility of GO by eliminating the fabricationof reactive oxygen species and tuning thestrong hydrophobic

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interaction between GO and organelles [99], which has beenconfirmed by integrating GO with various biocompatile molecules such as polymer macromolecules, serumprotein, antibodies, antigens, genes and others to reducetoxicity [100]. Zhi *et al.*[101]found that tumor necrosis factoralpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) increased significantly inthe presence of GO, leading to strong immunogenicity. While after functionalization of GO with polyvinyl pyrolidone (PVP), the apoptotic process of T- lymphocytes got delayedand improved the anti-phagocytosis aptitude of GO against macrophages. Thus, immunological evaluation has been a key factor for GO *in vivo* compatibility assessment. Furthermore, biocompatible polymer such as PVP, PEG or PVA (Poly Vinyl Alcohol) or macromolecule functionalized GO (as discussed in section 2) is expected to exhibit improved immunological compatibility and reduced toxicity than non functionalized GO. A synopsis of advantages and disvantages of GO has been summarized in Table 1.

4. Drug Targeting Strategies of GO

This section highlights both the physicochemical characteristics of the GO based nanocarriers and the physiological features and microenvironment of site of action to outline what strategies should be undertaken to deliver the molecules of interest specifically to certain targeted site. This segment discusses about the respective properties of carrier and targeted site, describingthe convenient choice between passive and active targeting mechanisms. Herein, we will discuss about the principles for both processesand their correlation with the tumor microenvironment. The previous literature illustrates how the nanocarriers and the enhanced permeationand retention effect (EPR) influence the passive targeting. Whereas the active targeting depends on the ligand-receptor binding, which improves selective accumulation to targeted sites. Here we highlight the passiveand active targeting processes to enable such nanoparticles to be targeted todesired bindings sites efficiently (Figure 2).

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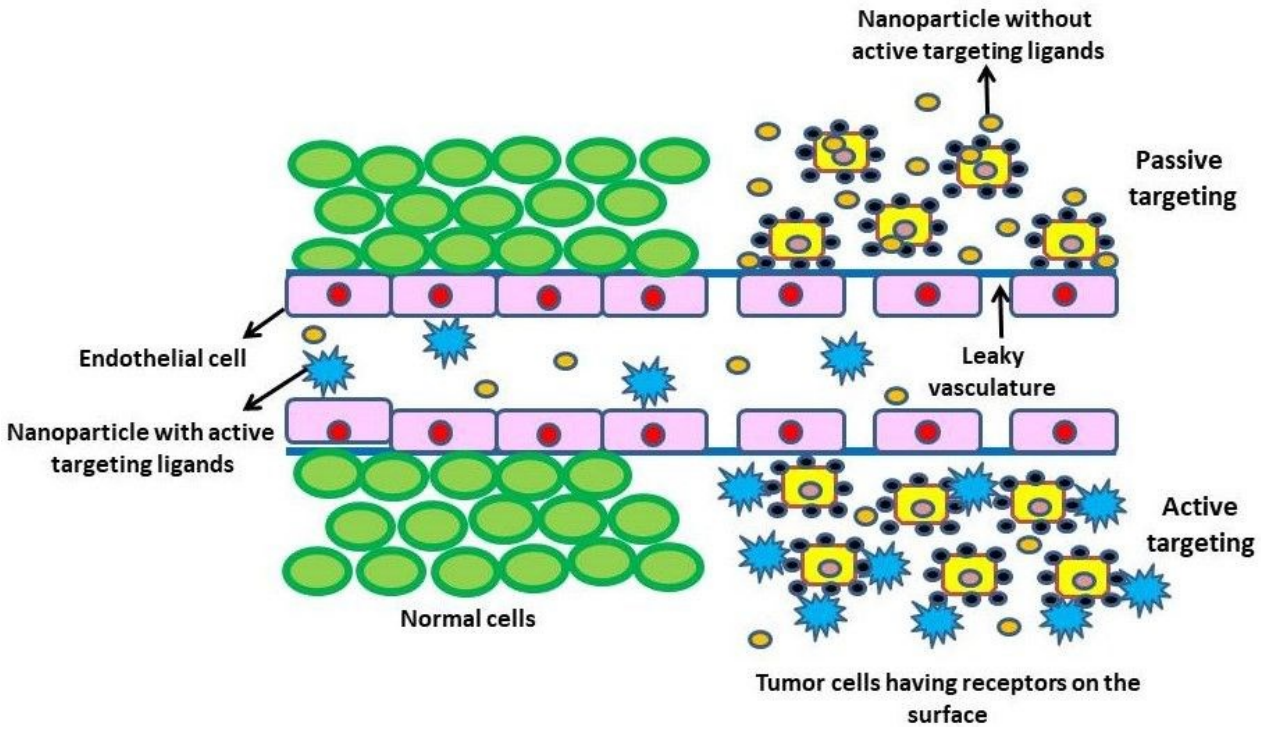

Figure 2. Passive and Active targeting of nanoparticles towards target cells.

4.1. Passive targeting

Passive targeting takes advantage of the unique pathosphysiological characteristics of tumor vessels, enabling nanodrugs to accumulate in tumor tissues. Passive targeting mechanisms are attractive to target drug delivery because this diffusion does not need any extensive functionalization, and these have been exploited using graphene. Passive targeting involves the transportation of nanocarriers through permeable tumor vessel into the tumor cells by means of passive diffusion. In passive diffusion, movement of molecules takes place within the fluids through selective accumulation of drug and nanocarriers follows by the EPR effect [102] which is effectively confirmed by many research groups [103-105].

By the use of additional physical methods the EPR effect can be made more specific in its work process. For example, graphene or GO has high infra-red absorption capacity which allow photothermal effects for localized cell killing through hyperthermia, where the infrared light is applied only to the area being targeted [106,107].

Thus generalized heating through photothermal radiation also increase cell permeability and transfection efficiency of the graphene complexes in the area where the infrared light is applied

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[108-110]. In the same way, prepared graphene-based magnetic nanoparticle composites helps graphene particles to target specifically using generalized magnetic fields [111].

Feng *et al.*[112]used the pH difference in the tumormicroenvironment for modification in their GBNs for efficient cellular uptake. The flakes were loaded with drug DOX and then it is conjugated with PEG and a pH responsive polymer. When neutral or basic environments were introduced the flakes become negatively charged and in an acidic environment their charge becomes positive which creating interaction with the negative cell membrane and subsequent endocytosis. The fluorescence imaging and flow cytometry is also used to increase in the cell death followed by DOX-loading, results in significant improvement in cell uptake and drug delivery in acidic conditions as compared to neutral conditions. Finally, photothermal heating was used to further enhance cancer cell killing, which shows additional improvements on rates of cell death.

4.2. Active Targeting

It is noteworthy that the active targeting is essential for the delivery of drugs, genes and theranostics to the location of interest avoiding the normal tissues and thereby enhances the therapeutic efficiency and limits the side effects. Active targeting is able to significantly increase the quantity of drug delivered to the target cell compared to free drug or passively targeted nanosystems. After accumulation in the tumour region, the drug efficiency can be even increased by the so-called active targeting. This is achieved through the decoration of the nanocarrier surfaces with ligands binding to receptors overexpressed onto the malignant cells. This strategy will improve the affinities of the nanocarriers for the surface of cancer cell and thus enhance the drug penetration. In addition to the EPR effect, active targeting represents another strategy for enhanced tumor uptake, which is generally achieved by conjugating or grafting a nanosystem with affinity ligands to enable the specific recognition of tumor cells [113]. The active targeting directs the nanoparticles towards the tumor sites through ligand-receptor interactions where antigens are over expressed on the tumor surfaces, thus facilitating specific drug release inside the tumor. The targeting ligands conjugated with graphene can be antibodies [114,115], peptides [116], aptamers [117] or small molecules [118]. In a study Liu *et al.* took Transferrin (Tf) an iron-transporting serum glycoprotein, as a ligand to develop Tf-conjugated PEGylated GO for loading and glioma targeting delivery of anticancer drug DOX (Tf-PEG-GO-DOX). Tf-GO shows a high DOX loading capacity. Tf-PEG-GO-DOX displayed greater intracellular delivery efficiency and stronger

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cytotoxicity against C6 glioma cells as compared (PEG-GO-DOX) and free DOX. This comparitive experiment reveals that Tf was essential to glioma targeting *in vitro*. The HPLC assay for DOX concentration in tumor tissue of the brain demonstrated that Tf-PEG-GO-DOX could deliver more drug at tumor site *in vivo*. Hence Tf-PEG-GO-DOX exhibited significantly improved therapeutic efficacy for glioma for both *in vitro* and *in vivo* [119]. Pursuing a similar study a double targeted GO based delivery system has been formulated coupling both FA and Tf onto a Pluronic F68 modified GO where DOX was loaded succesfully (TGFP-DOX) and has been target successfully against SMMC-7721 cancer cell line with improved therapeutic efficacy and lowered toxicity [120]. In another study, hyaluronic acid, with a high affinity for CD44 (hyaluronan) receptor, was conjugated onto GQDs for a targeted system using catechol as a linker. The *in vitro* and *in vivo* results showed significantly enhanced uptake of the hyaluronic acid-conjugated GQD system into cancer cells (A549) [121-124].

A vast number of receptors have been recognized as well as their antibodies were successfully synthesized and investigated *in vitro* and *in vivo*. Inducing very strong ligand/receptor binding, they can serve as potential models to promote active targeting technology. Among the classical examples of ligands, we can cite the FA that specifically binds to the folate receptor as well as present in TME. Folate itself has no toxicity and it is taken up via receptor endocytosis, through different non-specific routes [125].As a targeting ligand it provides a potential approach to cell therapy, and also an approach for receptor-mediated targeting and intracellular drug-targeting. Zhang *et al.* used graphene conjugated with carboxylated and sulfonated folate to target the breast cancer cell line MCF-7 and deliver complex anti-cancer drug DOX and CPT via graphene based nanocarrier [126].Then the flakes were also conjugated with Rhodamine-B and fluorescence microscopy images show that these flakes dispersed evenly in the cells with no definite intracellular localisation. The result shows that folate-conjugated flakes significantly induced greater toxicity than non-targeted flakes at similar level of cytotoxicity as free DOX. These graphene based targeted drug delivery results have been confirmed by other groups with different folate-receptor expressing cell lines such as HeLa and HepG2.

5. Drug Delivery Profiles and Systems

For a specific site-targeting approach, drug delivery profiles and systems must be précised and well organized along with the potential effective technologies. Now a days, site-directed

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targeted drug delivery is a key research to improve the drug efficiency and decrease the side effects of drugs. In this context, GO has emerged as a promising materials as it performs like drug delivery vehicles due to itsbiodegradable, cost-effective easy to fabricate and nonimmunogenic nature[127].Also, GO and its derivatives are able to facilitate systemic pharmacokinetics which are typically concerned with quantitative formulation of both carrier and load (any bioactive compound) withcontrolled release at the specific site.With this specific characteristic of being a carrier, GO based DDS can compete from the drawbacks ofconventional administration of the same drug by enhancing drug solubility, its prolonging duration, and retaining drug bioactivity [128,129]. At the same time owing to the particular characteristic of crossing cell membranes and potential delivery of bio-molecules like proteins, nucleic acids, and peptides into cells, GO promotes the cellular uptake of micro molecules (e.g., anticancer, antibacterial, or antiviral agents) and macromolecules [130]. This section is dedicated on and hasilluminated the potential applications of GO, especially the functionalized GO, as a nanocarrier insuch DDSs.

5.1. Delivery of single drug

The presence of abundant functional groups on the surface and edges, allow GO to conjugate with polymers and other biological moities. Therefore, compared to GO, functionalized GO has reactive groups which can provide the binding sites for some biological molecules such as antibodies, enzymes, nucleic acids to form multifunctional materials, thus functionalized GO provides a wide range of applications rather than pure GO [131].Previously hydrophilic biocompatible polymer PEG coated GO is the most common modification to improve the biocompatibility as it can be functionalized on GO surface via both covalent and non covalent approach [132].Sun and co-workers [133, 134] have revealed PEGylated nano GO (PEG-NGO) sheets that are soluble in buffers and biological media by covalently grafting PEG onto NGO for the first time. Later on, the applications of PEG-NGO in drug delivery and cell imaging are studied comprehensively. For the same, Wu *et al.*[135] reported that PEG-GO also has potential to be an immune modulator for antigen-specific immune responses. They explained that the exposure to PEG-GO significantly attenuated the serum level of ovalbumin specific immunoglobulin E. In addition, PEG-GO augmented the metabolic activity of splenocytes restimulated with OVA but not with the T-cell mitogen concanavalin A. Further Karki *et al.*[34] demonstrated the comparative study of the drug SN-38 with two biocompatible polymer (β-CD) betacyclodextrin and PVP.Figure

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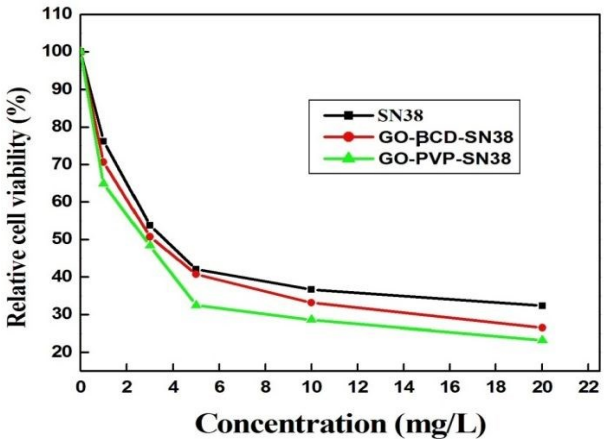
3.clearly convinces the drug targeting with the modified GO as both polymers show enhanced cytotoxicity against the MCF-7 cell.



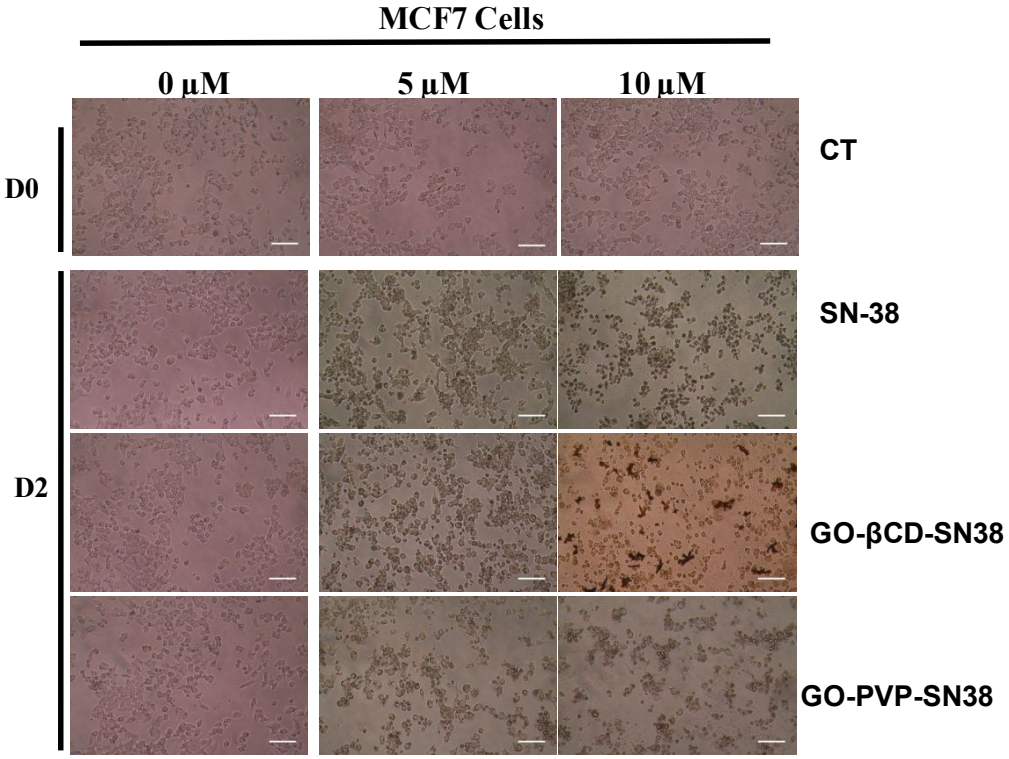


Figure 3. Morphological changes of MCF-7 cells after treatment with control, SN-38, GO-PVP-SN-38 and GO-β-CD -SN-38 and cell viability of MCF-7 cells with different concentrations ofSN-38, GO-PVP-SN-38, and GO-β-CD-SN-38. Reprinted from [34], Copyright 2018, with permission from Elsevier.

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Xu and coworkers [136] discovered, a citrate-stabilized coper sulphide (CuS) nanocrystals via NH_2-terminated aptamer of carcinoembryonic (CEA) antigen to fabricate aptamer-CuS complex *via* carbodiimide-activated coupling(Figure 4). Then, the complex was conjugated with graphene oxide (GO) to form aptamer-CuS/GO conjugates via π-π stacking interactions. Finally, glucosamine (Glu) was loaded into aptamer-CuS/GO conjugates to prepare aptamer-CuS/GO/Glu composites. The composites enabled targeted and pH-sensitive Glu release against embryonic carcinoma. They found that, under near-infrared light irradiation at 980nm, the composites have photothermal-accelerated release of Glu and chemo-photothermal synergistic therapy *in vitro*. Due to combined advantages from tumor biomarker-targeted, pH-sensitive, photothermal-accelerated drug release, as well as chemo- photothermal therapy, the composites could be developed towards multifunctional drug-delivery systems for highly efficient treatment against tumor cells. Thus functionalization of GO nanosheets has created unexpected properties for advanced potential applications.

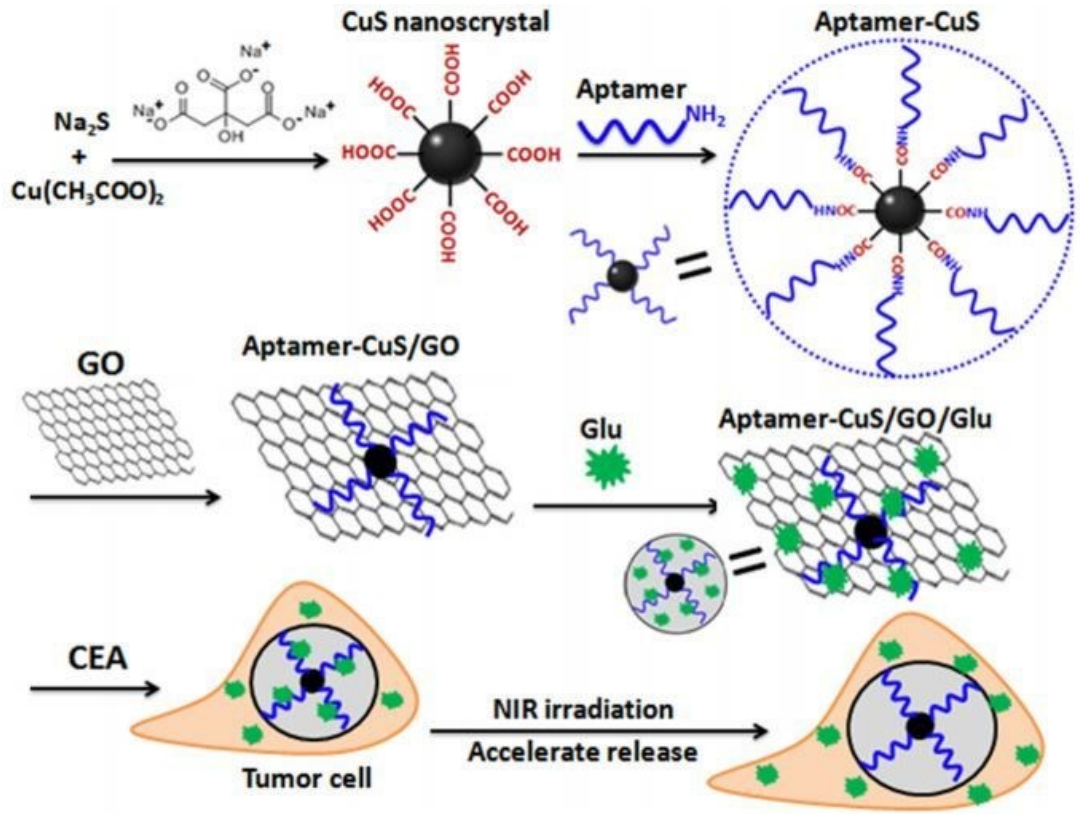

Figure 4. Schematic representation of the systemetic drug release of aptamer-CuS/GO/Glu composites. Reprinted from [136], Copyright 2017, with permission from Elsevier.

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Later on various metal and their respective oxide nanoparticles have also been proven as promising materials for drug delivery with solitary or functionalized GO [137, 138].Afzal *et al.*worked on the zinc oxide nanoparticle (ZnO) doped GO nanosheets using a facile chemical deposition method.The authors found significant increase in the absorption patternof ZnO doped GOin UV-Visible Absorption spectrum,which might have been due to the hydrogen bonding between functional groups ofGOand ZnO.Along with the high absorption spectra,GO doped ZnO had higher drug loading efficiency of about 89% compared to pure ZnO (82%). These results provided an efficient design of the drug delivery system for dissolution enhancement according to the required drug release [139].GO based nanometal composites have been emerged as a promising material for anticancer therapeutics. Owing to their high drug loading capacity, photothermal and synergizing effects, it is very important to exploit them for targeted chemo-thermal cancer therapeutics. Chauhan *et al.*[140]explainedthe targeting behaviorof gold nanoparticles (AuNPs) with FA decorated GO. Here AuNPs composite-folate conjugated GO(FA-GO-AuNPs) nano-platforms were synthesized and found to be NIR sensitivewhich results an intensified release of anticancer drug DOX. Simultaneous delivery of DOX and AuNPs in the cellular vicinity was further enhanced after localized NIR exposure which resulted in significantly improved cancer cell toxicity.Also pharmacokinetics and organ distribution studies were carried out in healthy mice tissues which further estimated the actual biological activity of these nanohybrids. *In vivo* studies showed substantial tumor regression in solid tumor model in Balb/c mice and NIR exposure induced photo-thermal effects further resulted in better tumor management. Yang *et al.* explorednanographene sheets (NGS)with polyethylene glycol (PEG). PEG coated NGS show severalinteresting in vivo behaviors including highly efficient tumor passive targeting and relatively low retention in reticuloendothelial systems. Thus formulated system shows strong optical absorbance of NGS in the NIR region for in vivo photothermal therapy, achievingultraefficient tumor ablation after intravenous administration of NGS [141]. Hence this study simultaneously provided substantial evidences for both*in vitro* and *in vivo* level to support the fact that this metal nanoparticle doped GO composite used as a tumor targeting tool.

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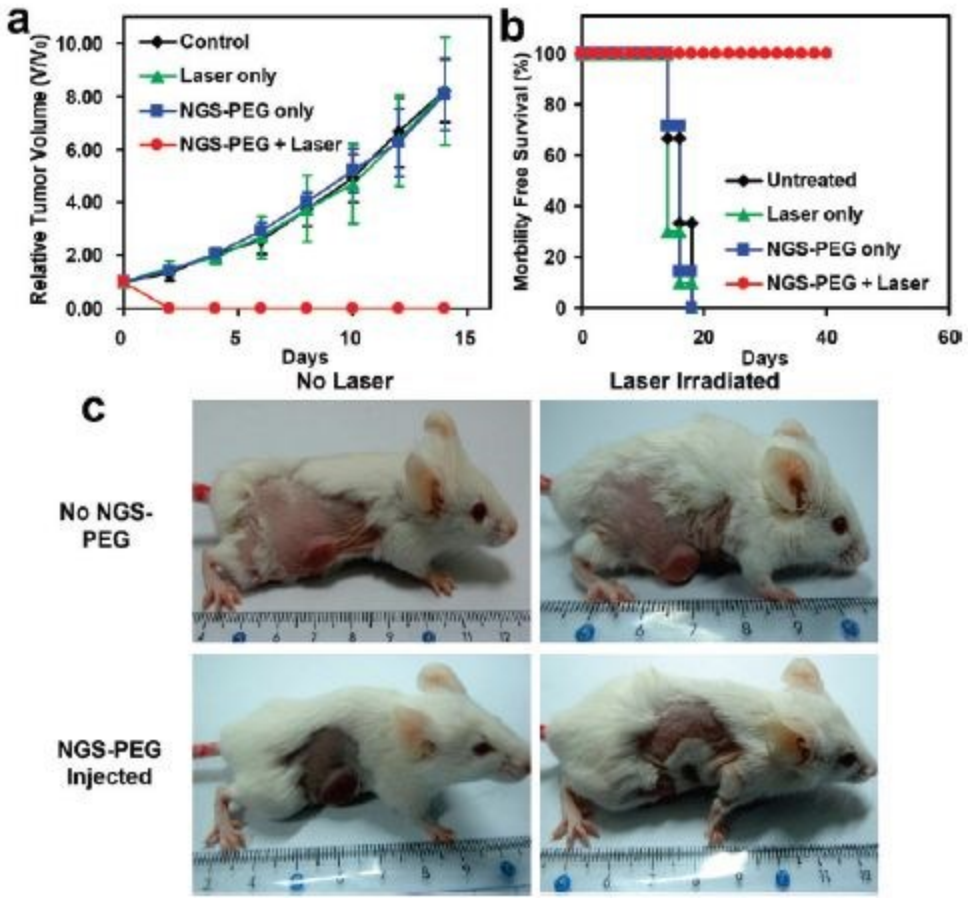

827

828 **Figure 5.** In vivo photothermal therapy study using intravenously injected NGS-PEG. (a) Tumor
829 growth curves of different groups after treatment. (b) Survival curves of mice bearing 4T1 tumor
830 after various treatments indicated. NGS-PEG injected mice after photothermaltherapy (c)
831 Representative photos of tumors on mice after various treatments indicated. Thelaser irradiated
832 tumor on NGS injected mouse was completely destructed. Reprinted from [141], Copyright 2010,
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836

837 However, few studies have been carried out on the applicationof GO as a gene delivery
838 system to treat various diseases caused by genetic disorders. In this regard,Dou and co-workers
839 developed a new type graphene-based miRNA transfection system in which they functionalized
840 graphene oxide with PEI.This complex was used to efficiently load miR-7b plasmid and deliver it
841 into bone marrow macrophages. The entire system was targeted towards cell–cell fusion in bone
842 marrow for inhibiting the formation of mature osteoclasts while preserving beneficial pre-
843 osteoclasts [142].Further, Huang *et al.* [143] reported PEI functionalized GO as the carrier of

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siRNA against C-X-C chemokine receptor type 4 (CXCR4) which was a biomarker for cancer cell metastasis to inhibit the cancer metastasis. Also we can conclude that the same DDSs can provide multisensing approach as our prerequisite.Another approach with the same polymer was done by Zhang *et al.*[144].They demonstrate a new non-viral gene carrier bipolymer-functionalized nanoscale GO (nGO-PEG-PEI) to increase the efficiency of plasmid DNA transfection in Drosophila S2 cells.Small targeting biomolecules are usually minuscule and can be easily digested in the body in a very short period. Therefore, it is critical to have carriers to convey these molecules safely to the desired target site, and graphene and GO are recognized to be an excellent choice for this particular issue. In this context several research groups reported that functionalized GO could effectively deliver molecular beacons (MBs) and aptamers into cells for in situ specific detection of biomolecules [145,146].

Recently antibacterial activity of GO has also received more attention in nanomedicine. Nowadays, several research groups are extremely focused to formulate antimicrobial products with GO. The synergistic effect of GO and silver (Ag) nanoparticle was examined by Ma *et al.*in order to fabricate antimicrobial products. They explained the antibacterial activity of Ag-modified GO materials through GO attachmentonto *E. coli* cell surface that occurred *via* the formation of hydrogen bonds between the lipopolysaccharidesof the bacterial cell and the oxygen-containing functional groups of GO [147]. They observed that GO decreased theintake of nutrition from the surroundings while increasing the interaction between Ag and thebacteria. Ag is also reported to disrupt the bacterial membrane, thereby inhibiting the respiration andreplication of bacteria, which eventually leads to cell death [148]. The Ag-modified GO exertsits antibacterial effect which increases the deposition of bacteria as well as the contact between the cells and Ag-modified GO nanoparticle. Thus Ag–GO is used as a novel antibacterial material, which exhibited a superior antibacterial activity towards *Escherichia coli (E. coli)* due to the synergistic effect of graphene oxide and silver nanoparticles [149].

The combination of carriers with specific ligands that can recognize corresponding receptors on the cancer cell surface or respond to the specific stimulations in microenvironment, has been widely used as an efficient approach to developing DDSs; this DDS strategy is called internal-stimulation targeting DDS [150-154].As the most commonly used magnetic stimuli material for magnetic field-controlled drug-carrier systems,Fe_3O_4 is well known for its supraparamagnetism, low toxicity, and favorable biocompatibility in physiological environments.

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By applying Fe_3O_4, Yang *et al.* [155]first prepared a supraparamagnetic GO- Fe_3O_4 hybrid through chemical precipitation method. This nanohybrid had a high drug loading capacity, high dispersion, and through the external magnetic field, it could move regularly to the action site. The supraparamagnetic property allowed the nanocomposite to easily disperse in solution with negligible magnetic interactions between each composite, avoid magnetic clustering, and deliver drugs with high efficiency and accuracy with the assistance of an external magnetic field.

Turcheniuk in his work explained the role of GO in insulin delivery with the development of insulin formulations that protected the native insulin from degradation under acidic pH in the stomach. For the first time, they showed that a GO based matrix can ensure the stability of insulin at low pH. GO doped with magnetic particle (MP) matrices loaded with insulin and the pH triggered release of the insulin was examined. The loading of insulin on the GO nanomaterials proved to be extremely high at pH < 5.4 with a loading capacity of 100 ± 3% on GO and 88 ±3% on GO–MPdope. The insulin-containing GO matrices were stable at acidic pH, while insulin was released when exposed to basic solutions (pH=9.2). These results suggest that GO based nanomatrices are promising systems for the protection of insulin [156].

As an alternative way to battle against bacterial drug resistance, antibiotic-nanoparticle combinations have been proposed by various research group [157-162]. However, studies on the property of sustained release of drugs with such materials are limited. Developing antibiotic graphene oxide nanocomposites to synergistically enhance the antibacterial activity and prolong its activity is a novel approach to combat antibacterial resistance. Antibacterial activity based nanocomposite for sustained release of Cephalexin (CEF) was explored by Katuwavila in his recent work [163]. The enhancement of antibacterial activity of CEF, with GO in the nanocomposite form, is observed.Encapsulation efficiency of 69% and a loading capacity of 19% are obtained with the optimized formulation of GO-PEG-CEF. *In vitro* CEF release profiles showed an initial burst release followed by a more sustained release over % days with cumulative release of 80%. The half maximal inhibitory concentration (IC_{50}) values have both dose and time dependent antibacterial activity for GO-PEG-CEF against both gram-positive and gram-negative bacteria while pure CEF showed only dosedependent antibacterial activity. The minimum inhibitory concentration values of GO-PEG-CEF have been 7.8 and 3.9 mg/mL against *S. aureus* and *B. cereus*, respectively, while it was 10 mg/mL with pure CEF against both gram-positive bacteria. This confirms the enhanced antibacterial activity of GO-PEG-CEF over pure CEF against

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gram-positive bacteria. These findings therefore confront GO as nanoantibiotic system for effective treatment against bacterial infections.

In a recent study, drug nanocarriers based on mesoporous silica-coated magnetic GO were synthesized for anti-cancer drug delivery of DOX [164]. The addition of mesoporous silica increases the surface area, thus drug loading efficiency, as well as the cellular uptake. Such carriers were designed with a dendrimer-like structure based on supramolecular poly-pseudorotaxane.Theywere commonly used in targeted drug delivery and acted as molecular gates storing the drugs that can be opened by an external stimulus e.g. pH change. Thus the resulting system, being pH-sensitive and positively charged, favored higher colloidal stability and improved cellular uptake.

By means of significance of GO, there is great interest in functionalized GO as a nanocarrier for both *in vitro* and *in vivo* drug delivery. Various works demonstrate the potential of GO derivatives as exciting nanocarriers for the loading and delivery of biological agents.

5.2. Delivery of binomial drugs

Combined therapy with two or more drugs provides a promising strategy through co-delivery of drugs within the same nanoparticle to increased synergistic effects of both the drugs. [165].It has been proved clinically that a variety of drug combinations can induce synergisms among them and prevent from disease reappearance [166].For achieving such therapeutic selectivity for DDSs has been a major obstacle [167] as it requires precise target modulation, which can be discontented by thecompensatory mechanisms available to complex biological systems [168]. To overcome this drawback high drug doses requires over and over again thatresults unwanted side effects in other healthy and uninfected tissues[169,170].

Cytotoxicity can in principle be maximized if drugs with different activities can be delivered simultaneously to the same cell. However, combination therapy with drugs having distinct properties such as solubility generally requires use of multiple carriers or solvents, limiting the likelihood of simultaneous delivery. Ahmed and his group briefly [171]described the *in vivo*application of biodegradable polymersomes for systemic delivery of an anticancer cocktail. These polymer-based shells exploit a thick hydrophobic membrane and an aqueous lumen to efficiently carry both hydrophobic drug paclitaxel and hydrophilic drugs doxorubicin. Polymersomes are long-circulating *in vivo* but also degrade and release their drugs on a time scale

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of about 1 day, by which time the tumors treated here will otherwise have almost doubled in volume. A single systemic injection of the dual drug combination shows a higher maximum tolerated dose than the free drug cocktail and shrinks tumors more effectively and more sustainably than free drug: 50% smaller tumors are seen at 5 days with polymersomes. The polymersomes cause two-fold higher cell death in tumors than free drug and show quantitatively similar increases in maximum tolerated dose and drug accumulation within the tumors.

However, one major challenge of combinatorial therapy is to unify the pharmacokinetics and cellular uptake of various drug molecules, which will allow the precise control of the dosage and scheduling of the multiple drugs, thereby maximizing the combinatorial effects. One of the most popular approaches to overcoming this challenge is to load multiple types of therapeutic agents onto a single drug-delivery vehicle and then concurrently deliver them to the sites of action [170-174]. Several drug-delivery systems, such as polymeric nanoparticles and liposomes, have shown the ability to co-deliver multiple drugs, but fine-controlling the comparative loading yield and release kinetics of the multiple-drug payloads remains an unmet need.

Herein, a combinatorial drug-conjugation strategy is to meet the aforementioned need by covalently conjugating multiple therapeutic agents through hydrolysable linkers to form drug conjugates prior to loading the drugs onto a delivery vehicle. In contrast to loading individual types of drugs separately, this drug-conjugates approach enables multiple drugs to be loaded onto the same drug carrier with a predefined stoichiometric ratio. The cleavable linkers allow the therapeutic activity of the individual drugs to be resumed after the drug conjugates are delivered into the target cells and unloaded from the delivery vehicles. In this regard Aryal and coworkers [175]demonstrated the conjugation of PCT and gemcitabine hydrochloride(GEM) with a stoichiometric ratio of 1:1 via a hydrolyzable ester linker, and have subsequently loaded the drug conjugates into lipidcoated polymeric nanoparticles. The time-dependent kinetics of hydrolysis and cytotoxic effect of the combinatorial drug conjugates against human pancreatic cancer cells are studied. It is shown that the synthesized drug conjugates can be readily encapsulated into a lipid-coated polymeric drug-delivery nanoparticle, which significantly improves the cytotoxicity of the resulting combinatorial drug conjugates against human cancer cells which was comparable to that of the corresponding free PCT andGEM mixtures after the conjugates were hydrolyzed.The cytotoxicity of the drug conjugates was significantlyimproved after their encapsulation into drug-delivery nanoparticles.

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Herein Shen *et al.* presented [176] a novel method of synthesizing ultra-fine graphene oxide (uGO) doped with (MNs composites is presented. This composite is fabricated by combination of a simple and effective chemical deposition with further oxidation of iron ions on a carboxylated uGO base, followed by coating oleic acid on MNs. Two anticancer drugs, camptothecin (CPT) and methotrexate (MTX), are separately bound to uGO sheets and the carboxyl terminals of uGO on the hybrid, forming a superparamagnetic & dual drug-loaded MTX-uGO–COOH-MNs-OA-CPT nanocomposite. The size of the composite is approximately 80 nm by DLS. The entrapment efficiencies of MNs, CPT, and MTX reach approximately 458 mg g^{-1}, 682 mg g^{-1}, and 896 mg g^{-1}, respectively. *In vitro* release and apoptotic assay results show that the nanocomposite can cause the apoptosis and death of HepG2 cells by preferentially releasing drugs to the tumor microenvironment. The tumor inhibitory rate of 73.9% in S-180 sarcoma-bearing Balb/c mice suggests that the combination of nanocomposite-mediated dual drug synergic chemotherapy with photothermal therapy has remarkable therapeutic potential against drug-resistant tumors.

Moreover, FA-conjugated chitosan oligosaccharide (FA-CO) functionalized GO (GO-FACO+) used for delivering DOX and siRNA was prepared for reversal of cancer drug resistance [177]. GO-FACO+ could effectively load DOX and siRNA simultaneously through p-p stacking and electrostatic interaction and specifically deliver toMCF-7 cells. siRNA could silence MDR gene which induced the expression of P-glycoprotein (P-gp) to reduce the efflux of chemotherapy drug DOX in MCF-7 cell. Therefore, this functionalized GO could be used as a novel drug carrier to enhance the effect of chemotherapy.

Recently Tiwari*et al.*[178]reported an excellent carbonaceous nanocarrier modified with polymer functionalities which was biodegradable and biocompatible, poly- vinylpyrrolidone (PVP), to load dual drug combination gefitinib (GEF) as well as QSR and compared with it individual drug therapy. The loading and cell cytotoxicity of both drug conjugated systems (i.e. GO-PVP-GEF/GO-PVP-QSR and GO-PVP-GEF-QSR were investigated in PA-1 ovarian cancer cells. They successfully showed that combined drug system loaded with modified nanocarrier, GO-PVP, is significantly more toxic than individual drug therapy to the PA-1 ovarian cancer cells compared to the toxicity toward IOSE-364 cells (Figure 6.). In another report of Yang and authors [179], GO was functionalized by carboxymethyl chitosan. Afterwards,it was conjugated with fluorescein isothiocyanate/ hyaluronic acid and subsequently anticancer drug doxorubicin was

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loaded onto this conjugate. Similarly Jang and coauthors [180] have demonstrated that the combinatorial system of simvastatin and irinotecan was more effective than their separate systems. Their combination synergistically slowed down colon cancer cell proliferation in HT-29 cells with/without irinotecan resistance. They also showed the various fixed ratio combinations of irinotecan and simvastatin and revealed that 1 : 2 molar ratio shows good potential effect on HT-29 cells with or without irinotecan resistance and clearly suggested that simvastatin may be play advantageous role in the treatment of colon cancers and to triumph over irinotecan-resistance.

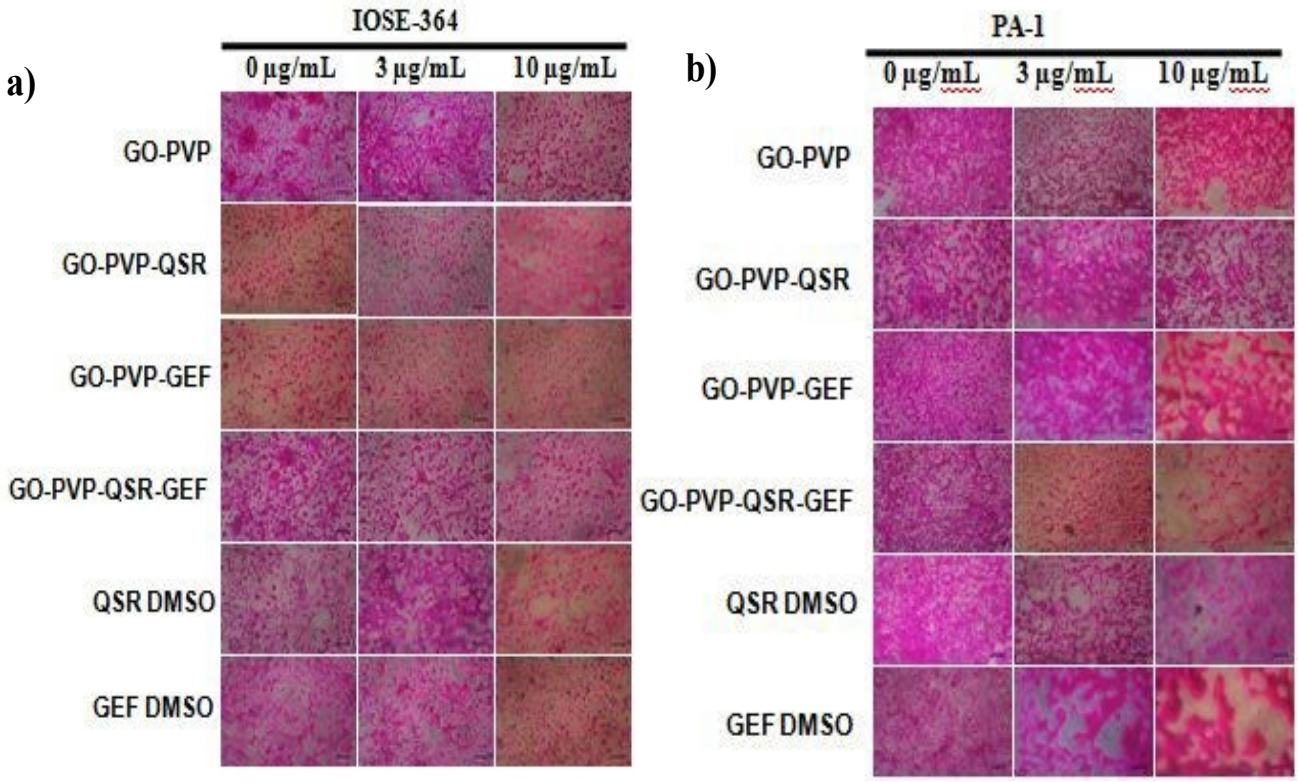

Figure 6. Phase contrast microscopic images showing the morphological changes of (a) IOSE-364, and (b) PA-1 cells after treatment with indicated drug-loaded nanocarriers at 0,1, 3, 5, and 10 ug/ml concentrations. Reprinted from [178], Copyright 2019, with permission from Elsevier.

Moreover, a multifunctional targeted delivery system based on GO that combined dual magnetic and molecular targeting was constructed by Song and co-workers [181].In their study, lactoferrin (Lf) was used as a brain-targeted molecule to modify GO because its ability of crossing the blood brain barrier and combining Lf receptors (LfRs) overexpressed on the glioma cells.

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Fe_3O_4 nanoparticles with magnetic targeting ability can also improve target delivery efficiency of drugs under external guided magnetic field. Doxorubicin hydrochloride was loaded on the Lf-GO-Fe_3O_4 nanocomposites via π-π stacking, and the drug loading capacity achieved 0.8 mg/mg when the DOX concentration was 1 mg/mL and the drugs exhibited pH-dependent release. At pH 5.5, DOX can be rapidly released from GO-Fe_3O_4 and Lf-GO-Fe_3O_4 because of the protonation of DOX under acidic conditions, and the cumulative release rates were 20% and 26% in 72h, respectively. However, at pH 7.4, the cumulative release rate of DOX in both solutions was less than 10% in 72h. C6 glioma cells incubated with GO-Fe_3O_4 and Lf-GO-Fe_3O_4 without drug loading exhibited no appreciable toxicity even within the 250 ug/mL concentration range for 72h, indicating that GO-Fe_3O_4 and Lf-GO-Fe_3O_4 can be a good carrier for drug delivery. Then C6 glioma cells were cultivated with free DOX, GO-Fe_3O_4-DOX and Lf-GO-Fe_3O_4-DOX (the loading ratio of DOX was 1 mg/mg). The IC_{50} of cells treated with GO-Fe_3O_4-DOX and Lf-GO-Fe_3O_4-DOX were found to be 31.30 μg/mL and 23.95 μg/mL, respectively.

Wang *et al*. integrated chitosan onto rGO-SPIONs nanosheets to enhance their balance, solubility and biocompatibility for most cancers chemotherapy and gene remedy [182]. The resulting nanocarrier validated an efficient drug loading ability, pH dependent launch and precise cytotoxicity. DOX was then absorbed at the surface and the ensuing composite turned into encapsulated with a reporter DNA series and green fluorescent protein (GFP) *via* their interplay with the undoubtedly charged chitosan. The transport of each DOX and DNA was studied *in vitro* and in tumor bearing mice and observed through MRI, and the outcomes proven that the very last composite DOX-(chitosan magnetic-G)-GFP-DNA became fantastically dispensed alongside the tumor. Furthermore, toxicity research confirmed that there has been no frame weight loss of the treated mice. Following this pursuit, Zhang *et al.* 2010 [183]co-loaded DOX and CPT for efficient inhibition of cancer cell through topoisomerase intercalation only using nanomolar quantity of CPT. Furthermore, Owonubi 2015 reported that reduced GO (rGO)-acrylamide (AAm) pH responsive nanoconjugate when fabricated in wheat protein isolate based hydrogel, showed remarkable drug loading of drug duo Proguanil and Chloroquine. The entire system interestingly showed antidiabetic activity when targeted *In vivo* against relevant neoglucogenic receptors [184]. Owonubi *et al* 2018 again reported that the same drug combination, when loaded simultaneously on functionalized rGO-whey protein based hydrogel, showed efficient activity as antimalarial with steady state release of both the drugs [185]. In 2019 Bullo *et al.* fabricated GO-PEG-FA (Folic

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acid) based target specific dual drug delivery system where protocatechuic acid and Cholorogenic acids were loaded and successfully delivered as antineoplastic combination [186]. As a beneficial cocktail Pei *et al.* 2017 reported that PEG functionalized GO when loaded with Cis-Pt and DOX, the therapeutic efficacy of the duo in cancer cell was higher than that of the individual candidate [187]. The authors also reported that the toxicity of the drug cocktail was also greatly reduced compared to the solitary ones. It has also been reported that GO or rGO can also be used to target other drug cocktails such as QSR-5 flurouracil or epirubicin-temzolomide to target complex neoplasia such as paediatric brain tumours.

However, the question lies that why dual drug loading onto GO/rGO or rGO-synthetic polymer conjugate improves stability or therapeutic efficiency? Computational studies revealed that much of the co-loading of drugs and synergistic release of the individual depends on drug-carrier or drug linker interaction. Alinejad *et al.* 2019 performed Density Functional Theory (DFT) and Molecular Dynamics (MD) simulation of DOX-CPT co-loaded system onto GO-FA hybrid and reported that the stability of this system has been a major contribution of drug-carrier interaction. They reported that DOX has reinforced stronger interaction with FA than CPT thus establishing the fact that FA has influenced DOX release kinetics in the medium more than CPT. Moreover, the major interaction between DOX/CPT-GO has been π-π stacking, while the interaction between DOX/CPT-FA has been hydrogen bonding (HB) due to heteroatom present onto the drugs and polar hydrogen present within the FA. Thus in this type of system, CPT adsorption is weaker facilitating faster release while DOX showed slower diffusion kinetics than CPT. FA improves both stability and therapeutic safety of the drug molecules [188]. Biomimetic peptides have emerged as a promising alternative tool of organic medicine which often binds with target cells due to high target specificity and produces potential therapeutic activity for their resemblance with actual protein or anti-protein in specific biochemical pathways. Exploiting this, a type of cell apoptosis peptide (KLAKLAK)2(KLA) had been impregnated on GO matrix through a disulfide bond to achieve GO-SS-KLA. Then, anticancer drug doxorubicin (DOX) was charged on the engineered GO through π–π conjugation and hydrogen bonding. Finally, bovine serum albumin (BSA) was used stabilize DOX-GO-SS-KLA/BSA. The authors reported that KLA and DOX were released based on the reductive and pH stimulation inside the cells, respectively, and reaped a synergetic remedy for most cancers [189].A summary of delivery systems has been summarized in Table 2.

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6. Dual Drug Delivery Systems over Single Drug Delivery Systems

Synergistic combinations of two or more agents can overcometoxicity and other side effects associated with high doses ofsingle drugs by countering biological compensation, allowing reduced dosage of each compound or accessing context-specific multitargetmechanisms [190-192].Thus combination of multiple drug components may offer a rational molecular basis in novel chemotherapeutic strategies. In current era, numbers of combinational therapies are in tradition in which the radiotherapy, immunotherapy with chemotherapy, hormone therapy and combination of multiple chemotherapeutic agents, are most common strategies for revolutionizing treatment of many diseases.

Limitations of single drug delivery system towards cancer therapy

Single chemotherapeutic system is limited to act on cancer survival pathways with little response rate and relapse of tumor for which when system treated on the cancer patients, it were found to fail in clinical setting [193]. The most important limiting factors are significant toxicity, multi-drug resistance (MDR) and uninvited side effects with single chemotherapeutic systems when treated in cancer patients. These factors are major aims of significant drug delivery systems. In individual drug delivery system with or without carrier or pro-moiety no synergistic effects are available which enhance targeting, therapeutic activity and helps to reduce side effects.

- Low drug loading
- Not proper release
- *In vivo* variability in single unit drug delivery system.
- Immediate withdrawal of drug is not possible.
- Drug dose manipulation in case of child and elder patients is not possible

Advantages of combinational strategies towards cancer therapy

Unlike individual drug therapy, combination or co-drug therapy not only can alter different signaling pathways but also triumph over toxicity or reduces individual drug-related toxicity and resulting in improved therapeutic effects . Moreover this combination strategy can act as a conqueror to the mechanisms of drug resistance associated with cancer treatment. Multiple drug

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effect/combination index (CI) isobologram analysis can be effective in calculating which drug combination is best therapeutic combination with maximum antitumor efficacy and also an efficient tool to demonstrate that therapeutics are performing synergistically [194]. Figure 7.shows the various advantages of combination drug delivery for cancer therapy. In recent years, the use of combination therapy has been well conventional to the different cancer treatment and its advantages in cancer therapy are pointed below.

- The overall therapeutic advantage of the drugs in co-drug system is found to be superior to the sum of the effects of individual drugs [195].
- Synergistic modulation can offer the opportunity to alter the doses of the parent therapeutic in order to improve efficiencies and reduce drug toxicities [196].
- Enhanced stability due to synergistic effect of partner drug without impairing its properties.
- Major and considerable advantage is to maximize release performance [197-198].
- Modulation of odour: for example, parent drug with a strong unsympathetic odour can be reduced by attaching a co-drug that increase BP (boiling point) so as to interpret it less volatile, thus reducing or removing odour [196].
- Modulation of taste: groups like carboxylic acid groups which bestow the sensation of bitterness can be reduced or modulated [196].
- Anchoring GO with drug along with anticancer peptide can improve the viability, target specificity and synergism of the therapeutic cocktail; in addition can reduce the toxicity of the dosage regimen.

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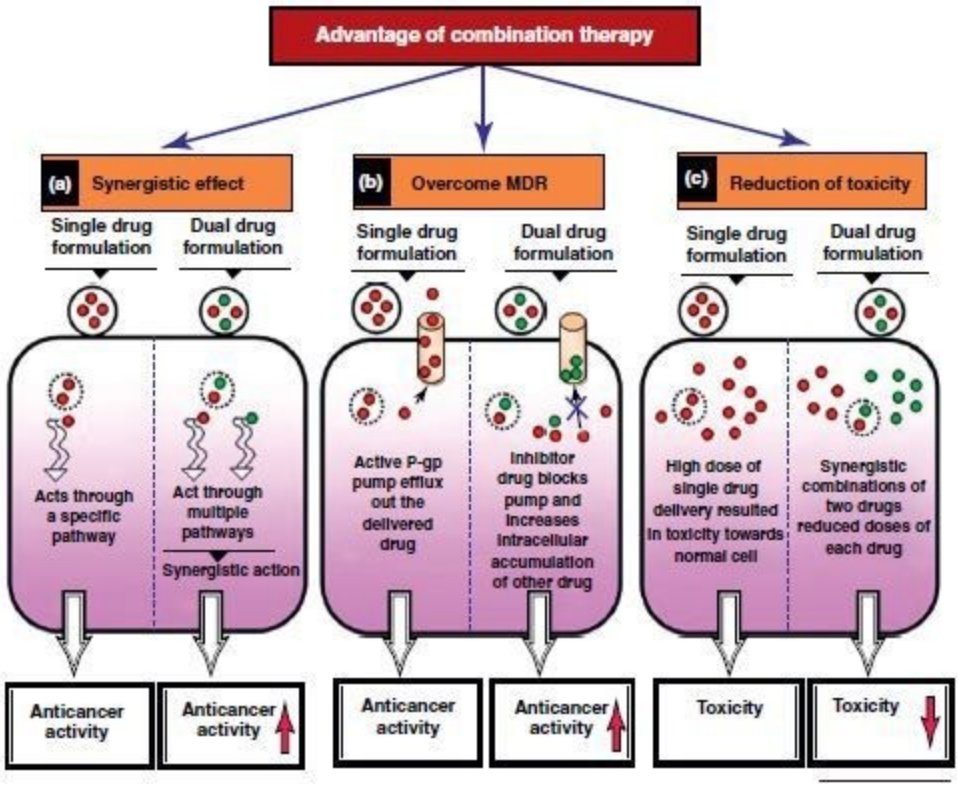

Figure 7. Schematic representation depicting various advantages shown by combination drug delivery for cancer therapy. Reprinted from [194], Copyright 2012, with permission from Elsevier.

7. Role of GO in Bioimaging

To improve the survival rate of patients suffering from cancer, early diagnosis is crucial. For different kinds of cancer imaging techniques, the development of contrast agents and imaging probes is essential. Bioimaging has a crucial role in both research and clinical practice.Owing to their unique physical/ chemical properties, extensive research has been devoted to carbon nanostructure (CNT, graphene, fullerene, and nanodiamond) based platforms for cancer imaging. The surfaces of these carbon nanostructures can be engineered via functionalization to manipulate their physicochemical/biological characteristics [199]. Many reports also explained that functionalized GO nanocomposites were utilized as a contrast agent in various biological imaging such as fluorescence imaging, photoacoustic imaging and magnetic resonance imaging (MRI) [104, 200].It has been observed that most commonly used imaging agents are unable to cross the cell membrane. On the other hand, carbon-based nanostructures (e.g. CNTs) can be helpful to deliver such contrast agents intracellularly for cell tracking with high selectivity, and great potential [201].

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7.1. Optical Imaging

Optical imaging is a passive technique, having superior advantages over other imaging techniques withcomparatively low-cost, high multiplexing capability, relatively high sensitivity and real time imaging [202].This technique provide the detailed images of micro organ's tissues and cells with the help of visible light and photons.Despite of these advantages, optical imaging is highly affected by the poor tissue penetration due to tissue auto fluorescence and light adsorption by macromolecules such as heme groups, proteins etc. To overcome these problems now a days, particularly, NIR wavelength is suitable when it was applied in organisms, because tissues are transparent to light at such a wavelength [203].

GOacquire strong photoluminescence characteristic in NIR region due to the presence of surface and edge defects, suitable band gaps and exceptional photostability [204]. Along with the above GNs have interesting chemical, mechanical, and optical properties which makes them excellent imaging probe in biomedical field [133,140,205].

Optical imaging is studied throughfluorescence imaging [FI], two-photon FL imaging [TPFI], and Raman imaging [RI] in which GBNs are functionalized with various molecular dyes, quantum dots, upconversion nanoparticles etc.

7.1. 1.Fluorescence Imaging

Fluorescence is a phenomenon in which fluorescent probes absorb the quanta of a significant energy, then stimulated from ground state to allowed excited state, where the exited electron stay for short period of ($\sim 10^{-9}$ s) and then come back to its ground state by simultaneously emitting the stored energy in form of photon which results emission of light. Owing to this emitted light FI enables the extensive range of interaction between the molecules in tissues and cells to observe the location and dynamics of gene, protein expression etc [206, 207]. Chauhan*et al.,* reported binding and recognizing of Raji B cells through PEGylated nano GO (NGO) in which NGO has been covalently conjugated by antibody Rituxan (anti-CD20) for selective *in vitro* killing of cancer cell. The photoluminescent property of NGO used in field of bioimaging application as it is NIR active and its π- π stacked structure further provide efficient loading of aromatic anticancer drug DOX [140].Based upon these specific properties of GBNs many researchers explore GBNs in bioimaging field. Recently, *Chetna et al.* in 2019 reported a greener and cost effective route for synthesis of potassium-doped GO using agricultural waste i.e. *Quercus ilex.* This nanomaterial shows low toxicity, good biocompatibility and strong PL properties and

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reflected as an excellent probe for bioimaging. To determine the cytotoxic effect of K-doped GO, they performed Sulphorhodamine B colorimetric assay using tumorigenic ovarian epithelial IOSE-364 cells (Fig. 8.) and the result showed greater than 90% cell viability at a concentration of 30 µg/mL, whereas inhibitory concentration (IC) value was greater than 50 µg/mL. Further, they confirmed its biocompatibility by using IOSE-364 cells, executed *invitro* MTT assay at 24 and 48 h and results indicated around 80% cell viabilities after treatment with K-doped GO for 48 h at highest concentration of 50 µg/mL, indicates its non-toxic nature for this cells. This material shows bright blue fluorescence when incubated with IOSE-364 cells for 4h, followed by washing the images was taken using fluorescence microscope, indicates material is excellent bioimaging probe for detection of IOSE-364 cells [208].

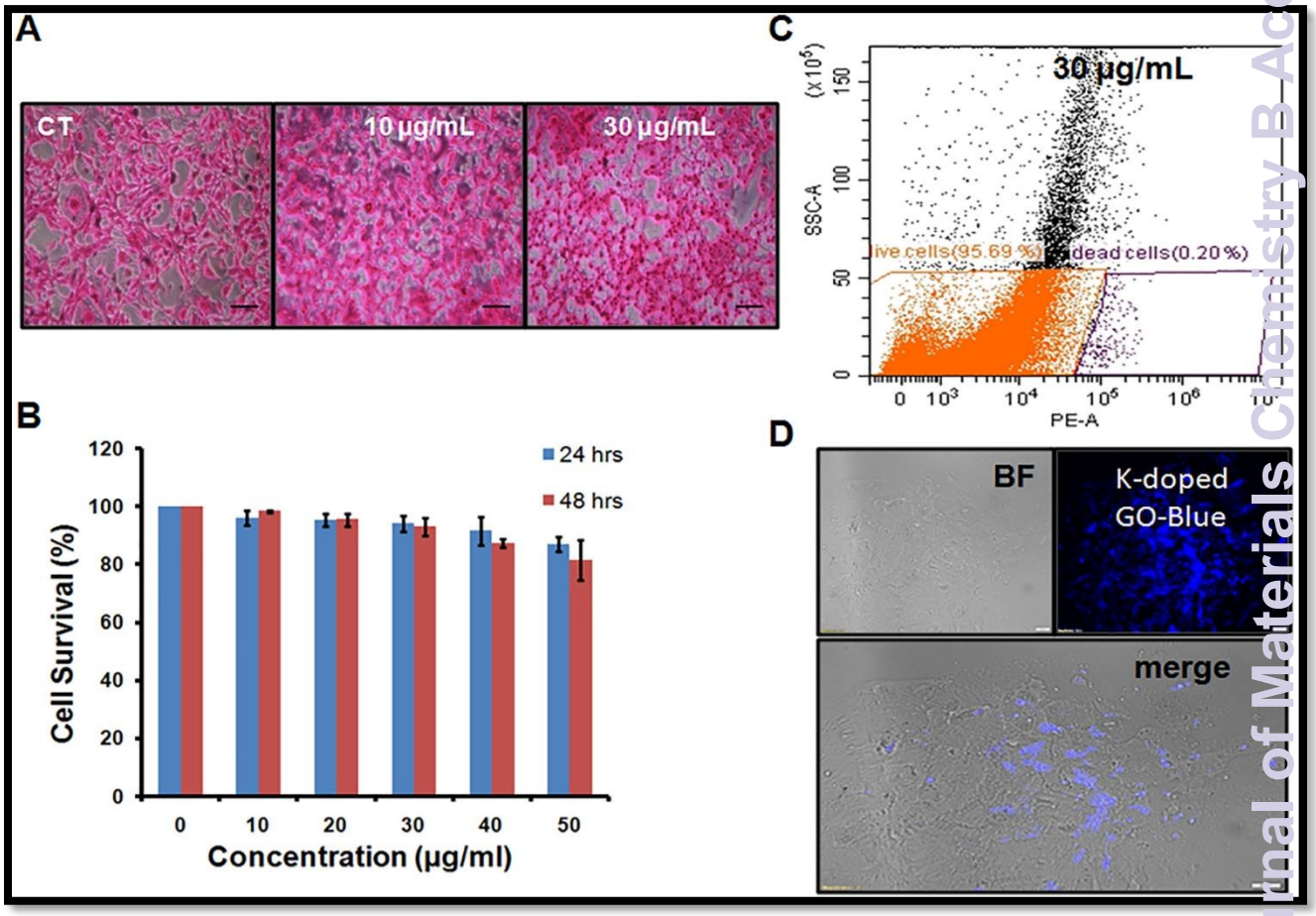

Fig. 8. Biocompatibility and bio-imaging studies of K-doped GO in non-tumorigenic ovarian epithelial IOSE-364 cells, (A) Representative light microscopic images of cells stained with

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Sulphorhodamine B after treatment with different concentrations of K-doped GO for 24 h. (B) Cell viability MTT assay of K-doped GO at different indicated concentrations using IOSE–364 cells at 24 and 48 h. (C) Flow cytometry data showing the live and dead cells populations after PI staining (D) Confocal microscopic images of cells using K-doped GO as fluorescent probe. Reprinted from [208], Copyright 2019, with permission from Elsevier.

In addition to FI, GQDs with quantum captivity and edge effects have possessed optical properties, hence used for bioimaging applications. GQDs have a wide optical absorption, from UV to NIR region, with the strongest peak located in the UV region. The size of the GQDs is the key factor responsiblefor the fluorescence color e.g. Pan*et al*. found GQDs with blue fluorescence[209]while Zhu*et al.* explained green colored GQDs in their experiment [210]. Dong*et al.* functionalized GQDs with dual biocompatible polymers i.e. poly (L-lactide) (PLL) and PEG for intracellular imaging of miRNA along with gene transport to provide improved therapeutic efficacy [211]. The PLL-PEG-decorated-GQDs showed excellent physiological stability withsteady photoluminescence. PLL-PEG-decorated- GQDs wereconjugated to agents targeting miRNA-21 and survive in as a gene vector into Hela cells; andas a result green fluorescence appeared inside the cells when observed under a confocal microscope. Thisallowed improved observation of regulation in gene delivery thorough GQDs. Fascinated with the properties of GQDs further, Wen *et al.* applied the fluorescent property of organosilane and fabricated them with GQDs (producing Si-GQDs) that were further encapsulate into mesoporous hollow silica nano-spheres [212]. The Si-GQDs hybrid nanospheres displayed blue and green colors in the visible range at cellular uptake in HePG2 cells. The results again demonstrate GQDs as promising candidates.

In addition to FI, in 2015 Li*et al.* reported a novel label-free highly sensitive transient imaging technique for the fast imagining and computable layer study of graphene and GO, along with the on time imaging of GO *in vitro* with cells and *ex vivo* in circulating blood, based on the transient absorption process [213].They used modulation range of MHz that effectuallydodged the low-frequency laser noise. With this imaging modality, they were able to attain high-speed as well as real time imaging of GO with quantitative analysis of the intracellular concentration of well-dispersed PEG-GO. This eventually opened up new windows for GO to emerge as a bioimaging markergrounded on the transient absorption imaging process.

7.1. 2. Two-Photon Fluorescence Imaging (TPFI)

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FI imaging has poor tissue penetration which somewhere limits its application in field of bioimaging. To overcome these complications, TPFI is used in field of medical diagnostics [214].TPFI is capable to get the more detailed information regarding the activities of deep located tumor targets. It generates high level of special resolution than FI by using its nonlinear excitation mode and results in photobleaching reduction. Now a days, GBNs are on high demand in TPFI based techniques. Li *et al.* demonstrated, GO as an excellent optical imaging probe due to their strong two-photon luminescence. They labelled the target cells with GO, which resulted in extremelyconfined and low energy therapy. Thus highly efficient GO, after combining with an ultrafast pulsed laser,proved to bepromising material for 3D TPFI [215]. In recent years, fluorescence resonance energy transfer (FRET) has been evolving asa fascinating tool to strategize novel two photon PDT (TP-PDT) bioimaging systems. In this perspective, Sun and his group [216] synthesized a system, in which nitrogen doped GQD was coupled with a photosensitive drug Rose Bengal (RB) and applied it for TP-PDT based FRET (Figure 9).They found that the system N-GQD-RB possessed high photostability as well as biocompatibility. The N-GQD helped to excite the photosensitive drug RB with one or two photon laser. Further TP-PDT was also examined *via* blocking the targeted blood vessels with high precision utilizing small amount of RB and low dose of two photon irradiations.

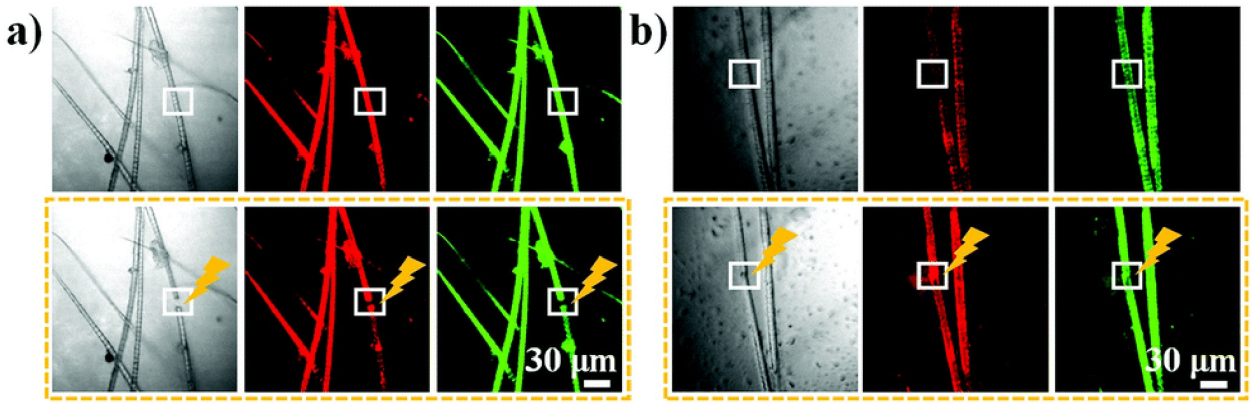

Fig. 9. Pre-irradiation and post-irradiation images of the ear blood vessels of mousetreated with a) N-GQD-RB or b) FITC-dextran and two-photon excitation. Reprinted from [216], Copyright 2018, with permission from John Wiley and Sons.

7.1.3. Raman Imaging

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This is a quantitative and qualitative technique to investigate the inelastic scattering of phonons originated from molecular vibration excitation mode of various molecules and biological samples [214].It works in a nonperturbing and nondestructive manner with high signal to noise ratio and negligible photobleaching. Both graphene and GO exhibit unique intrinsic Raman signals that can be further enhanced by integrating GBNs metal NPs [217]. Wang *et al.,* reported direct reduction of silver (Ag^{+}) on GO to form Ag-GO hybrids which exhibited an outstanding surface-enhanced Raman spectroscopy (SERS) effect [218]and further found it helpful for effective SERS imaging of cancer cells. Hence Raman spectra of GBNs have also been applied for bioimaging. In addition to this,Song*et al.*described the dual metal doped graphene (GP) NPs by developing multi layers of graphene onto the surface of silver (Ag) and copper (Cu) alloy NPs [219].The Ag-Cu-GP have been employed to develop characteristic Raman signals from the graphitic shell, making Ag-Cu-GP an ideal candidate for cell labeling, rapid RI and SERS detection. Further in this series Maand co-workers reportedgold nanoparticle (Au NPs) doped GOas an active imaging probe, (Figure 10) [220]. These GO-Au nanocomposites could be utilized for both intracellular bioimaging markers and DDSs.

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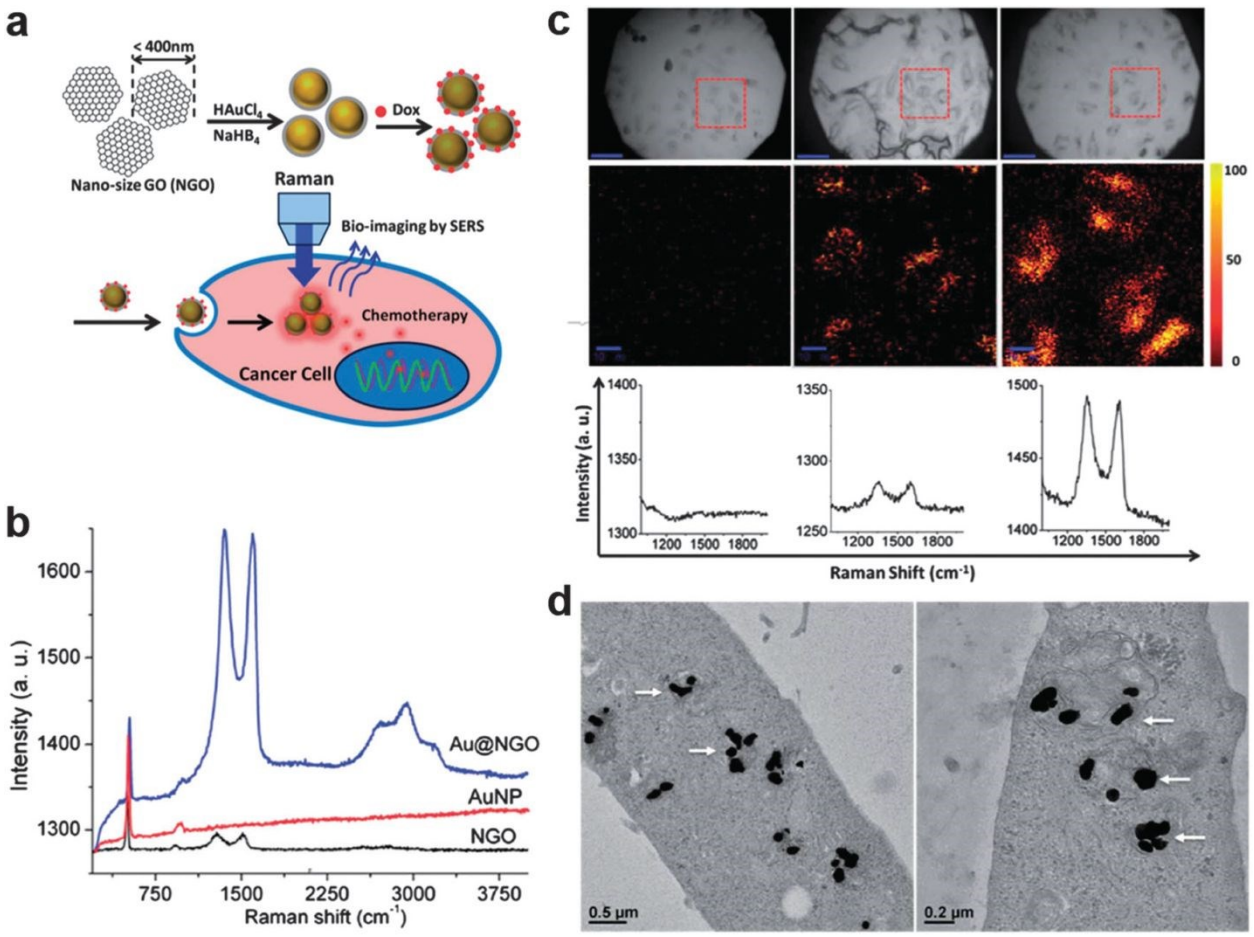

Figure 10. In vitro Raman imaging using the SERS effect. (a) Schematic diagram of Au nanoparticle–GO (Au@NGO) synthesis. (b) Raman spectra of Au@NGOand both bare materials (AuNP and NGO). (c) In vitro Raman imaging of HeLa cells. (d) TEM images of HeLa cells incubated with Au@NGO. Reproduced from ref. [220],with permission from The Royal Society of Chemistry.

7.2. Radionuclide Imaging

Optical imaging generally affect by auto fluorescence of tissues and cannot provide quantitative results, while the excellent properties such as negligible penetration and high sensitivity (~10^{-11}–10^{-12} mol/L)of Radionuclide Imaging (RAI) were extensively applied for labeling the substance *in vivo* and also for the quantitative analysis [221].Radio labeling method mainly contains positron emission tomography (PET) and single-photon emission computed tomography (SPECT) .The main difference between these two imaging method is based on the

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characteristic of radiotracers used. In PET scans positrons produced by a specific dye containing radioactive tracers while SPECT scans is based on gamma rays scanning [222].Hong*et al.* reported GO-PEG labeled with radioactive ^{125}I on the edges of GO. The radio labeling of nGO–PEG with^{64}Cu was explored for active tumor targeting and imaging [223].

Cao *et al.*[224]proposed an ultra-small NGO-PEG (usNGO-PEG) and NGO-PEG, then ^{125}I-radiolabeling was labeled on them for comparative retention of different sizes of NGO in the tumor via single SPECT imaging. After that six-arm branched PEG was modified to both system to compare their biocompatibility. According to longitudinal visualization of non-invasive SPECT imaging, us NGO-PEG showed longer and higher tumor accumulation than NGO-PEG, which was attributed to EPR effect and good passive targeting.

7.3. Magnetic Resonance Imaging (MRI)

Due to the high spatial resolution andnon-invasive feature, magnetic resonance imaging (MRI) has been widely applied in bioimaging field [221]. Owing to the nonselective coordination with biomolecules, paramagnetic metals ions i.e. manganese (Mn) and gadolinium (Gd) show high toxicity [225]. GO with various oxygen containing functional groups can be easily chelated with these toxic ions byclutching the ions between graphene layers, which moderate the toxic effect of these ions [214]. Gizzatov *et al*. 2014 chelated Gd^{3+} ions with carboxyphenylated graphene nanoribbons (GNRs) for enhanced MRI relaxivity [226]. Gd^{3+} ions and GNRs displayed better MRI contrast images in both longitudinal and transverse environment.

Ma and Yang*et al.* developed a model for specific gene-targeting and chemotherapeutic drugs by combining of dendrimer and gadolinium-functionalized NGO (Gd–NGO) [227].Gd–NGO can be controlled by MRI to locate the tumor area and justify quantitatively the concentration of therapeutics within the tumor.Nanosized ferrites spinel possessed supermagnetic properties and emergedas a promising contrasting agent for MRI. But due to small size, they show physiological instability. To overcome this problem, the spinelsrequired support ofdispersible agents as per authors' conclusion. Recently Alazmi *et al.* used GO as a precursor to make composites of cobalt ferrite ($CoFe_2O_4$) whichaffectedgreatly the average size, dispersion and magnetic behaviour of the grafted spinels nanoparticles. Results showed that GO, as a precursor, effectively enhancedthe proton relaxation rate by two folds in the proposed system [228].In addition, the aggregation of Fe_3O_4 NPs often leads to precipitation, causing shortening of circulation time in blood.

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HenceFe$_3$O$_4$ NPs coated ligands have been doped with GO to make it supermagnatic hybrid conjugate (GO/IONP), which are extensively used to shorten the relaxation time of protons. For example, Chena*et al.*[200] developed a GO based system for contrasting agent by forming the aggregates of aminodextran-capped Fe$_3$O$_4$NPs that can grip onto GO sheets to form clusters, allowing enhanced contrast for enhanced MRI compared to the isolated Fe$_3$O$_4$ NPs.

7.4. Photoacoustic Imaging

For diagnostic imaging, Photoacoustic Imaging (PAI) has generally employed due to its specific features of depth imaging and spatial resolution [229]. In PAI, the non ionizing laser pulses of lower energy are applied which causes low energy wave to penetrate deeper into the tissues and provide effective imaging.

As a new diagnosticimaging modality, PAI typically requires the contrast agents (CAs) to further improve their imaging performance. Nanoprobes with strong NIRabsorbances are generally regarded as the desirable CAs for this particular imaging [230].

Based on the excellent NIR-absorbance performance, Patel *et al.* (2013) synthesized microwave-enabled low-oxygen graphene (ME-LOGr) which could be easily dispersed in water and used to generate PA signals with the help of NIR excitation [231].Further, Wang and group prepared Indocyanine green (ICG) dye enhanced GO nanohybrid (ICG-GO).They found ICG–GO exhibited relatively high absorbance in the NIR region and displayed outstanding photothermal properties under NIR irradiation. After complexing the system with folic acid,i*n vitro* experiments revealed that the complex could be used for targeted photothermal cancer cell destruction and for PAI demonstration[232] where this complex was used as a CA.

Among GBNs, especially rGO and GO have cosmic application in field of medical science. Lalwani *et al*., in 2013 reported a comparative study in context of PA effect between oxidized singlewalled GNRs (O-SWGNRs) and oxidized multiwalled GNRs (O-MW-GNRs). They found 5-10 times intense signal for PAIand concludedO-GONRs as promising CAs for PAI [233].

7.5. Computated Tomography

Computated Tomography (CT) is a painless imaging technique through which detailed images of the inner organs are analysed by means of X-rays. In CT the anatomic details of inner parts are subjected under X-rays which provide the detailed images of the objects. With the help of CAs such as GBNs, the diagnosis of renal dysfunction is proposed by Li *et al.* (Figure 11). They

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developed the GO/AgNPs composite, by deposition of AgNPs on the surface of GO, and simultaneously injected with simvastatin to eliminate *in vivo* toxicity. They found GO/AgNPs at even very lower dose (≈0.5 mg per kg bw) could enhance the imaging of CT into the liver, lung, and kidney of mice for a long time period of approximately 1 day. Hence the modified GO has arisen as an imaging agent, for highly sensitive CT [234].

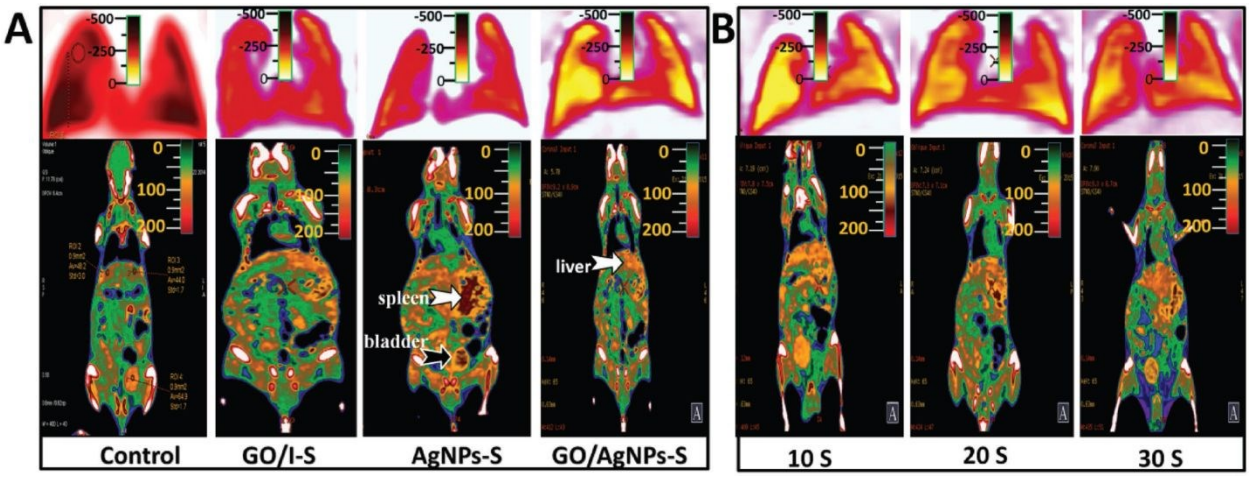

Figure 11.(A) CT imaging of Control, GO/I-S, AgNPs-S, and GO/AgNPs-S in mice in vivo after coinjection with simvastatin for 20 min. Doses of GO/I, AgNPs, and GO/AgNPs are 5 mg per kg bw, simvastatin dose is 20 mg per kg bw. (B) Effect of simvastatin dose on CT imaging of GO/AgNPs, 10 S is 10 mg per bw, 20 S is 20 mg per bw, 30 S is 30 mg per bw, white bone tissue is not included in color bar (1000 HU).Reprinted from [234], Copyright 2017, with permission from John Wiley and Sons.

Recently many literatures are published regarding the applications of GBNs sponges (GBNSs) in biomedical field including antimicrobial activity, bioimaging etc. The applicability and scope of their advantages depends on the post synthesis step in which the metals nanoparticles are introduced in the carbon matrix. Smith *et al.,* reported GBCS based CT after the uptake of silver and iron nanoparticles which provide information of nanoparticles deposition on the internal and external structure of 3D GBNSs[235].

7.6. Multimodal Imaging

Each imaging modality has some specific characteristics and drawbacks which somehow limits their application in bioimaging field. For overcome this issue and gathering the information provided by advantages of individual imaging modality, the idea of integration of several imaging modalities comes in the form of multimodal imaging (MI) [236]. MI provides signals *via* multiple

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imaging techniques simultaneously and gather all required information from various imaging modalities by eliminating the drawbacks generated due to the particular imaging technique [222].

Recently, GBNs are used as building blocks for multimodal imaging due to its multifunctional chemistry and large surface area. Bai*et al.,* group designed a multi modal imaging probe based on iron oxide nano particle (IONP) doped rGO with PEG for FL, PAI, and MRI[157].In 2014, Rong *et al.*reported the GO–PEG loaded with photosensitizer 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-alpha for PDT of tumors [237]. They found GO–PEG–HPPH complex allows dual-modality FL and PET imaging.

In addition to that, Zhang and group developed a system with BaGdF5 NPs directly grown on the surface of GO nanosheets in the presence of PEG. The comparative study between Iohexol (contrast agent) and GO/BaGdF5/PEG sheets reveals that GO/BaGdF5/PEG shows low cytotoxicity, positive magnetic resonance (MR) contrast effect and better X-ray attenuation property than Iohexol, which enables effective dual-modality MR and X-ray CT imaging [238].

8. Challenges and Outlooks

8.1. Prevention of drug from biological degradation

The drugs stability in GO is a big bottleneck in GO based drug delivery *in vivo*. The loading mechanics of drugs in GO nanosheets largely influences the drugs stability and its release which, in turn, depends on the molecular chemistry as well as their inter-matrix interactions. In pursuit, McCallion *et al*. 2016 acknowledged that various drugs can undergo binding with GO nanosheet by multivariate bonding interactions. For example, SN38 binds with GO with π-π interaction while DOX binds with GO largely due to its hydrogen bonding with GO based hydroxyl and carbonyl groups. Thus pH based stimuli govern the release of DOX under specific microenvironment which does not hold true for SN38 [239]. Furthermore, gene targeting has become an efficient tool for drug delivery which involves combining antisense oligonucleotides with drug co-loads. The stability of gene-drug combination has also scaled a different height exploiting GO based interaction cum protection. Lu *et al*. 2010 acknowledged that gene wrapped in GO matrix, either cross linked with molecular beckon or such kind of adaptors, remain stable *in vivo* and deliver payload on specific tissue targets. Linkers such as polyethylene amine (PEI) or polyamidoamine (PAMAM) may serve excellently to acquiesce such kind of gene-GO loading. The authors also discussed that the genes become resistant to the DNAse attack upon such kind of loading [240]. In

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addition, we have mentioned that computational studies have already discovered loading mechanism to improve stability of the drugs. For example, Molecular dynamics simulation on DOX loaded GO showed that functionalizing GO with polymer like PEG improves drug's stability in matrix [241]. Furthermore, stable loading of hydrophobic drug on GO may be achieved using supramolecular GO nanosheets where secondary carrier like beta cyclodextrin may be nested with the former. This augments drug stability due to its interactions with both GO and cyclodextrin. Thus for improving drug stability inside GO matrix, we may suggest the following key scores:

1) Choice of suitable polymer for functionalizing GO depending upon the drug molecule or combination.

2) Choice of Janus structure discussed earlier, if required, for concomitant loading of hydrophilic and hydrophobic drugs

3) Choice of linkers such as PEI or PAMAM for improving stability of loaded gene on GO matrix.

4). Consideration of secondary carrier such as cyclodxtrin nested in GO for improving stability and release of drugs.

8.2. Effective targeting

In last a few years, a lot of engineering approaches have been reported to formulate target specific drug delivery system, still the complexity of target tissue, disease specific gene or protein expression, system metabolomics, microenvironment of the target cell have been laying spectrum of challenges in designing target specific drug delivery. One of the potential outlooks of these challenges is to endow the delivery system with cDNA or siRNA whose shorter version has been the application of aptamers. Now, the stability of cDNA or siRNA inside body is another challenge to the scientists, so a balance between between target specificity and chemical modification of the cDNA or siRNA is required in the outlook to solve this challenge. RGD or other peptidomimetics based targeting has also been another alternative to this approach where surface of the GO is manipulated with such peptides to lead the delivery to the target. Cellular uptake and cleavage by proteolytic enzymes in blood or other body fluids are major challenges of using peptides in drug delivery system. Furthermore, using folic acid (FA) or other epitopes have evolved as potential outlooks herein, challenges have still remained to perturb myriads of complex cases *in vivo* through target specific drug delivery which is also true for our GO based system. For example, as per our previous discussion, Hyaluronic acid (HA) solitarily can be coated over GO nanodevice to target this towards cancerous cells. To add to this, HA conjugated with RGD peptide (Arg-Gly-Asp)

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could be effectively used to target DOX loaded GO towards cancer endosomes [242].Thus effective targeting of GO based drug delivery has been being studied through various avenues and reasonable success of these routes has promised solution of off-target accosting of such devices in physiological system.

8.3. Cost effectiveness

Cost effectiveness is always a challenge for engineered or smart drug delivery system (DDS) over the decades. The use of natural, synthetic or co-polymers; use of primary nanocarriers, operational or manufacturing cost have been some of the primary cost influencing factors which are unique for the process and product. Choice of drug depends on the target disease whereas distribution, marketing and such others are inevitable constant parameters which levy a fixed percentage of cost into the final product pricing. Thus, the first stated factors usually help to tune the cost of an engineered DDS. For fabrication of a nanodevice, the choice of nanomaterial and its procurement cost is a prime important factor. In this context, GBNs or GO has already been acknowledged by various authors as cheaper raw materials compared to other nano fabricating materials [35]. Recently Pandey *et al* 2019 has devised a new technology to synthesize graphene nanosheets from waste plastic in bulk scale which has showed a new way to produce graphene in considerably lower cost compared to other technologies [243]. The authors calculated the cost of produced graphene with all expense parameters and compared with that of commercially available graphene sheets in the market. While 1 gram of commercially available graphene costs around 100 USD-200 USD, the cost of 1 gram of graphene obtained from waste plastic recycling has projected around 1 USD [244]. The technology transfer is under process, and once finalized, may revolutionalize the graphene based industries round the globe by bringing down the production cost of raw graphene significantly.

9. Conclusion and future prospective

Hence from the entire review, it can be concluded that graphene oxide either its own or in reduced form can be an excellent carrier for fabricating various biomedical devices. Although GO due to its high carbonaceous structure, has significant toxicity *in vivo*, the toxicity is often minimized by covalently crosslinking it with biocompatible cosolvents such as PEG, PVA or PVP). The GO-PEG or GO-PVA/PVP system could be efficiently utilized as functionalized

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nanocarrier for further biomedical applications. Graphene oxide can be efficiently designed with various polymer and co-polymers (such as PAm, PNIPAAm, PMA) to deliver single drugs where depending on polymers surface chemistry, its π-electron cloud, ring opening reaction, electron donating or accepting capacity, various drugs of differential polarity can be loaded onto GO-polymer conjugate. While delivered *in vitro* or *in vivo*, the drug-carrier shows excellent stability, steady drug release and improved biocompatibility. This type of single drug delivery has been widely used in cancer therapy on trial basis, where the actual formulation of GO based anticancer therapy is yet to come. In addition, when used for administering combination drugs such as DOX-CPT, DOX-5FU, Epirubicin-Temozolomide, QSR-GEF, the synergistic action of the component drugs have been revealed. Although this type of dual drug loading is challenging and requires lots of surface engineering of GO-Polymer conjugate, the final dual drug-GO sandwich is one promising potential for tackling critical diseases since synergistic action of drugs herein improves therapeutic potential of them in many folds. To further engineer its release pattern, cell permeability and stability, various engineered GO micro devices such as GO nanosheets (GON), GO SPION nanosheets, GQDs, ZnO doped GO, GO-hyaluronic acid combination with peptide modification have been fabricated. Janus structured nano device based on GO and especially surface engineered GO (such as using SI-RAFT technology) have been blessings to potentiate combination drug loading of various polarity or to improve its biocompatibility. Stimuli responsive GO based drug delivery has been another promising toolkit of modern days biomedical sciences. Since GO or rGO can be surface engineered with temperature, pH or photosensitive polymers or dyes; subsequent thermoresponsive, pH responsive or photosensitive (leading to PDT) GO or rGO nanoboats have been fabricated which have shown promising drug release under special microenvironment inside body with controlled irradiation with laser or NIR in cases. The surface of GO can be further wrapped with various cell recognition genes or peptides which can redirect the GO-drug micromotor towards desired site, attach onto this and subsequently infiltrate within the cell or endosome. This finally leads to reduced toxicity and improved output of the candidate drug under the specific disease condition. Using the electrical property of graphene, its high conductivity and high energy electron emission, GQDs have been positively used in MRI imaging or photon based imaging. Using its ability to couple with other nanoparticles such as Ag or Fe_3O_4 nanoparticle, which leads to formation of supramagnetic nanohybrid, has facilitated MRI imaging in biomedical sciences. The GO has been further coupled with radioisotopes, fluorescent dyes to

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devise radionucleotide based or fluorescent based imaging. Recently FRET based imaging devices on GO have also been brought into limelight which has opened the gateway of finely tuned bioimaging operations under highly sensitive microenvironment inside body.

Graphene oxide has a huge potential in futuristic applications of biomedical field. The first application we can suggest is its antibacterial and antiviral potential. Due to its unique polygonal structure of catenated carbon atoms, GO possess the ability to damage cell membrane of microbes. Plus, Graphene oxide produces cluster of free radicals which may damage the microbial cell membrane as well as other organelles [245,246].This encompasses huge potential in devising non-antibiotic antimicrobial that may help in combating antibiotic resistance all over the globe.

Another future application of GBNs is heat-therapy which achieved by raising the surface temperature upon photon irradiation through tactful modification of the same. For example, Jiang *et al* 2019 reported that bacterial cellulosed entrapped graphene oxide can be used as antibacterial candidate, which upon reduction with chemical treatment forms nanocomposite of reduced graphene oxide (rGO) in cellulosic membrane. This nanocomposite upon irradiation with normal light undergoes thermal activation sensed by its temperature elevation. In Jiang's work this technology has been used to fabricate biomembrane which killed microbe and deterred biofouling [247]. We propose that this technology can be implemented *in vivo* to treat resistant microbe or acrid tumours (malignant or benign) which are otherwise hard to treat by simple chemical or peptide based therapies. In addition Chen *et al* 2019 showed that, the surface –COOH groups, if functionalized with sulfhydryl (-SH) groups of L-cysteine, becomes photothermally active. The authors reported that when challenged against microbes, this nanodevice efficiently invaded the microbial cells by first tearing their cell membranes with its knife edged polycarbonaceous surface, later subjected then to photothermal ablation [248]. This therapeutic supremacy may be utilized against various kinds of tedious infections such as pneumonia, gonorrhea, tuberculosis, blood poisoning and food borne diseases as suggested by the authors.

Next futuristic application of Graphene based polymer is application of the same in stimuli responsive form thus making it more target specific as well more potent in executing therapeutic payloads. As discussed earlier, pH responsive and temperature sensitive GBN has been successfully designed with selective polymer coupling and allowing their ring opening mechanism under the particular stimuli. Even electroresponsive graphene based nanomaterial has been undertaken as a drug carrier where pulsatile release of drugs under various voltages have shown

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great promise [249]. Furthermore, tagging the GBNs with photosensitizing eletrophores such as Ce6 or other porphyrin derivatives, PDT has been evolved. We suggest that this kind of therapy in future may be used to treat various form of malignant tumours or microbial endosome *in vivo* which in otherwise, be extremely difficult to treat. In this era of Covid-19 pandemic, where mutated microbe is endowed with indomitable penetration power in respiratory organs, this kind of therapy may be an alternative to virostatic or virocidal agents in respective organs.

Surface engineered Graphene oxide with oligonucleotides can also be used as successful candidate to design target specific therapy in future. As described earlier, aptamer functionalized Graphene oxide was successfully used to deliver drug molecules in target specific fashion. The abundant –COOH groups on GO help in aptamer conjugation through cross linking of its –NH_2groups with former's carboxyl terminal. We also propose that this target specific therapy can be effectively utilized to deliver any kind drug molecules to desired targets.

Another promising application of using GBNs in biomedical field lies in strategic and smart drug loading within its matrices. For example, GO so far has been used mostly to incorporate hydrophobic drug such as DOX in solitary fashion. However, one of the most interesting and potential approach of loading combination drugs is application of Janus based nanostructure over GBNs which has already been discussed in this review. Since two drug molecules often have differential polarity, simultaneous loading of both the drugs on symmetrically functionalized GBNs have often faced with adverse interactions. This kind of orthodox loading has led to either poor loading of drugs or sub-optimum release of drug particles in the solution. However, in Janus structure GBNs where two surfaces of GO matrix have been functionalized with two differently polar polymers, loading of hydrophilic-hydrophobic drug combination has reached new efficiency with potential release kinetics as described earlier. The application of Janus based GBNs in drug delivery has started in recent past and holds great promise in vast pool of biomedical sciences in future.

Apart from Janus nanostructure, how could the drug loading and release be improved in future GBNs applications? In this pursuit, Molecular dynamics (MD) simulation has been a popular approach to delineate drug-polymer dynamics and thus design the future drug-nano conjugate for improved stability and bioavailability. In a few MD simulation studies related to GO and its cross linking polymer and drugs like DOX has revealed that functionalization GO with PEG has a great impact on stability and release of DOX from the polymer matrix [241]. In other MD simulation

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studies, it has also been revealed that the diameter of the guest molecules together with dimension of GO nanosheets greatly influences binding energy, molecular cross-walking of the candidates, diffusion and release of them in liquid mediums. Not only that but also it is the molecular structure and chemistry of the candidates which determine their best loading mode on GO through either single or double surfaces of it [250]. Although it has been explored only on a few molecules and functional polymer such as PEG, there is a huge scope to explore such kind of molecular dynamics between other kinds of drug molecules and various functional polymers such as PVA, PVP, PAm. NIPAAm, DDMAT. This would help to design properly functionalized GBNs, choosing right drug molecules, resulting in better loading of drugs with improved release kinetics both *in vitro* and *in vivo*.

The next futuristic application of GBN is to build supramolecular GO using nested GO structure using cross linking with inclusion complexes with cyclodextrins (CD). Since cyclodextrins have excellent capacity to accommodate hydrophobic molecules in its inner core using hydrogen bonding with its multiple functional –OH groups, tethering GO with such channel lattice or clathrates may revolutionize drug delivery in future application. Since β-CDand Hydroxypropyl propyl beta cyclodextrin (HP-β-CD) have improved aqueous solubilities, drug release from both these matrices are steady and efficient in biological fluids. Exploiting this property, poor drug release from functionalized GO which is largely hydrophobic and hold the drug with π-π interactions, could be solved. The supramolecular β-CD-GO nanocage would have the amalgamated potential of efficient targeting the drug within the cell, trigger its stimuli responsive performance and release the molecule efficiently at the end. One of such attempt has already been discussed by Cruz and Coworker at 2019.

Genetherapy has been a promising target now a days where genes are delivered within the recipient cell either to correct a malfunctioning gene or to make the gene act as a therapeutic candidate within the cell. Exploiting this, attempts have been made to co-administer gene and drug together in order to potentiate each other's action. As discussed earlier in our review, siRNA or plasmid DNA protected gene therapy *via* GO based carrier can efficiently deliver the genes to the target cell. It has been acknowledged that suitable grafting of GO by PEI or polyamidoamine (PAMAM) together with GO-PEG or GO-chitosan can successfully deliver gene towards target cell. We are hopeful that this gene delivery can be very beneficial in future to boost administration of gene-drug cocktail to various diseases such as cancer, AIDS, different viral infections, various

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gene associated disorders such as Glucose-6-phasphate deficiency, haemolytic anemia, autoimmune disorders, Huntington's disease and many others.

Due to its unique chemistry and energy potential GO or its derivative can effectively cross blood brain barrier. As reported in our earlier discussion, using this potential epirubicin and temzolomide combination has been effectively targeted to treat paediatric brain tumour. Thus, there is a great future potential of treating various brain related disorders using functionalized GO encapsulating drug-drug or drug gene combination.

Although acknowledged in review, graphene oxide has been mainly studied so far against cancer and tumours in medical science field, Owonubi *et al* in 2015 and 2018 studied the effect of GO with binomial drug cocktail against other diseases such as diabetes and malaria. Following this, Ge *et al* 2019 reported that bionomially coated GO, one surface with docetaxel and other surface with anticoagulant heparin, was successfully used in Cardiovascular stent which showed no noticeable aggregation or thrombosis when implanted inside zebrafish body [251]. In addition, various scientists are trying to envisage the pulmonary application of GO by studying its toxicity and biotransformation within alveolar fluids, subsequently the effect of biotransformation on the drug delivery pattern of GO [252]. Another interesting observation has been revealed by Afzali and coworkers that GO could increase the number of Kupffer cells in liver when tested in mouse embryo [253]. Thus it is very encouraging to observe GO applications being studied in other biomedical fields apart from cancer with a strategic effort to down-regulate its toxicity. These efforts can be further channelized in future to utilize GO against various other diseases such as atherosclerosis, liver disfunction, cardiovascular diseases, coronary thrombosis, bone regeneration, osteoporosis, Type I and II diabetes mellitus and many others.

In bioimaging field, plethora of improved technology have been being tried with GO and as in the review, all of them have been reported to provide image of target organ or organelles successfully. Radionucleotide based imaging, photoacoustic imaging, two photon based imaging, fluorescence based imaging, MRI, CT to FRET based imaging all have provided scientists wider windows to capture snapshots of various organs or cell stages at different diseased conditions. We hope that exploiting various unique properties of GO as discussed above, the imaging science can galore to a different benchmark with holograms of captivating various life patterns with or without abnormalities. This would aid scientists propose various dogmas, technicalities and solutions of biological sciences which world has not witnessed so far.

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Overall, we would like to be optimistic in utilizing GO and its multifaceted grafted systems for multimodal applications in biomedical field which has yet to be explored in coming eras. As a part of that, in this review we have tried to revisit some recent advancements of it involving polynomial drug delivery from single GO based switches, its current status and mechanisms, advancements in grafting technology to manufacture such smart switches and various alleys of bioimaging sciences that has progressed to ultrasensitivity in capturing different microcosms of life. We have also discussed the unfathomable opportunities to explore GO based nanodevices in biomedical applications which may scale a different height in drug delivery or bioimaging science. We hope, our small attempt of this review would help scientists to plan, dive and progress in this ocean in future days.

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Table 1. Summary of advantages and disadvantages of GO

Advantages	Disadvantages
Pi- Conjugated Structure: ➢ GO and its derivatives provide the high π electrons density on itheir basal plane which favor the addition of different therapeutics in their surface [1-4].	**Hydrophobic Nature:** ➢ Primitive graphene is hydrophobic in nature. ➢ Surfactants or stabilizing agents are required for making stable suspension or preventing from agglomeration in water or biological fluids [13].
Polar basal plane: ➢ GO makes stable suspension in water due to the presence of polar basal plane and negative charge (carboxylate group) on edge site [9-12]. ➢ GO acts as an amphiphilic molecule and can be used as surfactant to stabilize hydrophobic species in water [13].	**Aggregation in Biological Media:** ➢ GO has poor colloidal stability in buffered saline and cell cultured medium. ➢ GO exfoliates and shows stable dispersions in polar organic solvents [67]. ➢ Functionalization can be utilized to improve the stability of these species [68].
Optical Properties: ➢ GO has excellent optical properties. ➢ Single layer of GO transmits 97.7% of incident light. Their high light transmittance and charge	**Toxicity:** ➢ GO produces singlet oxygen species which destroy or affect the function of normal tissues [52].

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mobility tendency and photoluminescence property make graphene a valuable material for MRI [26-28]. ➢ GO has ability to manage separation or recombination of surface electrons can be fully utilized in the development of Bio- imaging applications [29-30].	➢ High zeta potential of GO damges cells. On the other hand if electronic charge is too high then toxicity is not affects strongly [53]. ➢ *In vivo* and *in vitro* experiments have shown that GO displayed observable dose-dependent toxicity [78].
Interaction with DNA: ➢ GO and functionalized GO have affinity to interact with DNA or RNA, which make these species as an attractive candidate for DNA or RNA sensing and delivery carrier [34-36]. ➢ GO preferentially adsorbs ssDNA over to double strand (ds) DNA and protects them it from nuclease enzyme [37].	

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2067 **Table 2. Summary of therapeutic applications of GO.**

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Functionalized Graphene Oxide (FGO)	Therapeutic Agents (TA)	Interaction between FGO and TA	Highlights of the study	Stimuli	Ref.
GO-β-CD and GO-PVP	SN-38	Noncovalent	Functionalized GO with both polymers show enhanced cytotoxicity against the MCF-7 cell.	pH	[13]
GO-CuS	CEA and Glu	Noncovalent	GO-CuS-CEA-Glu shows photothermal accelerated release of Glu and *in vitro* chemo-photothermal synergistic therapy.	NIR, PTT, pH	[91]
GO-ZnO	DOX	Noncovalent	GO=ZnO shows higher drug loading efficiency(89%) compared to pure ZnO (82%) and provides enhanced	pH	[94]

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			dissolution according to the drug release.		
GO-FA-AuNPs	DOX	Covalent and Noncovalent	Substantial *in vivo* tumor regression in solid tumor model in Balb/c mice	NIR-PTT	[95]
GO-PEI	mi-RNA	Covalent	Efficient loading of miR-7b plasmid and delivers it into bone marrow macrophages for inhibiting the formation of mature osteoclasts while preserving beneficial pre-osteoclasts.	NIR	[97]
nGO-PEG-PEI	Plasmid DNA	Noncovalent	Efficiency of plasmid DNA transfection in Drosophila S2 cells increased	pH	[99]
GO-AgNPs	*E. coli*	Noncovalent	Antibacterial activity of GO-AgNPs on*E. coli* surface decreases the intake of nutrition from the surroundings while increases the interaction between Ag and the bacteria.	pH	[102]
GO- Fe_3O_4	DOX	Noncovalent	GO- Fe_3O_4 shows a high drug loading capacity, high dispersion through the external magnetic field.	External Magnetic Field	[110]
GO-FeNPs	Insulin	Noncovalent	GO-FeNPs-Insulin is stable at acidic pH, and released when exposed to basic solutions.	pH	[111]
GO-PEG	CEF	Covalent	GO-PEG-CEF shows both dose and time dependent antibacterial activity against both gram-positive and gram-negative bacteria while pure CEF shows only dosedependent antibacterial activity	pH	[118]
GO-Fe_3O_4–$mSiO_2$	DOX	Noncovalent	The addition of $mSiO_2$on GO-Fe_3O_4–increases the surface area, thus drug	pH	[119]

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			loading efficiency, as well as the cellular uptake.		
GO-Fe_3O_4	CPT and MTX	Noncovalent	*In vitro* shows that the nanocomposite can cause the apoptosis and death of HepG2 cells by preferentially releasing drugs to the tumor microenvironment	NIR-PTT	[131]
GO-FA-CS	DOX and siRNA		GO-FA-CS could effectively load DOX and siRNA simultaneously through p-p stacking and electrostatic interaction and specifically delivers to MCF-7 cells.	pH	[132]
GO- PVP	GEF and QSR	Noncovalent	The loading and cell cytotoxicity of GO-PVP-GEF, GO-PVP-QSR and GO-PVP-GEF-QSRin PA-1 ovarian cancer cells are significantly more cytotoxic than individual drug therapy to the PA-1 ovarian cancer cells compared to the toxicity toward IOSE-364 cells.	pH	[133]
rGO-AAm	PRG and CHL	Noncovalent	The rGO-AAm-PRG-CHL system interestingly shows antidiabetic activity when targeted *In vivo* against relevant neoglucogenic receptors	pH	[139]
GO-PEG	Cis-Pt and DOX	Noncovalent	GO-PEG-Cis-Pt-DOX shows more tumor cell growth inhibition than pure drug alone.	pH	[142]
GO-FA	CPT and DOX	Noncovalent	DOX has reinforced stronger interaction with FA than CPT thus establishing the fact that FA has influenced DOX release kinetics in the medium more than CPT.	pH	[143]

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Table of Content

Functionalized Graphene Oxide as a Vehicle for Targeted Drug Delivery and Bioimaging Applications

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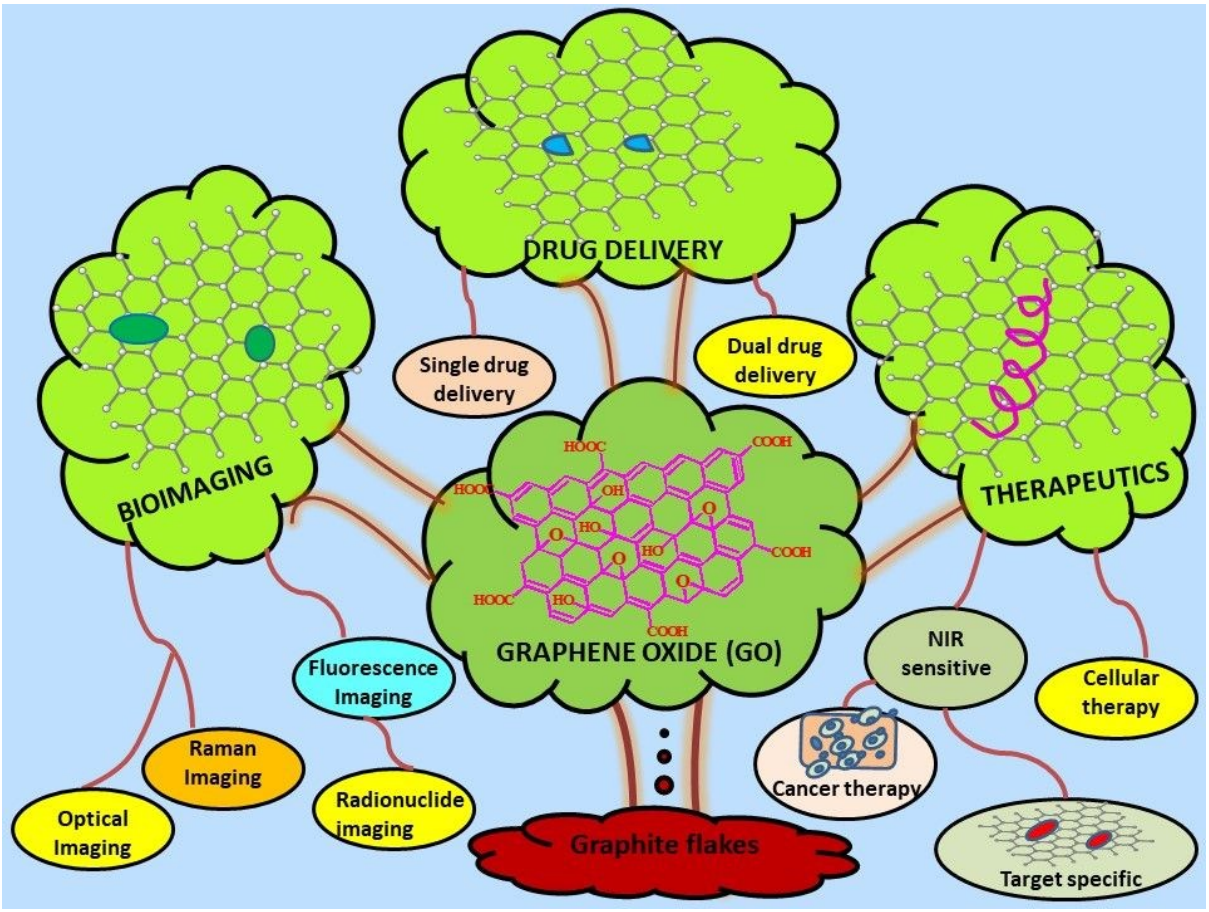

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