Bioorganic & Medicinal Chemistry Letters 24 (2014) 1638–1649

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

BMCL Digest

Graphene for multi-functional synthetic biology: The last 'zeitgeist' in nanomedicine

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ARTICLE INFO

Article history:
Received 19 October 2013
Revised 13 January 2014
Accepted 18 January 2014
Available online 10 February 2014

Keywords:
Nanotechnology
Nanomaterials
Drug delivery
Carbon

ABSTRACT

The high versatility of graphene has attracted significant attention in many areas of scientific research from electronics to physics and mechanics. One of the most intriguing utilisation of graphene remains however in nanomedicine and synthetic biology. In particular, the last decade has witnessed an exponential growth in the generation of novel candidate therapeutics of multiple biological activities based on graphene constructs with small molecules, such as anti-cancer drugs. In this Digest, we summarise the different synthetic strategies and routes available to fabricate these promising graphene conjugates and the opportunities for the design of multi-functional tools for synthetic biology that they offer.

Introduction: The landscape for discovering and developing drugs is evolving at an increasing pace as the direction for new diagnostic and therapeutic strategies heads towards personalised medicine. Personalised medicine aims at improving healthcare by integrating early detection of disease, preventive medicine, rational drug discovery and development, and monitoring of therapy.[1] Since the early 1970s, the use of nanotechnologies in general seems to have refashioned the pharmaceutical and drug delivery field and in particular the administration of medicines. Prior the use of 'nanomedicines' that is the development of nanomaterials containing medicines, it was inconceivable to administer a dispersion of solid particles by the route of intravenous injection considering the high risk of embolism. Nowadays, nanoparticles and nanomaterials have allowed the improvement of solubility, therapeutic activity and reduction of toxicity by increasing uptake to target sites and changing pharmacokinetic profiles of many traditional drugs. The design and production of these sophisticated nanoscale vectors has generated a fundamental technological and medical breakthrough, allowing the progression over many milestones in pharmaceutical development.

Despite the astonishing progress made in the development of disease-targeted nanoparticles, much effort is still needed in the design and engineering of more efficient and safer materials for enhanced targeting and delivery of drugs for which there are no effective delivery systems. In addition, the creation of multifunctional nanomaterials combining for example an imaging and a therapeutic agent (theranostic agent) or different therapeutic agents with complementary biological targets would certainly be the next challenging step in nanomedicine development towards personalised patient medicine.[2]

To date, many types of nanomaterials have been engineered for developing multifunctional platforms that can fulfil many tasks in cancer therapy and diagnosis and in synthetic biology.[3] The discovery of graphene in 2004 has generated significant interest in many areas of scientific research and has opened up a plethora of potential applications of its exciting properties encompassing various areas such as solar cells, transistors, super-capacitors, and memory devices.[4–7] Graphene is a single atom thick and a two dimensional arrangement of conjugated sp^2 carbons in a honeycomb structure.[8] Thanks to its unique properties such as an enhanced electrical and thermal conductivity, high charge carrier concentration and mobility, optical transparency, and mechanical strength, graphene is expected to reform the world of digital technology in a similar way as silicon has transformed the field of electronics. The high surface to volume ratio and the great chemical versatility of graphene make this exclusive material an ideal candidate for the development of a new generation of nanomedicines with applications encompassing the detection of biomarkers to imaging and cancer therapy.

To date, the exploitation of graphene properties for biomedical applications remains at its infancy. Could graphene bring solutions to the challenges faced by the current nanomedicines and offer an

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http://dx.doi.org/10.1016/j.bmcl.2014.01.051
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efficient platform for theranostics and synthetic biology? Without major breakthrough, the answer to this question is no. Graphene, in its unmodified state, suffers from one major drawback for its potential use in biomedical applications. The physical handling of graphene sheets is very delicate as it is not dispersible in aqueous conditions and most organic solvents.[9] Graphene tends to form irreversible aggregates or to restack by van der Waals interactions and π–π stacking. As a result, the conjugation of graphene with therapeutically active molecules or active biological components is extremely challenging.

Chemical functionalisation of graphene sheets may be one way to address these concerns. The hydrophobic character of graphene can be altered by the nature of the molecules and the extent of chemical functionalisation on the graphene sheets, improving the biocompatibility of this material. Graphene oxide (GO), the 'hydrophilic derivative' of graphene has attracted significant attention for its enhanced aqueous dispersibility and colloidal stability over other carbon-based nanomaterials. GO preparation from the Hummers method is now common knowledge and is currently widely explored for the development of nanomedicines.

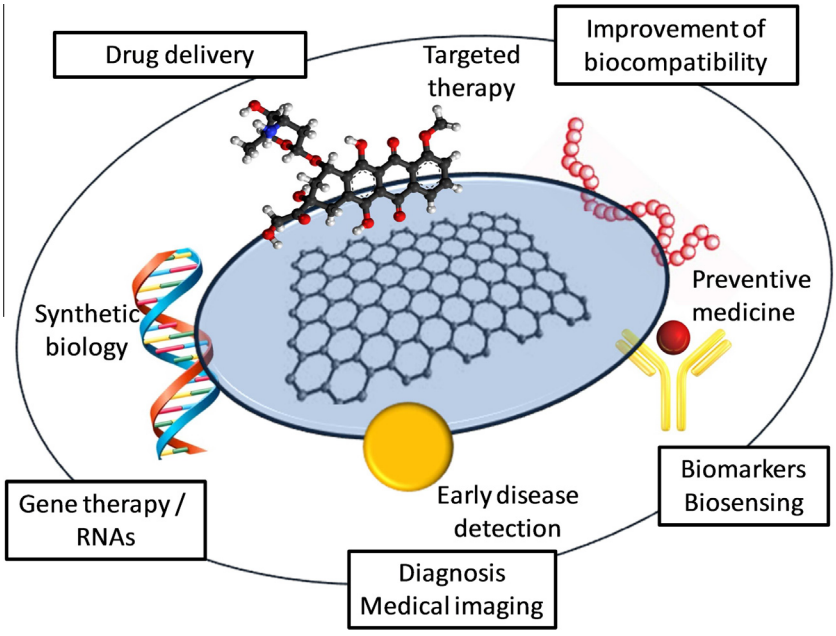
In this brief review, we aim at providing a general coverage of all the chemical functionalisation routes and strategies employed for the fabrication of these bioactive molecule/graphene conjugates and the opportunities for the design of multi-functional tools for synthetic biology that they offer[10](Scheme 1). In a first part of this Digest, the conjugation of bioactive molecules onto 'pristine' graphene platforms will be presented and in a second part, the addition of molecules using the chemical modification of GO will be discussed.

Conjugation reactions of bioactive molecules onto graphene: Two main synthetic routes are available to covalently attach molecules onto graphene platforms the first one is by using pristine graphene, or reduced graphene oxide (rGO), the second involving chemical modification of graphene oxide (GO). In this paragraph, the possibilities of chemical conjugation of molecules onto pristine graphene including rGO are described.

The most popular route to prepare graphene solutions is through graphite exfoliation in organic solvent by continuous sonication. This method, which is very attractive due to its simplicity, relies on the fact that strong interactions between carefully selected organic solvents and the layers can overcome the Van der Waals interlayer forces involved in graphite and result in graphene single layer formation.[9] To date, graphene layers can be characterised using several techniques such as electron microscopy and Raman spectroscopy that provide useful information on the structure and morphology of graphene sheets and allows an accurate evaluation of the sp^2 carbon network integrity following graphene preparation process (Fig. 1). Thanks to the high chemical versatility of the sp^2 carbon atom network, a variety of synthetic routes are currently developed to attach bioactive molecules onto the graphene plane including nucleophilic, electrophilic and free radical additions, and cycloadditions (Table 1).

Nucleophilic additions: Using a nucleophilic addition mechanism based on the Bingel cyclopropanation reaction, Economopoulos et al. covalently attached the electro-active extended tetrathiafulvalene (exTTF) moiety onto graphene layers.[11] In this Letter, graphene layers were obtained by ultrasonication of bulk graphite in benzylamine as a solvent. The resulting exTTF graphene conjugates contained one exTTF moiety every 198 carbon atoms. Functionalised graphene was characterised using energy dispersive X-ray (EDX) that confirmed the presence of sulfur on the material, and Raman spectroscopy that demonstrated the covalent addition onto graphene layers. The electrochemical activity of the exTTF graphene conjugate was measured by cyclic voltammetry suggesting the formation of a radical ion pair with a one-electron reduction from graphene and a one-electron oxidation from the exTTF active moiety. Tetrathiafulvalene and its derivatives are very attractive molecules as they are organic electron donors and acceptors. Such molecules have been used extensively in the fabrication of amperometric biosensors and have demonstrated that associated in monolayers onto conductive platforms, the resulting biosensors displayed higher sensibility for the detection of sugars like fructose.[12] The association between TTF molecules and graphene sheets seems therefore very promising to create functional platforms for the detection of bioactive components and biomarkers.

Cycloadditions. The most frequent synthetic route to covalently connect bioactive molecules to sp^2 graphene carbon network is



Scheme 1. Graphene–conjugate map for the generation of novel multifunctional nanomedicines paving the way to applications in personalised medicine and synthetic biology.

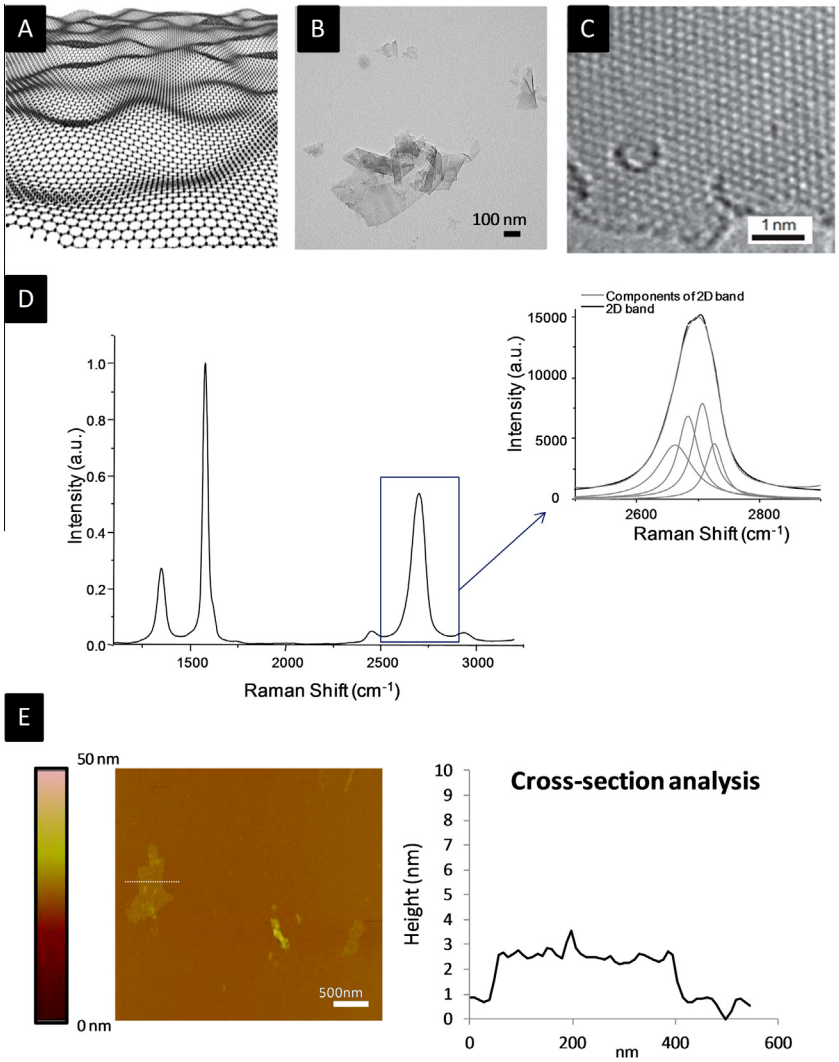


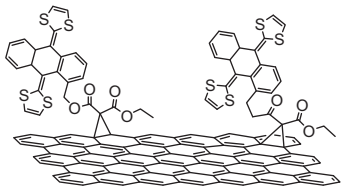
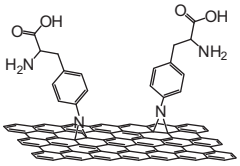
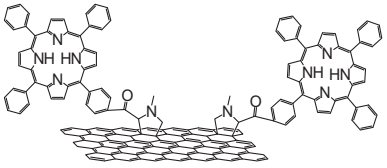
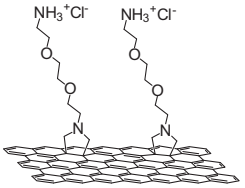
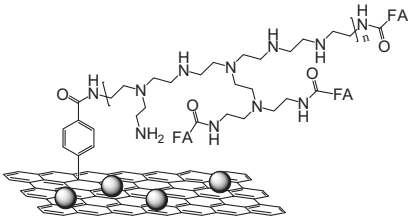
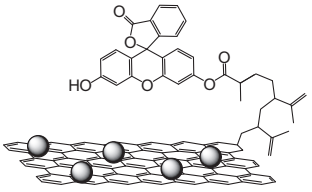
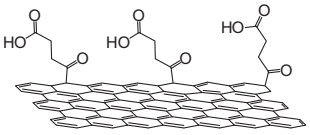
Figure 1. *Graphene structure and physical characterisation;* (A) molecular structure of a graphene plan,[42] (B) transmission Electron Microscopy image, and (C) high resolution TEM image; (D) Raman spectroscopy spectrum of few layer graphene; the de-convolution of the 2D band at 2700 cm^{-1} confirms that it is a few layer graphene from chemical exfoliation.[43,44] (E) Atomic Force Microscopy image of a single layer graphene. The cross-section analysis confirms the thickness of bilayer graphene around 3 nm.

through cycloaddition. Cycloaddition reactions differ from nucleophilic and electrophilic additions due to the fact that there is no intermediate formation such as anions or cations as bond rearrangement and bond formation processes occur simultaneously. Cycloadditions have been widely used for the functionalisation of fullerenes and carbon nanotubes, and their use on graphene to modulate graphene solubility in solvents seems promising.[13,14] To date various types of cycloadditions are reported to functionalise graphene layers; however the two main reactions employed for attaching bioactive molecules onto the sp^2 carbon atom network remain the cycloaddition through the formation of aziridine adducts and the 1,3-dipolar cycloaddition.

The insertion of an aziridine moiety onto graphene occurs via the formation of a nitrene intermediate, which is structurally related to a carbene molecule. This nitrene intermediate is generally obtained through thermal or photo-decomposition of an azide group and is subsequently conjugated to the sp^2 carbon atoms of graphene via a cycloaddition mechanism (Fig. 2). Using this reaction, Strom et al. described the conjugation of graphene layers with a modified phenylalanine.[15] In this work, Phe was modified with an azido group in *para* position of the phenyl ring prior coupling with the graphene through nitrene addition. The final construct was characterised using standard techniques such as thermo-gravmetric analysis (TGA) and an elemental analysis (XPS), demonstrating high functionalisation with one Phe moiety per 13 carbons. Adding sequences of amino acids onto graphene layers can be advantageous for improving water solubility and biocompatibility of graphene. Moreover, amino acid/graphene conjugates can serve as efficient platforms for the fabrication of novel therapeutic agents as peptide sequences can be cleaved specifically by enzymes accessible in cancer cells, facilitating drug targeting and delivery. This strategy was explored previously with multi-walled carbon nanotubes to deliver methotrexate to breast cancer cells MCF-7.[16]

The 1,3-dipolar cycloaddition of azomethine ylides has been widely applied to functionalise fullerenes and this chemical functionalisation of graphene towards a five-atom ring occurs through a six-electron cycloaddition between a 1,3-dipole and a dipolarophile (graphene) (Fig. 3 A).[17] This reaction was used to incorporate porphyrin molecules (TTP) onto graphene layers.[18] Graphene-TTP constructs were prepared and the presence of porphyrin moieties were confirmed by UV–vis spectroscopy and XPS with a relatively high loading estimated at one TTP group per 235 carbon atoms. Porphyrins are very attractive aromatic molecules as they display

Table 1
Bioactive molecule/graphene constructs prepared from pristine graphene

Constructs	Active molecule	Chemical reaction route
	Extended tetrathiafulvalene (exTTF)	Nucleophilic addition: Bingel reaction
	Phenylalanine	(2+1) Cycloaddition: Nitrene addition
	Porphyrin	(3+2) Cycloaddition: 1,3-dipolar cycloaddition
	Modified amino acid	(3+2) Cycloaddition: 1,3-dipolar cycloaddition
	Polyethylene diimine and folic acid (FA)	Aryl diazonium addition
	*o*-Methacrylate fluorescein (FMA)	Peroxide free radical addition
	Butyric acid	Electrophilic addition: Friedel–Crafts reaction

exceptional optical and electronic properties and have been used in nanomedicine as imaging agents for tumour detection. Due to their ability to generate singlet oxygen upon radiation porphyrin nanocomposites are being explored for photodynamic therapy.[19,20]

Quintana et al. reported the attachment of gold nanorods onto graphene using the 1,3-dipolar cycloaddition of azomethine ylides. The inserted amino groups selectively bound to gold nanorods, allowing to highlight the active sites created onto the graphene sheets.[21] These gold nanorod graphene constructs are very versatile platforms and could achieve a variety of biological tasks as drug carriers, photothermal agents, imaging agents and radio-sensitizers (Fig. 3 B).

Free radical additions. Free radical additions for functionalisation of carbon materials has attracted significant interest due to the possibilities of using green chemistry and working in aqueous media. Free radical additions are mainly achieved by both photo-thermal and photochemical processes and are now utilised for functionalising graphene layers to induce band gap opening and to alter graphene solubility.[22,23]

The most common approach employed for conjugating molecules onto graphene is by using aryl diazonium salts. In this reaction, graphene acts as an electron transfer agent that donates an electron to the aryl diazonium ion to form subsequently a radical species following the elimination of N_2[24](Fig. 4 A). Wei et al. covalently attached to rGO polyethyleneimine (PEI) and folic acid (FA) to specifically target CBRH7919 cancer cells using aryl diazonium salt approach.[25] The functionalised graphene was designed to deliver chemotherapeutic drugs such as Doxorubicin (DOX) and elsinochrome A (EA), which molecules were tied to graphene backbone via π–π stacking. The introduction of FA and PEI to graphene sheets was initially achieved by forming diazonium ions through the diazotisation of *p*-aminobenzoic acid. Following the coupling

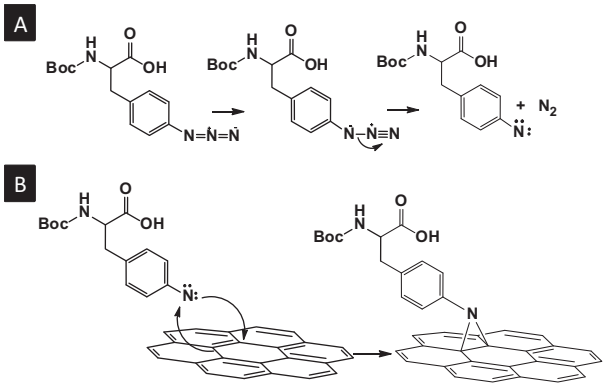


Figure 2. *Conjugation of amino acids onto graphene platform by nitrene addition;* (A) formation of the intermediate nitrene moiety; (B) mechanism of cycloaddition of the nitrene adducts onto graphene.

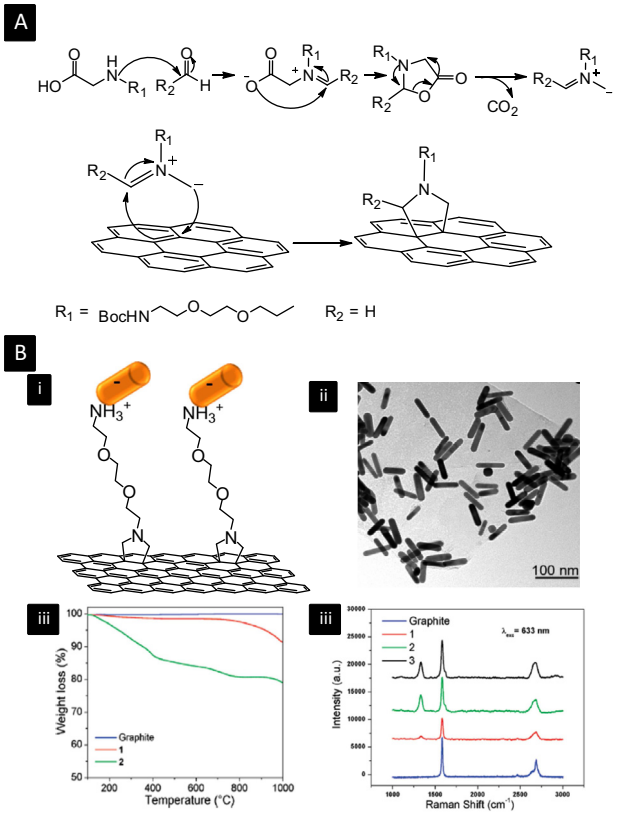


Figure 3. *Chemical functionalisation of graphene via 1,3-dipolar cycloaddition;* (A) mechanism of 1,3-dipolar cycloaddition onto graphene platform; (B) characterisation of the gold nanorods onto graphene single layers; (i) schematic of the construct, (ii) TEM picture, (iii) thermogravimetric analysis, (iv) Raman spectra of the final construct (3), the reaction intermediate (2), graphene (1) and graphite. Reproduced with permission.[21] Copyright 2011, American Chemical Society.

with ramified polymer, FA was subsequently added to the amino groups of PEI to form amide bonds (Fig. 4 B i). The chemical functionalisation of rGO sheets with *p*-aminobenzoic acid was characterised with TEM, FTIR and Raman spectroscopy (Fig. 4 B ii, iii, iv). EA and DOX loading onto the graphene constructs were calculated to be around 46% and 29% for EA and DOX, respectively. Cell internalisation of the graphene constructs was examined by confocal laser scanning microscopy (CLSM) and the images revealed a good cell uptake. Moreover, it was demonstrated that these constructs could effectively enhance the apoptosis of liver cancer cells and increase the sensitivity of these cells towards drugs and radiation (Fig. 4 B v, vi). However, cell toxicity was induced by the graphene conjugates in the absence of the drugs above the concentration of 12 μg/ml. This could be a serious limitation for translating this delivery system pre-clinically.

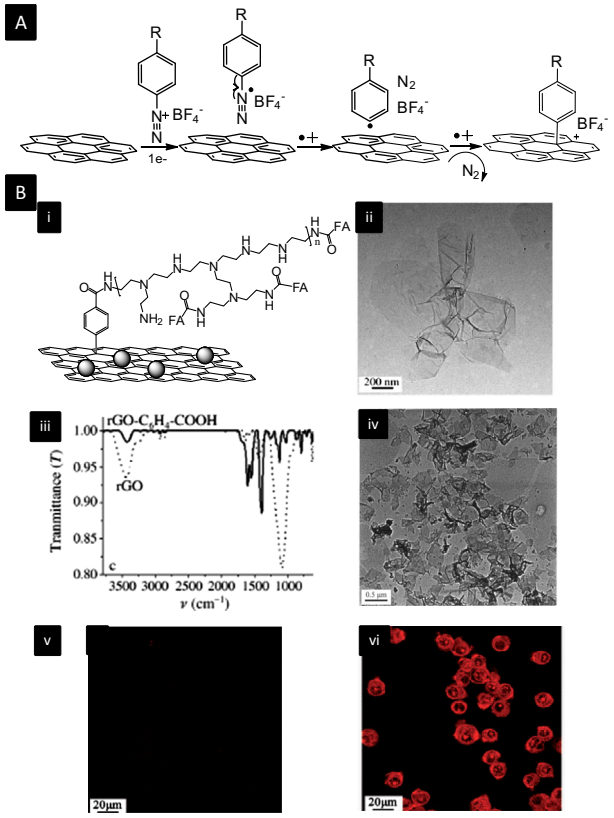


Figure 4. *Chemical functionalisation of graphene by free radical reaction using diazonium salts;* (A) mechanism of graphene chemical functionalisation with diazonium salts; (B) addition of folic acid (FA) onto graphene for cell targeting using diazonium salts; (i) FA/graphene construct, (ii) TEM images of rGO, (iii) FTIR spectra of rGO and rGO/p aminobenzoic acid, (iv) TEM image of rGO/p amino benzoic acid constructs; FA/graphene cell internalisation, (v) untreated cells, (vi) cells treated with FA/Cy3 graphene constructs. Reproduced with permission.[25] Copyright 2012, Wiley-VCH Verlag 14708 GmbH&Co. KGaA, Weinheim.

The other synthetic route to covalently attach molecules via free radical addition is using peroxides. Peroxides are a common source of radicals, which are formed under photochemical treatment. Covalent grafting of polyacrylic acid (PAA) and fluorescein *o*-methacrylate (FMA) onto rGO was reported by Gollavelli and Ling using benzoyl peroxide under microwave irradiation[26] (Fig. 5 A, B). These constructs were designed for the generation of promising markers for in vitro and in vivo imaging. In this Letter, GO was converted into 'magnetic' graphene (MG) using in situ microwave assisted reduction and subsequent magnetisation with adsorbing metallic iron nanoparticles prior the covalent addition of PAA and FMA molecules. The presence of the metallic nanoparticles onto the sp^2 carbon atom network was confirmed by TEM and AFM (Fig. 5 C, D), while the saturation magnetisation of MG was measured indicating superparamagnetic properties of MG sheets. The in vitro and in vivo fluorescence of the multifunctional graphene (MFG) was demonstrated in HeLa cells and zebrafish, respectively (Fig. 5 E, F). The constructs resulted biocompatible and resistant to degradation in the lysosomal compatements. These sophisticated constructs due to the presence of the metallic iron nanoparticles are very versatile and could be exploited for Magnetic Resonance Imaging (MRI), magnetically guided drug/gene delivery and photothermal/photodynamic therapy, simultaneously serving several tasks.

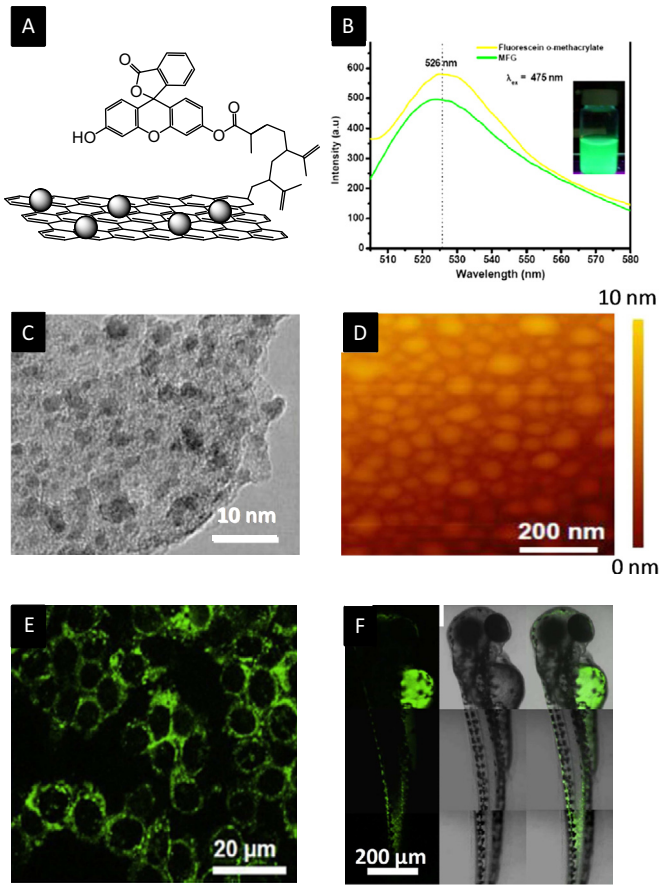


Figure 5. *Chemical functionalisation of graphene with FITC by free radical reaction using benzoyl peroxide;* (A) schematic representation of the FITC/iron oxide/graphene construct; (B) fluorescence spectra (λ_{ex} 475 nm) of MFG (green line) and fluorescein o-methacrylate alone as a reference (yellow line). Inset representing the green fluorescence image of MFG aqueous solution upon exposure to UV light; (C) TEM image of FITC/iron oxide/graphene construct at high magnification; (D) AFM image of FITC/iron oxide/graphene construct; (E) CLSM images of treated FITC/iron oxide/graphene construct (incubated with 20 μg/ml of graphene for 24 h) HeLa cells; (F) distribution of functionalised graphene inside a fully developed zebrafish using CLSM, fluorescence image of MFG with a FITC filter, DIC image, and overlay image. Reproduced with permission.[26] Copyright 2011, Elsevier Ltd.

Electrophilic additions. The electron rich nature of graphene allows the addition of molecules onto the carbon backbone by electrophilic substitution. Graphene sheets display high reactivity towards strong electrophilic molecules. To date, Friedel–Crafts acylation continues to be one of the widely used method to add an aryl ketone onto graphene platforms and has been successfully performed to introduce 4-aminobenzoic acid onto graphene layers.[27] The introduction of carboxylic acid moieties onto graphene was achieved via Friedel–Crafts by Chen et al.[28] to form stable dispersion of exfoliated graphene with grafted butyric acid (Gs-BA). These constructs were designed for developing electrochemical biosensors and in particular for the detection of glucose and choline. The authors demonstrated that this type of constructs combined with polyacrylic acid-benzoxazole (PAA-BO), a hydrogen peroxide sensitive probe, displayed higher sensitivity and response time than traditional biosensors making graphene hybrid materials a valuable platform for biosensors with higher capabilities.

Conjugation reactions of bioactive molecules on graphene oxide. GO platforms have been widely exploited as starting materials for covalently attaching bioactive molecules, as GO individualised sheets can be obtained from bulk graphite with relatively high yields. There are different methods to prepare GO, however the most popular synthetic route remains the 'Hummers' method developed by Hummers and Offeman in the fifties.[29] GO sheets are highly functionalised with oxygenated groups such as hydroxyl, epoxide, ketone and carboxylic acid moieties resulting in remarkably stable dispersions in water and organic solvents. The structural differences and notably defects on graphene sp^2 carbon networks can be easily evidenced using Raman spectroscopy, where the disappearance of the 2D peak at 2700 cm^{-1} and the higher D peak at 1380 cm^{-1} than the G peak at 1576 cm^{-1} highlights the large presence of sp^3 carbon atoms on GO (Fig. 6).[30] The various oxygenated groups attached on GO layers, due to their difference in reactivity allow surface modification via numerous synthetic routes including electrophilic and nucleophilic substitutions, free radical addition and condensation reactions; however the most common routes employed for functionalising GO remain through nucleophilic and condensation reactions (Table 2).

Condensation reactions. Condensation reactions are reactions during which two molecules are combined together, resulting in one single molecule. In case of graphene, this reaction occurs via the two reactive oxygenated groups present on the GO sheets such as the hydroxyl and carboxyl functionalities. The amidation reaction between an activated carboxyl group from GO and an amino group remains nevertheless the most popular reaction for introducing bioactive molecule onto GO platforms. Many groups have utilised this synthetic route to attach covalently molecules resulting in graphene conjugates for biomedical applications encompassing medical imaging and diagnosis, biosensors, and drug and gene delivery, as described in the next paragraph.

The introduction of biocompatible polymers using condensation reaction has been widely applied in order to develop graphene conjugates with improved solubility and biocompatibility. This strategy was used by Zhang et al. to engineer functional graphene sheets for investigating the biodistribution of biocompatible graphene conjugates.[31] In this work, the authors present the conjugation of GO sheets with dextran (DEX) by condensing the amino groups of DEX with the carboxylic groups of GO. The size of the GO conjugates was characterised by AFM and TEM and was found to be ranging from 50 to 100 nm compared to the lateral size of the GO sheets that was comprised between 100 and 500 nm, suggesting size reduction resulting from the DEX condensation reaction process. The DEX/GO conjugates demonstrated long lasting stability in aqueous media and mice serum and reduced cell toxicity compared to unfunctionalised GO sheets. Biodistribution studies were conducted in order to investigate the in vivo behaviour of these constructs by radiolabelling the DEX/GO sheets with radioactive ^{125}I. The graphene hybrids accumulated in the reticulo-endothelial system including liver and spleen after intravenous injection and demonstrated relatively long blood circulation times. In addition, the constructs were cleared from the animal body within a week from the injection without significant signs of toxicity.

Other functionalised GO constructs, fabricated through the formation of an amide group were reported for the development of therapeutic agents for drug or gene delivery by conjugating onto the graphene structure thioflavin-S (ThS).[32] This molecule is known to bind specifically to amyloid-β peptides forming the conjugate GO-ThS-Aβ. This construct was designed for the treatment of Alzheimer's disease by photothermal therapy. Photothermal therapy is often presented as good alternative to the traditional chemotherapy and radiotherapy as it has demonstrated remarkably reduced side effects and improved selectivity with limited healthy tissue exposure to the treatment.[33] In this work, GO sheets were first treated with chloroacetic acid for converting the hydroxyl functions into carboxylic functions. The condensation between a hydroxyl group of GO and another electrophilic molecule such as chloroacetic acid has been extensively used for modifying GO surfaces as a synthetic step for covalently coupling bioactive

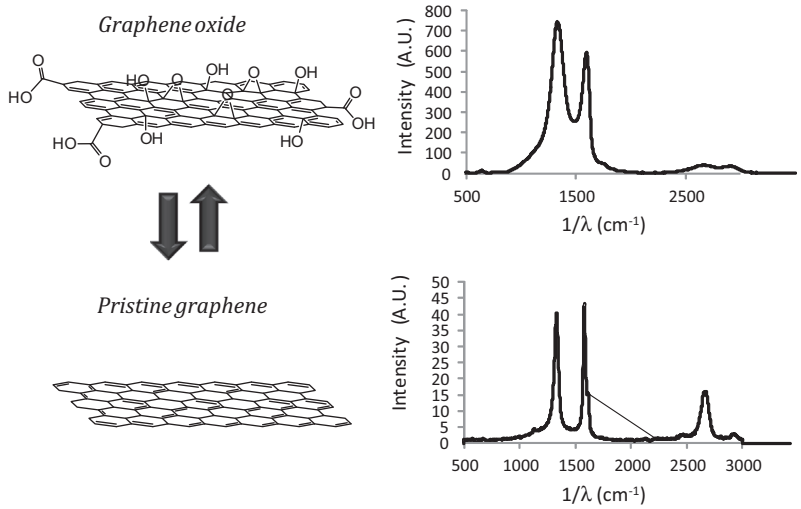


Figure 6. *Graphene oxide versus graphene;* the oxidation of graphene introduces defects onto the graphene sp^2 carbon atom network evidenced by Raman spectroscopy by the disappearance of the 2D band at 2700 cm^{-1} and the rise of the D peak at 1380 cm^{-1}.

molecules onto graphene layers.[34] This initial condensation reaction allows in fact the conversion of all the hydroxyl moieties of GO into carboxylic acid groups (Fig. 7A). The resulting GO-COOH material was then converted into an amino functionalised GO by grafting diaminoethylene glycol by amidation reaction (Fig. 7B). GO-ThS was then prepared by condensing the amino-modified GO groups with the ThS molecule, previously activated with $SOCl_2$ (Fig. 7C). Each step of GO functionalisation was characterised by FTIR and TGA analysis that confirmed the addition of each molecule onto the graphene layers. AFM was used to demonstrate that the constructs were mainly formed of single and double graphene layers. The rationale behind the fabrication of these constructs was to inhibit the Aβ aggregation and destabilise the pre-formed Aβ fibrils that are sought to be a critical step in the pathogenesis in Alzheimer's disease.[35] Using NIR laser irradiation, the author demonstrated the possibility of remotely heating and dissociating amyloid aggregation.

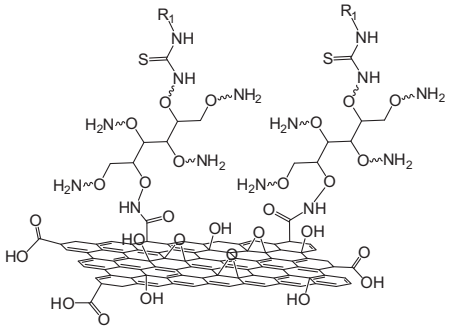
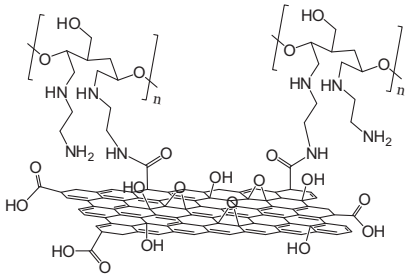
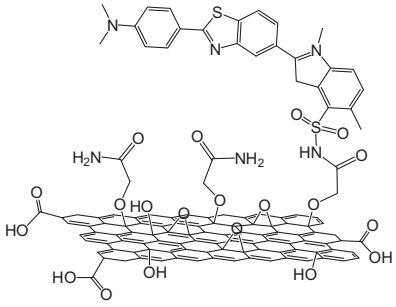
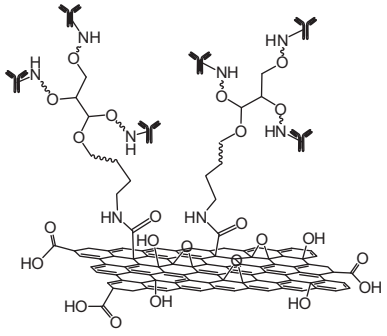
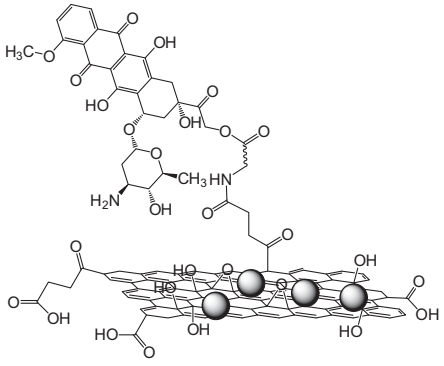
A similar strategy was developed by Yang et al. for the fabrication of EGFR conjugated PEGylated GO sheets for targeted chemotherapy and photothermal therapy.[36] After conversion of the hydroxyl moieties into carboxylic acids using sodium hydroxide, the modified GO-COOH was reacted with amino terminated PEG chains in order to obtain GO-CONH-PEG-NH_2 constructs. These constructs were then characterised with AFM, TGA and XPS demonstrating the successful functionalisation of GO sheets with amino terminated PEG chains via formation of NHS-esters followed by on GO. Following this step, the graphene conjugates were chemically functionalised with anti-EGFR antibody (C225) via a sulfo-succinimidyl 4-*N*-maleimidomethyl cyclohexane1-carboxylate (sulfo-SMCC) linker (Fig. 7C). The loading of C225 onto the modified graphene GO-PEG-NH_2 sheets was determined by UV–vis analysis measuring the concentration of unbound C225. The constructs were designed to achieve multiple tasks such as targeting glioma cells and inhibit their propagation with the presence of anti-EGFR antibody, chemotherapeutic drug delivery with the release of Epirubicin (EPI) non-covalently attached (π–π stacking) onto the functionalised graphene sheets, and photo-irradiation to burn residual tumour cells.

Sun et al. reported the conjugation of PEG functionalised GO with Rituxan, a B-cell lymphoma specific antibody.[34] The conjugation was aiming at generating individualised GO sheets with high solubility in water and compatible in biological environments. GO was initially treated with chloroacetic acid, converting the hydroxyl functionalities into carboxylic acids. Subsequently, the modified GO sheets were functionalised with PEG by condensing PEG-NH_2 onto the carboxylic groups of the graphene material. The resulting PEG functionalised GO (PEG/GO) displayed stable dispersions in aqueous media and serum, without agglomeration of the GO layers. Thiolated Rituxan was covalently attached to the amine groups of PEG/GO via a sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane1-carboxylate (sulfo-SMCC) linker. Using the intrinsic photoluminescence properties of GO, these constructs were designed for cellular imaging and drug delivery. GO hybrids were able to bind selectively to B-cell membrane, discriminating T-cell binding and were detectable by NIR photoluminescence imaging. The release of non-covalently bound DOX from these constructs to B-cells was also described, indicating the potential of selective killing of cancer cells in vitro using multifunctional functionalised graphene.

Zhang et al. reported the functionalisation of GO sheets with folic acid (FA-GO) with the aim of fabricating efficient nanovectors for targeted drug delivery.[37] The GO sheets were initially treated in order to convert all hydroxyl moieties into carboxylic acid groups. Folic acid was subsequently attached to the graphene material through diimide activated amidation. Two anticancer drugs, doxorubicin (DOX) and camptothecin (CPT) were loaded onto the FA-GO constructs by π–π stacking onto the sp^2 carbon network, controlling accurately the ratio between the two adducts. The DOX/CPT loaded FA-GO sheets demonstrated specific targeting to MCF-7 breast cancer cells and greater cytotoxicity, outperforming the non FA functionalised GO sheets loaded with both anticancer drugs. These multifunctional graphene constructs offer the opportunity of specifically deliver different drugs with complementary biological tasks allowing an enhanced therapeutic efficacy.

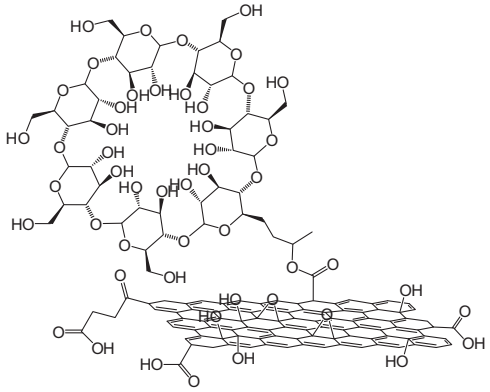
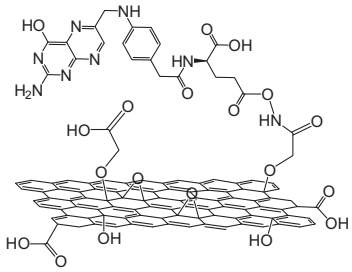
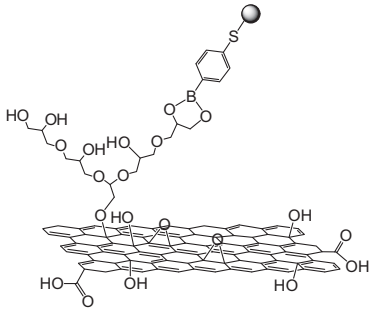
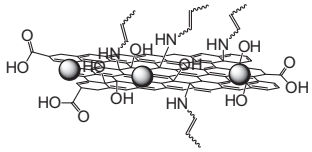
Esterification reactions, which are condensation reactions involving a molecule displaying a carboxylic acid group and another molecule with a hydroxyl group, have been used to attach bioactive molecules onto GO platforms (Fig. 8). Yang et al. developed a nanosized magnetic PEG functionalised GO drug carrier that can transport and deliver Epirubicin (EPI) to the target site by magnetic dragging.[38] EPI is known to be an anthracycline drug that intercalate into DNA and RNA, inhibiting DNA synthesis inducing cell apoptosis. The release of EPI was activated by low power focus ultrasound (FUS), resulting in deep-seated targeted hyperthermia and drug delivery. GO sheets were initially conjugated with PEG molecules via amino/carboxylic acid condensation reaciton. EPI

Table 2
Graphene constructs prepared from graphene oxide

Construct	Active molecule	Reaction
	NOTA ligand	Amidation reaction
	Dextran	Amidation reaction
	Thioflavin S	Amidation reaction
	Anti-EGFR antibody	Amidation reaction
	Epirubicin (EPI)	Esterification reaction

(*continued on next page*)

Table 2 (*continued*)

Construct	Active molecule	Reaction
	β-cyclodextrin	Esterification reaction
	Folic acid	Condensation through an hydroxyl
	Polyglycerol	Nucleophilic substitution
	Oleylamine	Electrophilic substitution

was then covalently attached to the GO constructs by esterification with the end carboxylic group of carboxylated PEG chains. The functionalisation of the GO sheets was characterised and confirmed by FTIR and XPS, and the lateral size was determined by TEM ranging from 120 to 150 nm (Fig. 8 a, b, c). The constructs were successfully designed as multifunctional nanocarriers with higher capabilities including superior heat absorption resulting in highly effective hyperthermia, high drug loading, specific targeting capability, molecular imaging (Fig. 8 d, e, f).

Another example of GO functionalisation with a bioactive molecule by condensation reaction onto graphene carboxyl moieties was reported by Xu et al., where the introduction of β-cyclodextrin onto GO platform was described.[39] β-cyclodextrin functionalised GO was prepared by condensing hydroxypropyl-β-cyclodextrin (HPCD) with thionyl chloride modified GO sheets. GO sheets were initially treated with thionyl chloride $SOCl_2$ in order to convert the carboxylic acid functionalities into acyl chloride groups, resulting in the graphene conjugate GO-COCl. The conjugation between HPCD and GO-COCl was characterised with AFM, FTIR and UV–vis spectroscopy that indicated the presence of HPCD functional groups onto the constructs. Raman spectroscopy confirmed the covalent addition of β-cyclodextrin by the increase in the D/G band ratio, characteristics of relative disorder in the graphitic sp^2 carbon network. The constructs were successfully designed for the development of biosensors with higher sensitivity and detection power for haemoglobin.

Nucleophilic substitutions. Nucleophilic reactions using the hydroxyl moieties on GO sheets have been explored to functionalise graphene-based nanomaterials and develop new nanomedicines. Pham et al. exploited the nucleophilic character of hydroxyl moieties of GO to introduce polyglycerol.[40] GO was initially treated with potassium methoxide in methanol to activate the hydroxyl functions and then mixed together with glycidol resulting in polyglycerol modified graphene conjugates PG/GO. Subsequently magnetic nanoparticles were covalently introduced on the surface of the PG/GO sheets via boron–ester bond. The characterisation of the graphene constructs was thoroughly performed using standard techniques and suggested high functionalisation of the GO sheets and

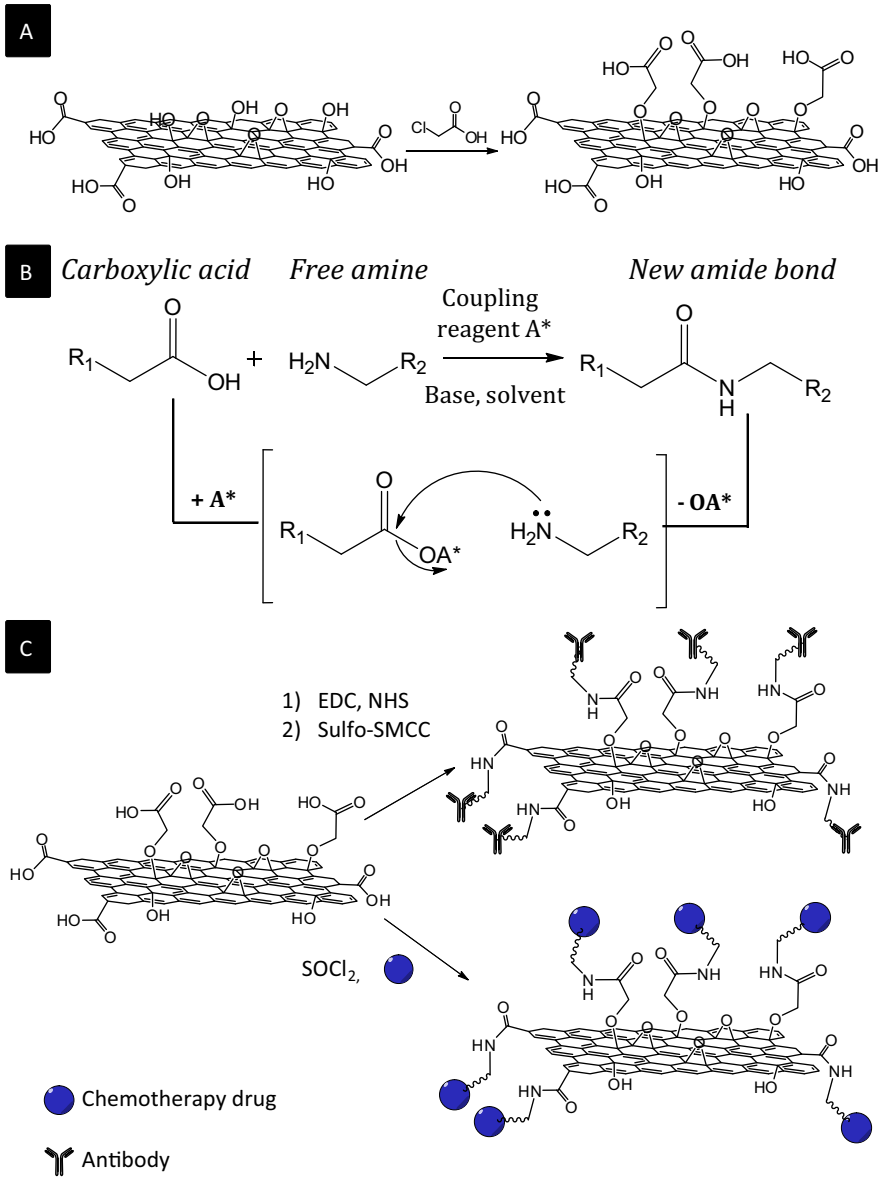


Figure 7. *Bioactive molecule conjugation onto GO layers via amide formation;* (A) conversion of the hydroxyl moieties of GO into carboxylic acids using chloroacetyl acid; (B) schematic of amide bond formation between carboxylic and amine groups; (C) addition of bioactive molecule onto GO via amide formation using (EDH, NHS) and Sulfo-SMCC.

the saturation magnetisation was measured and revealed the character super paramagnetic of these constructs. These magnetic graphene conjugates could be used as photodynamic agents.

Electrophilic substitutions. Introduction of bioactive molecules via electrophilic substitutions occur through the epoxide moieties of GO, resulting in the opening of the epoxide ring. Peng et al. utilised this strategic route to covalently attach oleylamine onto GO platform for the generation of magnetic nanoparticle decorated GO as new contrast agents for MRI.[41] Subsequently to the addition of oleylamine, magnetic iron oxide nanoparticles could be conjugated to the amphiphilic oleylamine/GO construct by non-covalent interactions. The functionalised GO sheets were characterised by FTIR spectroscopy and electron microscopy, confirming the successful attachment of oleylamine. The size of the resulting magnetic GO sheets could be tuned by adjusting the ultrasonication time and magnetic GO sheets as small as 56 nm could be obtained. In order to improve the colloidal stability of the graphene constructs in physiological environment, the magnetic GO were further functionalised with PEG. These constructs were found to display MR contrast efficiency in T_2 weighted images. In addition, the contructs could generate heat upon exposure to an alternating magnetic field making them promising candidates for hyperthermia treatments.

Future perspective of graphene platforms in nanomedicine. In this review, we have covered the different synthetic routes that have been used over the past few years for attaching bioactive molecules onto graphene-based nanomaterials. The high chemical versatility of graphene, GO and rGO allows the conjugation of a vast variety of small molecules, macromolecules, and bioactive agents, bringing promising biomedical opportunities to graphene platforms. Naturally, more needs to be done in the chemical functionalisation of graphene and in particular developing new methodologies to reduce possible chemisorbed and physisorbed reagents and/or contaminants. The success of this niche will pave the way to the development of novel multifunctional nanomedicines with higher efficiency and capabilities making closer to a reality the development of personalised medicines.

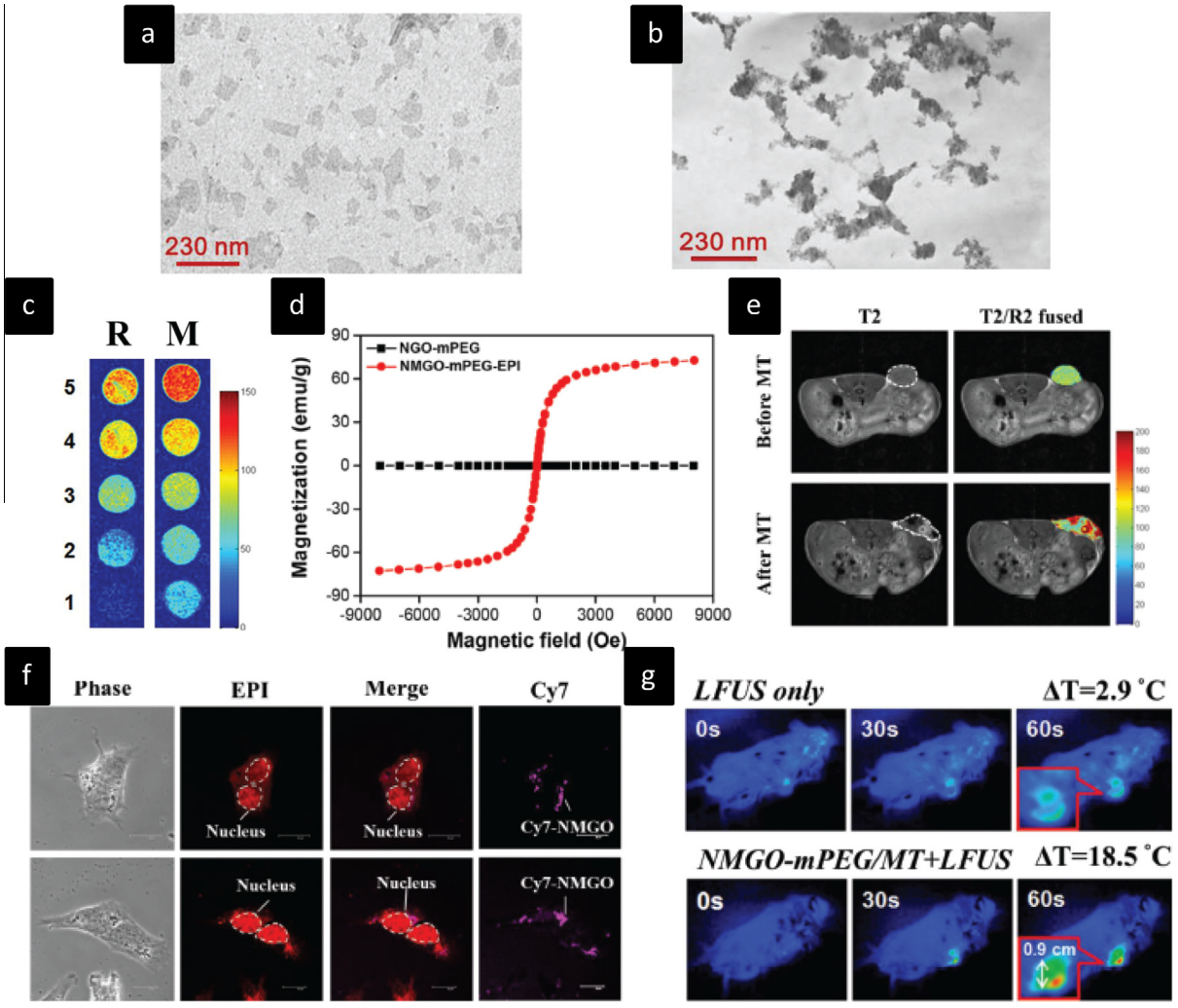


Figure 8. *GO chemical functionalisation for in vivo imaging and photo-thermal therapy through esterification reaction process;* TEM images of (a) PEG functionalised GO (GO–mPEG) and (b) iron oxide PEG functionalised GO (MGO-mPEG); (c) MRI phantoms of spin–spin relaxivity (R_2) imagesof Resovist (R) and NMGO–mPEG–Epirubicin (EPI) (M). (1: 0.1 m M Fe; 2: 0.2 m M Fe; 3: 0.4 m M Fe; 4: 0.6 m M Fe; 5: 0.8 m M Fe); (d) Superconducting quantuminterference device (SQUID) spectra of GO–mPEG and MGO–mPEG–EPI at room temperature; (e) In vivo imaging of NMGO–mPEG–EPI distribution in hypodermic tumors before and after magnetic targeting (0.4 T) for 36 h (left,T_2-weighted images; right, combined R_2 maps and T_2-weighted images); (f) Phase contrast and fluorescence images of GL261 cells exposed to 5 m M of Cy7-labeled NMGO–mPEG–EPI in the absence (top) and presence (bottom) of a magnetic field (900 Gauss). EPI can be visualized in GL261 cells by confocal microscopy with excitation at 488 nm. (Red color: EPI; purple color: NMGO); (g) NIR thermal images of tumors in mice after being treated with iron oxide PEG functionalised GO (MGO-mPEG)and guided by magnetic targeting (MT)(LFUS: 2 W; laser: 2 W cm^{-2}). Reproduced with permission.[38] Copyright 2013, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Acknowledgments

We would like to acknowledge the 7th RTD Framework Programme - Specific Programme Cooperation (FP7-ICT-2013-FET-F-604391) for partial sponsorship of this work under the Graphene Flagship project.

References and notes

1. Jain, K. K. *Med. Princ. Pract.* **2013**, *22*, 209.
2. Couvreur, P. *Adv. Drug Deliv. Rev.* **2013**, *65*, 21.
3. Brigger, I.; Dubernet, C.; Couvreur, P. *Adv. Drug Deliv. Rev.* **2012**, *64*, 24.
4. Novoselov, K. S.; Geim, A. K.; Morozov, S. V.; Jiang, D.; Zhang, Y.; Dubonos, S. V.; Grigorieva, I. V.; Firsov, A. A. *Science* **2004**, *306*, 666.
5. Geim, A. K.; Novoselov, K. S. *Nat. Mater.* **2007**, *6*, 183.
6. Geim, A. K. *Science* **2009**, *324*, 1530.
7. Novoselov, K. S.; Geim, A. K.; Morozov, S. V.; Jiang, D.; Katsnelson, M. I.; Grigorieva, I. V.; Dubonos, S. V.; Firsov, A. A. *Nature* **2005**, *438*, 197.
8. Bianco, A.; Cheng, H.-M.; Enoki, T.; Gogotsi, Y.; Hurt, R. H.; Koratkar, N.; Kyotani, T.; Monthioux, M.; Park, C. R.; Tascon, J. M. D.; Zhang, J. *Carbon* **2013**, *1*, 1.
9. Hernandez, Y.; Nicolosi, V.; Lotya, M.; Blighe, F. M.; Sun, Z. Y.; De, S.; McGovern, I. T.; Holland, B.; Byrne, M.; Gun'ko, Y. K.; Boland, J. J.; Niraj, P.; Duesberg, G.; Krishnamurthy, S.; Goodhue, R.; Hutchison, J.; Scardaci, V.; Ferrari, A. C.; Coleman, J. N. *Nat. Nanotechnol.* **2008**, *3*, 563.
10. Krishna, K. V.; Menard-Moyon, C.; Verma, S.; Bianco, A. *Nanomedicine* **2013**, *8*(10), 1669.
11. Economopoulos, S. P.; Rotas, G.; Miyata, Y.; Shinohara, H.; Tagmatarchis, N. *ACS Nano* **2010**, *4*, 7499.
12. Campuzano, S.; Escamilla-Gomez, V.; Herranz, M. A.; Pedrero, M.; Pingarron, J. M. *Sens. Actuators, B-Chem.* **2008**, *134*, 974.
13. Chua, C. K.; Ambrosi, A.; Pumera, M. *Chem. Commun.* **2012**, 5376.
14. Georgakilas, V.; Bourlinos, A. B.; Zboril, R.; Steriotis, T. A.; Dallas, P.; Stubos, A. K.; Trapalis, C. *Chem. Commun.* **2010**, 1766.
15. Strom, T. A.; Dillon, E. P.; Hamilton, C. E.; Barron, A. R. *Chem. Commun.* **2010**, 4097.
16. Samori, C.; Ali-Boucetta, H.; Sainz, R.; Guo, C.; Toma, F. M.; Fabbro, C.; da Ros, T.; Prato, M.; Kostarelos, K.; Bianco, A. *Chem. Commun.* **2010**, 1494.
17. Georgakilas, V.; Bourlinos, A.; Gournis, D.; Tsoufis, T.; Trapalis, C.; Mateo-Alonso, A.; Prato, M. *J. Am. Chem. Soc.* **2008**, *130*, 8733.
18. Zhang, X. Y.; Hou, L. L.; Cnossen, A.; Coleman, A. C.; Ivashenko, O.; Rudolf, P.; van Wees, B. J.; Browne, W. R.; Feringa, B. L. *Chem.-a Eur. J.* **2011**, *17*, 8957.
19. Hayashi, K.; Nakamura, M.; Miki, H.; Ozaki, S.; Abe, M.; Matsumoto, T.; Ishimura, K. *Adv. Funct. Mater.* **2012**, *22*, 3539.
20. Josefsen, L. B.; Boyle, R. W. *Theranostics* **2012**, *2*, 916.
21. Quintana, M.; Spyrou, K.; Grzelczak, M.; Browne, W. R.; Rudolf, P.; Prato, M. *ACS Nano* **2010**, *4*, 3527.
22. Huang, P.; Zhu, H. R.; Jing, L.; Zhao, Y. L.; Gao, X. Y. *ACS Nano* **2011**, *5*, 7945.

23. Wang, Q. H.; Jin, Z.; Kim, K. K.; Hilmer, A. J.; Paulus, G. L. C.; Shih, C. J.; Ham, M. H.; Sanchez-Yamagishi, J. D.; Watanabe, K.; Taniguchi, T.; Kong, J.; Jarillo-Herrero, P.; Strano, M. S. *Nat. Chem.* **2012**, *4*, 724.
24. Pinson, J.; Podvorica, F. *Chem. Soc. Rev.* **2005**, *34*, 429.
25. Wei, G. C.; Yan, M. M.; Dong, R. H.; Wang, D.; Zhou, X. Z.; Chen, J. F.; Hao, J. C. *Chem.-A Eur. J.* **2012**, *18*, 14708.
26. Gollavelli, G.; Ling, Y. C. *Biomaterials* **2012**, *33*, 2532.
27. Chua, C. K.; Pumera, M. *Chem.-An Asian J.* **2012**, *7*, 1009.
28. Chen, H. C.; Chen, Y. H.; Chen, S. L.; Chern, Y. T.; Tsai, R. Y.; Hua, M. Y. *Biosens. Bioelectron.* **2013**, *46*, 84.
29. Hummers, W. S.; Offeman, R. E. *J. Am. Chem. Soc.* **1958**, *80*, 1339.
30. Kuila, T.; Bose, S.; Mishra, A. K.; Khanra, P.; Kim, N. H.; Lee, J. H. *Prog. Mater Sci.* **2012**, *57*, 1061.
31. Zhang, S. A.; Yang, K.; Feng, L. Z.; Liu, Z. *Carbon* **2011**, *49*, 4040.
32. Li, M.; Yang, X. J.; Ren, J. S.; Qu, K. G.; Qu, X. G. *Adv. Mater.* **2012**, *24*, 1722.
33. Huff, T. B.; Tong, L.; Zhao, Y.; Hansen, M. N.; Cheng, J. X.; Wei, A. *Nanomedicine* **2007**, *2*, 125.
34. Sun, X. M.; Liu, Z.; Welsher, K.; Robinson, J. T.; Goodwin, A.; Zaric, S.; Dai, H. J. *Nano Res.* **2008**, *1*, 203.
35. Yamin, G.; Ono, K.; Inayathullah, M.; Teplow, D. B. *Curr. Pharm. Des.* **2008**, *14*, 3231.
36. Yang, H. W.; Lu, Y. J.; Lin, K. J.; Hsu, S. C.; Huang, C. Y.; She, S. H.; Liu, H. L.; Lin, C. W.; Xiao, M. C.; Wey, S. P.; Chen, P. Y.; Yen, T. C.; Wei, K. C.; Ma, C. C. M. *Biomaterials* **2013**, *34*, 7204.
37. Zhang, L. M.; Xia, J. G.; Zhao, Q. H.; Liu, L. W.; Zhang, Z. J. *Small* **2010**, *6*, 537.
38. Yang, H.; Hua, M.; Hwang, T.; Lin, K.; Huang, C.; Tsai, R.; Ma, C.; Hsu, P.; Wey, S.; Hsu, P.; Chen, P.; Huang, Y.; Lu, Y.; Yen, C.; Feng, C.; Lin, C.; Liu, H.; Wei, K. C. *Adv. Mater.* **2013**, *25*, 3605.
39. Xu, C. H.; Wang, X. B.; Wang, J. C.; Hu, H. T.; Wan, L. *Chem. Phys. Lett.* **2010**, *498*, 162.
40. Pham, T. A.; Kumar, N. A.; Jeong, Y. T. *Synth. Met.* **2010**, *160*, 2028.
41. Peng, E. W.; Choo, E. S. G.; Chandrasekharan, P.; Yang, C. T.; Ding, J.; Chuang, K. H.; Xue, J. M. *Small* **2012**, *8*, 3620.
42. Otakar, F. http://www.nanocarbon.cz/.
43. Ferrari, A. C.; Basko, D. M. *Nat. Nanotechnol.* **2013**, *8*, 235.
44. Ferrari, A. C.; Meyer, J. C.; Scardaci, V.; Casiraghi, C.; Lazzeri, M.; Mauri, F.; Piscanec, S.; Jiang, D.; Novoselov, K. S.; Roth, S.; Geim, A. K. *Phys. Rev. Lett.* **2006**, *97*, 187401.