



Biosensors based on graphene oxide and its biomedical application☆



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ABSTRACT

Graphene oxide (GO) is one of the most attributed materials for opening new possibilities in the development of next generation biosensors. Due to the coexistence of hydrophobic domain from pristine graphite structure and hydrophilic oxygen containing functional groups, GO exhibits good water dispersibility, biocompatibility, and high affinity for specific biomolecules as well as properties of graphene itself partly depending on preparation methods. These properties of GO provided a lot of opportunities for the development of novel biological sensing platforms, including biosensors based on fluorescence resonance energy transfer (FRET), laser desorption/ionization mass spectrometry (LDI-MS), surface-enhanced Raman spectroscopy (SERS), and electrochemical detection. In this review, we classify GO-based biological sensors developed so far by their signal generation strategy and provide the comprehensive overview of them. In addition, we offer insights into how the GO attributed in each sensor system and how they improved the sensing performance.

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1. Introduction

1.1. Brief introduction on graphene oxide (GO)

In 2004, Andre Geim and Konstantin Novoselov reported a methodology for isolation of graphene and were awarded the Nobel Prize in Physics in 2010 for “ground breaking experiments regarding the two dimensional material graphene” [1]. Since its isolation, this two-dimensional (2D) carbon sheet has drawn immense attention and researches revealed its high planar surface area calculated as $2630 \text{ m}^2 \text{ g}^{-1}$, superior mechanical strength with a Young's modulus of 1100 GPa, remarkable thermal/electrical conductivity, and optical property [2,3]. Owing to these exceptional properties, graphene and its derivatives have been actively employed in a lot of applications such as transparent electrode, energy storage, cell scaffold, biosensor, drug delivery system, and catalysis to date [3–6].

Graphene oxide (GO), generally obtained by oxidation of graphite in a mixture of strong acid and oxidizing agent, is a water-dispersible graphene derivative (Fig. 1a) [7–10]. The oxidation process results in partial breaking of the sp^2 hybridized structure of graphite and increasing the distance between carbon layers [11,12]. The precise structure of graphite oxide is still under debate to date, but it is certain that GO possesses both hydrophobic part from pristine graphite structure and hydrophilic part with oxygen containing functional groups such as hydroxyl, epoxy, carbonyl, and carboxyl groups on the basal plane and at the edge, generated by oxidation process (Fig. 1b,c) [13,14]. In terms of its fabrication, GO is commonly produced by using the Brodie, Staudenmaier, and Hummers methods or these methods with minor modifications [8]. The partial breaking of the conjugated structure localizes π -electrons, resulting in the decrease of the overall electrical conductivity. However, the remaining sp^2 domains with added hydrophilic groups during oxidation process make GO exhibit unique properties, such as affinity for aromatic rings and fluorescence-quenching capability, while maintaining high dispersibility in aqueous solvents. The hydrophilic nature of GO generated from the oxygen containing functional groups gives good water dispersibility and biocompatibility, which are highly important features in bio-applications. These properties of GO have provided a lot of opportunities for the development of novel biological sensing systems [11,12,15–18].

GO can sometimes serve as a sensing element itself due to its own properties such as Raman signal or fluorescence observed in some specially prepared GO. Frequently, GO can be employed as a precursor of reduced graphene oxide (rGO) possessing chemical structures closer to pristine graphene or graphene nanocomposites [3,18,19]. The rGO can be prepared starting from GO by the treatment of various reducing agents, such as hydrazine monohydrate, sodium borohydride, and hydroquinone [20–22], which eliminate most of the oxygen containing functional groups of GO and partially restore the electrical conductivity of GO. For example, reduction process can lower the sheet resistance up to $14 \text{ k}\Omega/\text{sq}$, which is about 2-order higher than that of pristine graphene, whereas as-synthesized GO exhibits a sheet resistance of about $10^{12} \Omega/\text{sq}$ or higher [23]. In addition to rGO, graphene nanocomposites produced by the combination of rGO with other functional nanomaterials exhibited significant enhancement in the original properties of graphene or synergistic addition of new function to the properties of graphene [3,17,18,24–26]. In this review, we focus on the bioanalytical applications of GO and its derivatives and discuss the significance, shortcoming, and future perspectives of the GO-based bioanalytical systems.

1.2. General strategies for GO-based biosensors

We first offer brief overview on the role of the GO adapted in each sensor system and how it improved the sensing performance. Basically, properties of GO have been utilized in many different types of biosensors, which can be representatively classified into biosensors based on (1) fluorescence resonance energy transfer, (2) laser desorption/ionization mass spectrometry (LDI-MS), (3) surface-enhanced Raman spectroscopy (SERS), and (4) electrochemistry (Fig. 2, Table 1). First of all, the excellent efficiency of energy/charge transfer from dye to GO enabled the development of a lot of FRET-based biosensors. Common strategy in these applications relies on the high energy/electron transfer capability and the amphiphilicity of GO [12,27,28]. These properties make GO capable of (1) strong binding with biomolecules through π - π stacking and/or hydrogen bonding interactions and (2) the fluorescence quenching of nearby fluorescent dye by the process of energy transfer from the excited state of the dye to GO [29–34]. Particularly, preferential binding of single stranded nucleic acid (NA) on GO

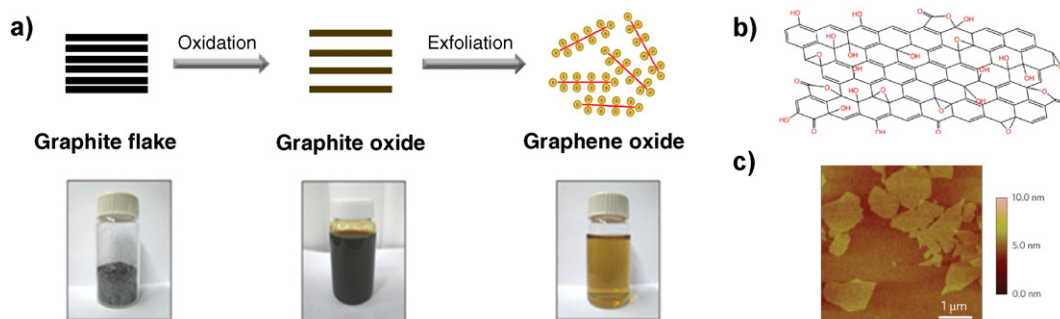


Fig. 1. (a) Chemical route to the synthesis of aqueous graphene dispersions. (b) The expected chemical structure of a single sheet of GO. (c) AFM image of the GO on a silicon substrate showing an average thickness of around 1 nm. Adapted and reproduced with permission from (c) Gao et al. [14], Copyright 2009, Nature Publishing Group.

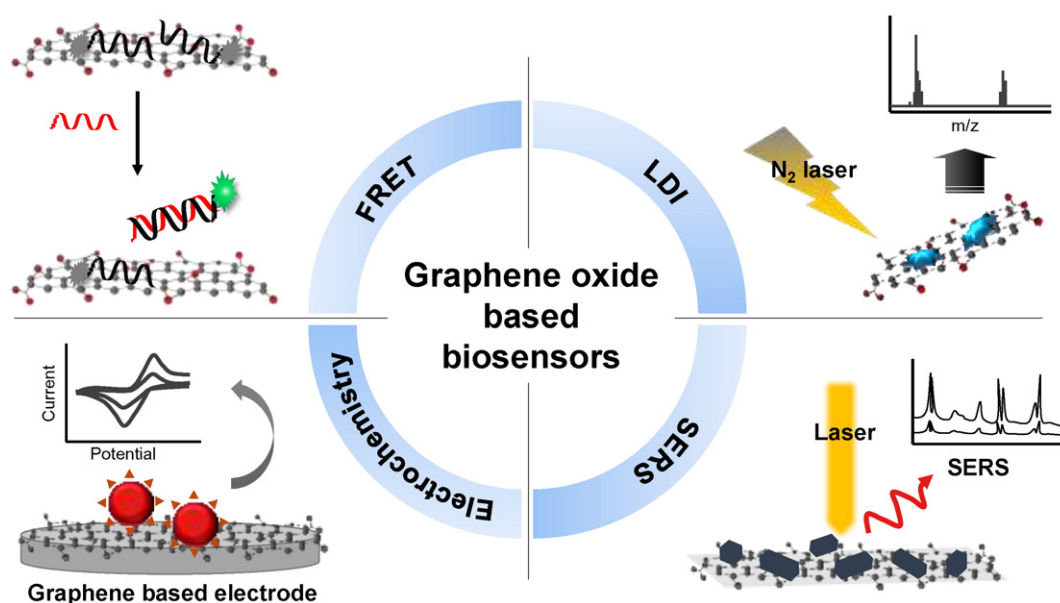


Fig. 2. GO-based biological sensors classified depending on the type of the detection signal.

compared to double-stranded NA was one of the most popular principles in the fluorescent GO utilized biosensors [35–38]. Second, GO is considered as a potential matrix for LDI-MS that can be employed as an alternative to conventional small molecule matrix in bioanalysis. GO has strong absorbance at the excitation laser wavelength of 337 or 355 nm and high affinity toward various amphiphilic biomolecules [39,27,40]. Also, oxygen containing functional groups generated by oxidation of graphene make GO readily soluble in aqueous solvents while maintaining its ability for hydrogen bonding with other molecules and facile proton donation toward analytes [39]. These properties of GO enable efficient energy transfer followed by ionization/desorption of the analytes, which is similar to the process associated with typical energy absorbing matrix. In addition to the highly efficient energy transfer and ionization of analytes, the GO utilized as a matrix provides clean MS spectra by minimizing undesired interference peaks in the low

molecular weight region that are generally observed in MS spectra obtained by using typical organic matrix [41]. Third, GO and rGO have been favorably received in the field of electrochemical sensors due to their outstanding performance in electro-catalysis, significantly lower charge-transfer resistance compared to graphite, and glassy carbon electrodes (GCEs) [26]. The electrochemical applications of GO can be further broaden through fabrication of its hybrid composites with other conducting or semiconducting materials, which enhances the electron transfer capability in the electrode [6]. Moreover, GO has been actively employed in SERS-based biosensors. Many studies revealed that the GO can quench the background fluorescence signal as well as enhance the Raman signal by chemical enhancement induced by electron transfer [42–44]. Recently, a lot of SERS-based sensors have been demonstrated with hybrid structures of graphene and noble metal nanoparticles (e.g., Au and Ag) which can induce electromagnetic Raman enhancement [45,46]. The hybrid-nanocomposites can generate the synergistically enhanced SERS signal through two kinds of enhancement mechanisms. In this review, we classify the GO-based biological sensors by its sensing strategy and its purpose in the applied fields, and discuss comprehensive overview covering the latest development.

Table 1

Summary of GO-based biosensors using various detection techniques.

Detection technique	Characteristics of GO	Purpose
FRET	(1) Strong binding with biomolecules through pi-pi stacking and/or hydrogen bonding interactions, (2) the fluorescence-quenching capability of nearby fluorescent dye	Biomolecule detection [47–65], signal amplification [106–115,117–123], enzyme assay [124–135], cell/tissue imaging [141–149], high-throughput screening [152]
LDI-MS	(1) Strong absorbance at the excitation laser wavelength of 337 or 355 nm, (2) high affinity toward various amphiphilic biomolecules (electrostatic/hydrophobic/pi-pi stacking interaction), (3) easy protonation of analytes by functional groups on GO	Biomolecule detection [66–78], enzyme assay [136,137], cell/tissue imaging [74,76], high-throughput screening [136]
Electrochemistry	(1) Outstanding electrocatalytic ability, (2) low charge-transfer resistance	Biomolecule detection [79–95], signal amplification [116], enzyme assay [138–140]
SERS	(1) Quenching the background fluorescence signal, (2) chemical enhancement in SERS induced by electron transfer	Biomolecule detection [96–105], cell/tissue imaging [150,151]

2. Biomolecule detection

2.1. FRET-based biosensors

2.1.1. Fluorescent biosensors using natural DNA probe

In 2009, Lu *et al.* firstly reported a GO-based fluorescent biosensor using two kinds of DNA probes, complementary sequenced DNA for target DNA detection and aptamer DNA for protein detection (Fig. 3a) [47]. The sensors demonstrated that each quenched fluorescence signal of the dye conjugated DNA probe adsorbed on GO could be effectively recovered when the probe formed a duplex with its corresponding DNA or protein target, which allowed the release of the probe from the GO. Since then, there has been increasing interest in GO utilized biosensors based on fluorescent signal detection. For example, He *et al.* reported a GO-based multicolor DNA sensing system that allowed selective detection of multiple DNA targets in homogeneous solution and molecular dynamics (MD) simulation of the interactions between ss- and dsDNA with GO (Fig. 3b) [48]. The large planar surface of GO allowed simultaneous quenching of three DNA probes labeled with different dyes, leading to a multicolor sensor for the detection of multiple DNA targets in one solution with limit of detection (LOD, defined as $LOD = 3.3$

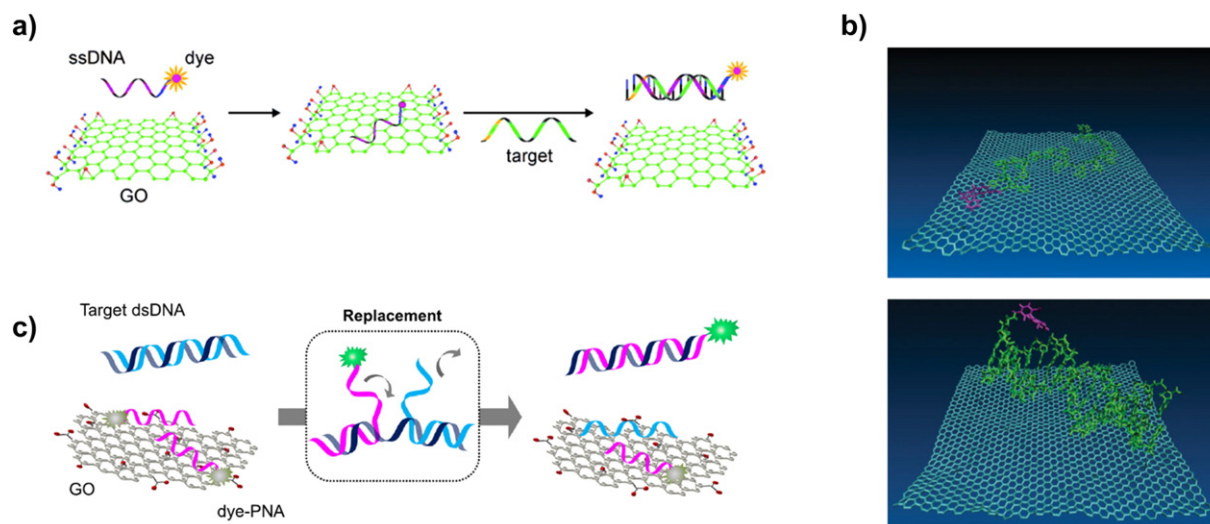


Fig. 3. (a) Scheme of the ssDNA target-induced fluorescence change of the DNA probe-FAM-GO complex. (b) Representative snapshots from MD simulation showing that fluorescent dye tagged ssDNA, which is tightly adsorbed at the surface of GO (upper), and dsDNA, which does not adsorb at the surface of GO (bottom). (c) Scheme of the direct detection of dsDNA using PNA and GO. Adapted and reproduced with permission from (a) Lu et al. [47], Copyright 2009, Wiley-VCH; (b) He et al. [48], Copyright 2010, Wiley-VCH; (c) Lee et al. [63], Copyright 2014, Elsevier.

(standard deviation of the response (σ)/slope of the calibration curve (S)) of 100 pM. They also demonstrated that the GO-based sensing platform is feasible to detect metal ion as well as small molecules when complemented with the use of each known aptamer. This preferential adsorption of ssDNA over DNA target complex onto GO has been broadly utilized to detect and quantitatively analyze biologically meaningful target molecules including DNA, RNA, protein, and small molecule [49–57]. Unfortunately, although DNA probe can precisely recognize its complementary target, it appears that DNA probe adsorbed on GO can be nonspecifically desorbed by other biomolecules such as proteins and other NA strands which also showed high affinity to GO, resulting in generation of the noise or high background signal. Therefore, there have been many attempts to overcome the critical shortcoming in practical uses. For example, quantum dot, upconversion nanophosphor or fluorescent Ag nanocluster have been introduced in FRET-based sensors instead of the organic dye conjugated DNA probe to enhance the sensitivity and signal to noise [58–61]. Besides, the use of artificial probe showing higher affinity to GO and the introduction of signal amplification strategies have been also carried out for improving the original FRET biosensors.

2.1.2. Fluorescent biosensors using artificial PNA probe

As mentioned above, researchers started to employ artificial nucleic acids as a probe in the GO-based sensor system to improve the DNA probe used system. Peptide nucleic acid (PNA) probe, one of the DNA analogues, has uncharged amide bonds instead of phosphate backbone, which enable its binding with not only complementary DNA or RNA with extremely high sequence specificity, but also negatively charged GO surface strongly, compared to natural DNA probes [62]. These two key features enable for the GO-based sensor utilizing PNA as a probe to detect NA with high sensitivity and low background signal even in biological samples such as blood serum and cell lysate in which a lot of biomolecules such as proteins and lipids can interact with GO and thus, possibly induce nonspecific desorption of the single stranded probes from GO if the binding affinity of the probes to GO surface is relatively weak. Above this, Lee *et al.* reported a GO-based direct dsDNA detection system utilizing invasive binding property of PNA to DNA duplex in which PNA replaces one DNA strand in DNA duplex, without requiring any thermal control for dsDNA denaturation (Fig. 3c) [63]. They also developed a strategy for recognizing one base difference in a miRNA family using PNA probe, presenting extremely high sequence specificity [64]. The combination of PNA and GO allows the quantitative

fluorometric NA sensing even in the biological samples without a wide variation of LOD, which was impossible to achieve by using natural DNA probe. To improve the performance of the PNA utilized FRET sensors, bovine serum albumin (BSA) was used as an additive for minimizing nonspecific adsorption of biomolecules to GO surface and for effectively desorbing the target/PNA duplex from GO surface while keeping the interaction between ssPNA and GO is unaffected [65].

2.2. LDI-MS-based biosensors

2.2.1. LDI-MS-based biosensors using soluble GO

In 2010, Dong *et al.* revealed that GO can be adapted as a novel matrix for analysis of small molecule compounds using LDI-MS [66]. Typical organic matrix exhibits low salt tolerance, low reproducibility, and high background interference peaks due to fragmentation of analyte especially in the low molecular weight range in the mass spectra. By employing GO in place of typical organic matrix in LDI-MS, the researchers successfully analyzed biologically important small molecules such as amino acid, steroid, anticancer drugs, and nucleoside, exhibiting little fragmentation of analyte, high salt tolerance, low background interferences, and good reproducibility. Gulbakan *et al.* established a smart platform using GO-based affinity probes for LDI-MS analysis of trace amount of analytes (Fig. 4a) [67]. By covalent modification of GO with target specific aptamer, GO could capture the target, energy transfer, and ionized analyte selectively even in biological samples. Tang *et al.* reported that GO can be used in LDI-based bioanalysis for enrichment and detection of protein [41]. They demonstrated that GO can interact with proteins by electrostatic interaction and enable to extract and detect the target with femtomolar-level LOD. Besides, the GO dispersed in aqueous solution was also used for LDI-MS analysis of other small molecules, environmental pollutant, and even polymer [68–70]. Moreover, there have been recent attempts to develop multifunctional matrix by adding other materials such as Fe_3O_4 , TiO_2 , CoNP, and AuNP to the GO scaffold [71, 74]. These GO-based nanocomposite matrix could provide additional functions including target enrichment for determination of the low abundant analytes and functionalization of targeting agent and signal transducer for tumor tissue imaging.

2.2.2. LDI-MS-based biosensors using the GO derivative coated solid substrate

GO is available in the form of LDI substrate which presented the technical simplicity and high reproducibility compared to the typical

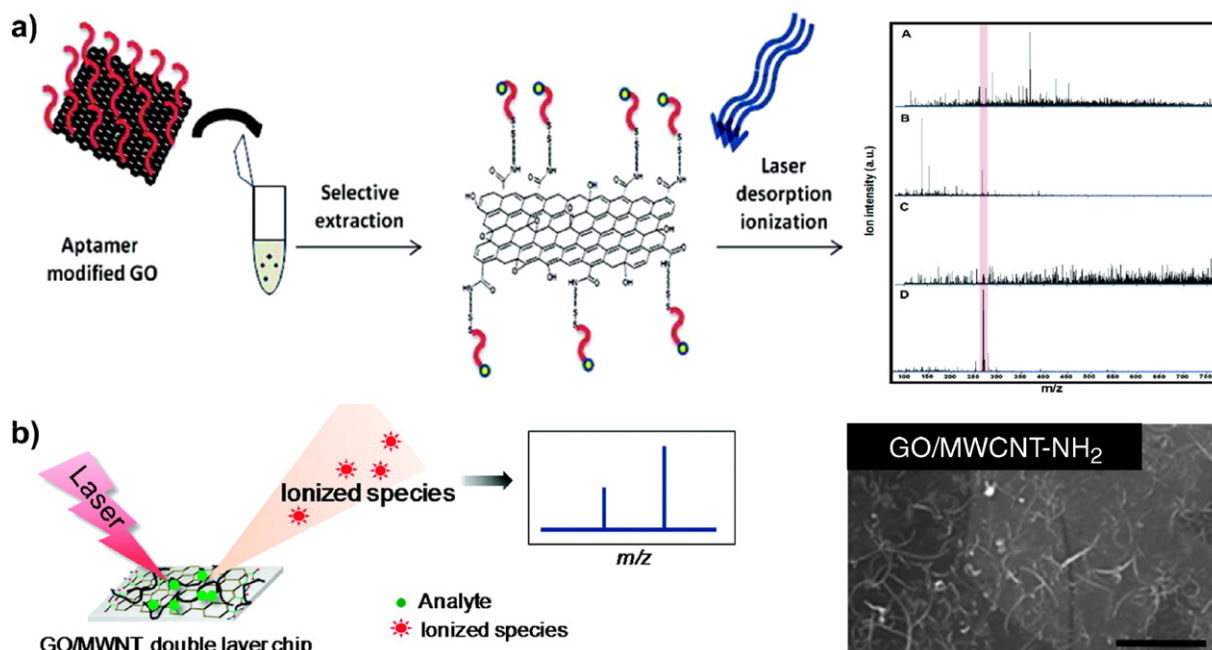


Fig. 4. (a) Scheme showing aptamer modification and GO-assisted target capture and analysis. (b) Scheme of LDI-MS based on GO/MWCNT double-layer film-coated substrate and scanning electron microscope (SEM) image of GO/MWCNT film. Adapted and reproduced with permission from (a) Gulbakan et al. [67], Copyright 2010, American Chemical Society, and (b) Kim et al. [75], Copyright 2011, American Chemical Society.

matrixes such as organic small molecules and other nanomaterials usually applied in the form of solution or suspension during sample preparation. Kim *et al.* developed a novel sensing platform using solid LDI substrate coated with GO and multi-walled carbon nanotube (MWNT) to enhance the surface roughness and area for analyte adsorption and, thus, increase LDI efficiency (Fig. 4b) [75]. By depositing small molecule analytes on the GO/MWNT double-layer films, the surface enabled efficient LDI-MS-based analysis of analytes with little fragmentation, high salt tolerance, and relatively regular mass intensities at any region of the sample spot. In this work, they showed that the film fabricated on solid substrates had excellent durability against mechanical force, photoagitation, and prolonged exposure to ambient conditions, and applicability to tissue imaging mass spectrometry. The same research group also reported the multilayer films of GO/MWNT, and gold nanoparticle (AuNP)/GO hybrid LDI film for the sensing of small molecules including glucose, mannitol, and cellobiose [76,77]. In addition, Qian *et al.* reported matrix free MS detection and imaging system using the laser engineered graphene paper produced by filtration of rGO sheets. The graphene paper possessed enhanced stability, hydrophobicity, and conductivity, which enabled hydrophobic analyte detection with high reproducibility by using only 50% of typical laser intensity [78].

2.3. Electrochemical sensors

In 2009, Zhou *et al.* firstly reported the simultaneous electrochemical sensing of all four DNA bases on the rGO-based electrode (Fig. 5a) [79]. Without the need of pre-hydrolysis procedure required for efficient oxidation of each base, four bases in both ssDNA and dsDNA were detected at physiological pH by using the rGO/GC electrode, which showed higher electrochemical activity than graphite and bare GC electrode. Furthermore, the electrochemical detection of single-base difference was demonstrated at physiological pH. For another example, Feng *et al.* reported an electrochemical impedance sensor (EIS) for label-free detection of cyclin A₂, a prognostic indicator in early stage cancers, using a porphyrin functionalized graphene modified electrode [80]. The attachment of cyclin A₂ on the electrode hampers the proximal interaction of the redox probe closer to the electrode, resulting in the increase of the electron transfer resistance and decrease in the change of

electrochemical impedance signal. Bonanni *et al.* developed a label-free DNA EIS using stably adsorbed hairpin probe on graphene through pi-pi stacking interaction, which enabled detection of a single nucleotide polymorphism (SNP) related to the development of Alzheimer's disease (Fig. 5b) [81]. When the hairpin pre-adsorbed on rGO recognizes and binds to target strands, charge-transfer resistance (R_{ct}) value significantly decreases. The sensor could sensitively recognize its target DNA even at the femtomolar concentration level due to the synergistic effect of the integrated PXa-rGO nanocomposite. Furthermore, many researchers developed a lot of different versions of electrochemical sensors by combining GO-based electrode with polymer or metal nanoparticle and demonstrated its applicability in the detection of protein, small molecule, metal ion, cell, and NA [82–95].

2.4. SERS-based biosensors

The nanocomposites composed of graphene and noble metals have been widely utilized as Raman signal enhancing substrate in various SERS-based biosensors [43]. These hybrid systems showed synergistically enhanced sensing performance compared to the typical systems with one kind of metal. For example, Ren *et al.* reported a GO – poly (diallyldimethylammonium chloride) (PDDA)–AgNP hybrid structure utilized to enhance the Raman signal of folic acid (FA), an important biomarker for cancer and heart attack [96]. Due to the presence of positively charged PDDA, the negatively charged FA could be adsorbed on the GO–AgNP hybrid structure, inducing the enhancement of Raman signal of FA. This sensor exhibited a linear response between 9 and 180 nM with LOD of 9 nM even in biological samples. Fan *et al.* developed another type of hybrid SERS probe consisting of GO and popcorn-shaped gold nanoparticle to detect HIV DNA and bacteria (Fig. 5c–e) [97]. This system enabled the label-free detection of HIV DNA with the femtomolar concentration level and MRSA bacteria with LOD of 10 CFU/mL. In addition, the various GO-noble metal nanocomposites have been widely applied to detect extremely small amount of pigments in food, H₂O₂, glucose, and polar antibiotics [98–103]. In addition to its role in the enhancement of SERS signal, the combination of graphene with metal nanoparticles has been used as a favorable hybrid structure for preventing the surface oxidation of metal nanoparticles [104,105].

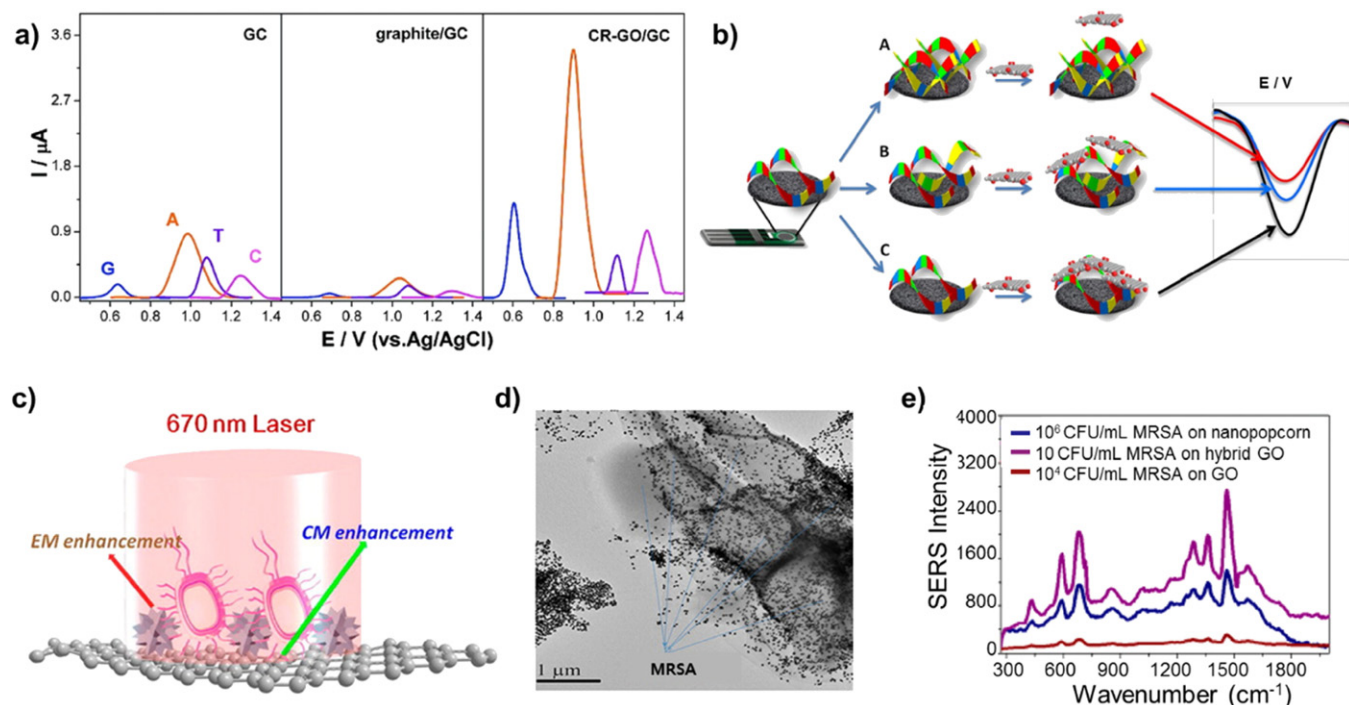


Fig. 5. (a) Differential pulse voltammograms obtained by using GC, graphite/GC, or CR-GO/GC electrodes for all four DNA bases. (b) GO nanoplatelets (GONPs) as electroactive labels for DNA analysis. The hybridization step was performed with complementary target (A), one-mismatch target (B), and noncomplementary target (C). (c) Hybrid GO (GO/popcorn-shaped gold nanoparticle) as ultrasensitive SERS probe for label-free biosensing through both electromagnetic mechanism (EM) and chemical mechanism (CM) enhancement. (d) TEM image of hybrid GO sheet, which binds to methicillin-resistant *Staphylococcus aureus* (MRSA). (e) SERS spectra of MRSA with three kinds of probes. Adapted and reproduced with permission from (a) Zhou et al. [79], Copyright 2009, American Chemical Society, (b) Bonanni et al. [81], Copyright 2012, American Chemical Society, and (c, d) Fan et al. [97], Copyright 2013, American Chemical Society.

3. Signal amplification

3.1. Enzymatic signal amplification in the GO-based fluorescent biosensors

Conventional GO-based FRET sensors have hardly detected the extremely low amount of analyte under femtomolar concentration level and, therefore, not been actively applied in practical diagnosis. To overcome this limitation, many researchers have challenged to introduce enzymatic signal amplification strategy in typical GO-based sensors (Table 2). Cui *et al.* designed cyclic enzymatic signal amplification for sensitive fluorescent miRNA detection in biological samples [106]. They used DNase for selective digestion of DNA probe hybridized with target miRNA, which induces recycling of the miRNA and the consecutive desorption of multiple probe strands from the GO surface (Fig. 6a). This sensor achieved the detection sensitivity of about three orders of magnitude higher than non-amplified system, which became suitable for sensing of the extremely low amount of miRNA in real biological samples. The so-called target recycling strategy was generally based on the formation of DNA/target hetero complexes and the enzymatic digestion of probe by DNA nuclease such as endo/exonuclease and DNase, which was demonstrated in the detection of various biomolecules such as protein, small molecule as well as RNA [107–112]. Other

research groups reported different types of strategies for enzymatic signal amplification using the catalytic activity of polymerase which produced huge amount of DNA duplex by triggering the strand displacement polymerization reaction and recycled short single stranded DNA [113–115]. These strategies utilizing the enzyme mediated digestion of DNA probes were also employed for amplifying the electrochemical signals in GO-based biosensors [116]. For another example of polymerase mediated amplification, Liu *et al.* reported a highly sensitive enzymatic signal amplification method by adopting rolling circle amplification (RCA) strategy using polymerase in the GO-based sensing system (Fig. 6b) [117]. In the presence of small molecule target, the DNA probe consisting of target binding aptamer part and polymerase recognizing primer part was desorbed from GO and started to generate long DNA products with thousands of repeating sequences. The repeated sequence acted as a template to hybridize with MB, followed by intermolecular hybridization between target and MB, exhibiting intense fluorescent signal. The protein enzymes were replaced by DNA enzyme, so-called DNAzyme, which recognized specific sequences of NA as well as catalyzed specific chemical reactions on NA (Fig. 6c) [118,119]. In fact, while protein enzymes showed higher catalytic activity than DNAzyme, the DNAzyme composed of DNA is highly stable compared to protein in general and hardly induces nonspecific desorption of

Table 2
Advantages and disadvantages of different signal amplification strategies.

Amplification strategy		Advantages	Disadvantages	Reference
Enzymatic amplification	Enzymatic recycling	Fast recycling by high catalytic activity	Requirement of additional cofactors and high cost	106–115
	DNAzyme mediated recycling	Simple components and process	Slow recycling	118, 119
	Rolling circle amplification	Fast production of repeated sequence	Requirement of large DNA circles at least 100 nt in length	117
Enzyme-free amplification		Applicability in in situ imaging	High cost in general	120–123

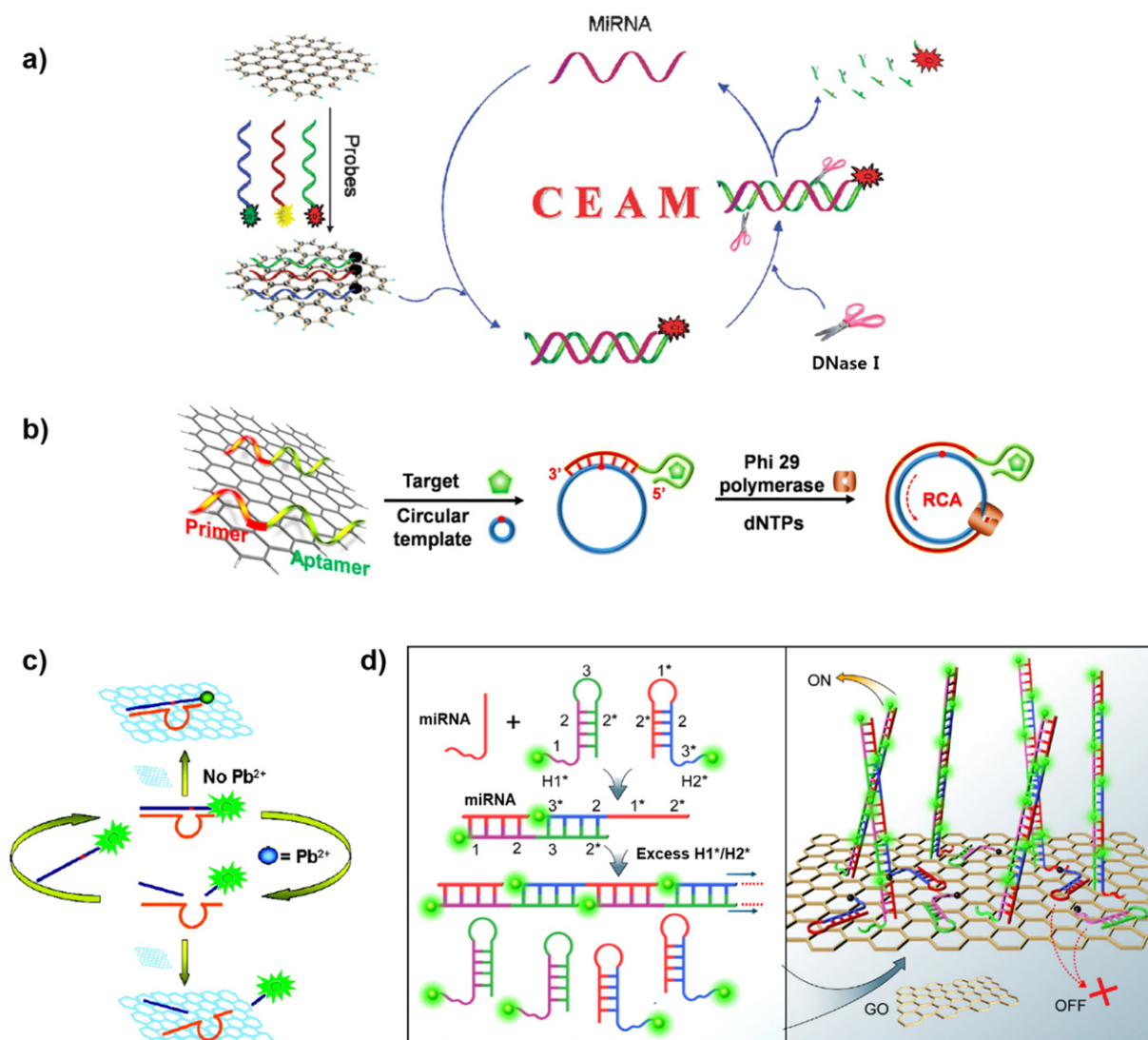


Fig. 6. (a) Cyclic enzymatic signal amplification method (CEAM) for sensitive miRNA detection. (b) Signal amplification method by using rolling circle amplification (RCA). (c) DNAzyme-based signal amplification for the detection of Pb^{2+} ion. (d) Enzyme-free signal amplification strategy using hybridization chain reaction (HCR). Adapted and reproduced with permission from (a) Cui et al. [106], Copyright 2012, Royal Society of Chemistry, (b) Liu et al. [117], Copyright 2014, American Chemical Society, (c) [118], Copyright 2011, American Chemical Society, and (d) Yang et al. [120], Copyright 2012, American Chemical Society.

probes from GO compared to protein enzymes. Therefore, to develop efficient enzymatic signal amplification, it is important to consider both advantages and disadvantages of two kinds of enzymes.

3.2. Enzyme-free signal amplification in GO-based fluorescent biosensors

In addition to the enzymatic signal amplification strategies, the enzyme-free signal amplification strategies were practiced in the GO-based fluorescent sensors (Table 2). For example, hybridization chain reaction (HCR) was developed, in which two species of dye-labeled DNA hairpin probes, amplification reagents, were polymerized into a long nicked double-stranded DNA (dsDNA) structure only in the presence of target (Fig. 6d) [120]. The dye-labeled hairpins participating in the formation of long dsDNA were desorbed from GO and the fluorescence signal was recovered while the closed hairpins were adsorbed on GO with maintaining quenched fluorescent signal. The detection sensitivity of the proposed HCR/GO sensing platform was at least 2 orders of magnitude higher than the GO-based typical 1:1 target/probe binding system. The HCR strategy was also utilized in signal amplified sensing of metal ion or small molecule [121,122]. Unlike enzymatic amplification strategy, HCR does not require any additional cofactor molecule or

high concentration of multivalent cations to amplify the target specific signal. More recently, the HCR/GO strategy was applied in situ imaging of miRNA in living cells to monitor the dynamic expression and distribution of miRNA [123]. In this work, GO enabled effective intracellular delivery of amplification reagents as well as activation of the signal switch. Up to date, various fluorescence-based sensing strategies utilizing signal amplification with or without enzyme have been developed, broadening the boundary of practical application of GO-based sensors.

4. Enzyme activity assay

4.1. FRET-based assay platforms

Along with biomolecule detection, researchers have developed a number of enzyme activity assay platforms using GO on the basis of the common features employed in the GO-based biosensors. The first GO-based enzyme activity assay method was reported by Jang *et al.*, demonstrating a new analytical method for measuring real-time duplex DNA-unwinding activity of helicase in 2010 (Fig. 7a) [124]. The sensor quantitatively monitored helicase mediated unwinding reaction of dsDNA by measuring the change in fluorescence signal that was

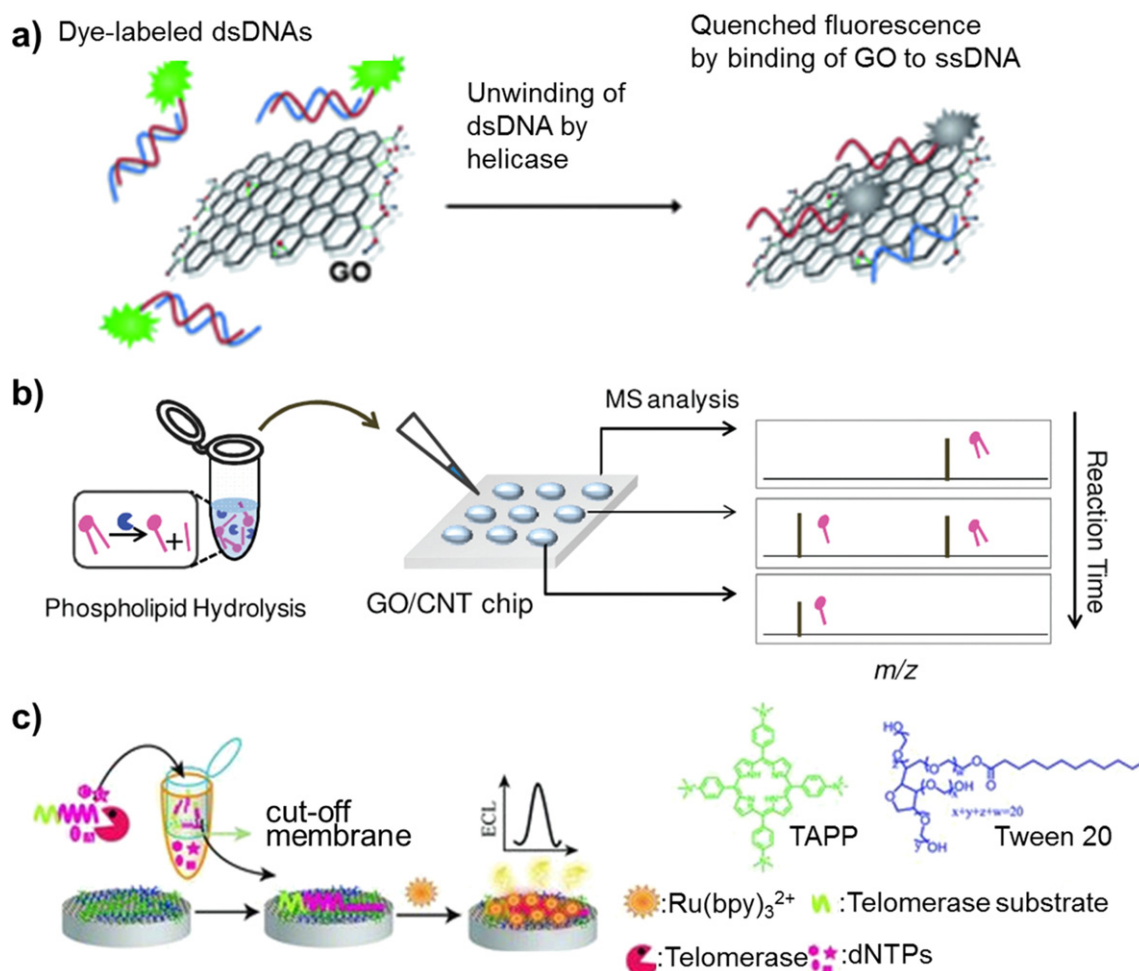


Fig. 7. (a) GO-based assay for DNA-unwinding helicase activity. (b) Mass spectrometric analysis for lipase activity assay based on GO/MWNT films. (c) Electrochemiluminescence assay based on graphene modified electrode for telomerase activity measurement. Adapted and reproduced with permission from (a) Jang et al. [124], Copyright 2010, Wiley-VCH, (b) Lee et al. [137], Copyright 2010, American Chemical Society, and (c) Wu et al. [140], Copyright 2012, Wiley-VCH.

modulated by the adsorption of the ssDNA generated from dsDNA by helicase activity on the GO surface, followed by quenching of the fluorescence of the dye conjugated to ssDNA. Starting with this report, many studies were devoted to the development of fluorescent enzyme activity assay platforms applicable for various types of enzymes such as DNA dependent kinase, endonuclease, methyltransferase, exonuclease, polymerase, uracil DNA glycosylase, and telomerase [125–130]. All these studies utilized NA as a probe interacting with GO surface. In particular, Park *et al.* established an assay platform to monitor RNA synthesis by RNA polymerase using a PNA probe [127]. The strong hybridization between PNA and RNA product made the assay independent of the harsh reaction environment such as high temperature and high salt concentration.

In another example of the GO-based enzyme activity assay, Zhang *et al.* demonstrated a thrombin activity assay platform using peptide as probe biomolecule, which served as a substrate of target enzyme [131]. The dye conjugated peptide was mainly composed of positively charged amino acids (e.g., lysine, arginine, histidine) and hydrophobic amino acid residues (e.g., tryptophan, tyrosine, phenylalanine) and, thus, could be adsorbed on the negatively charged GO and the fluorescence of the dye was quenched. Upon the cleavage of the peptide by thrombin, dye containing short peptide was desorbed from GO, generating fluorescence recovery dependent on the protease activity. On the basis of the electrostatic interaction between positively charged peptide and negatively charged GO, other platforms were also developed, for example, to measure the activity of protein kinase and

phosphatase [132,133]. In addition, another example was reported in which the GO covalently linked to the fluorophore-labeled peptide was utilized as a kind of FRET substrate for monitoring peptide cleavage activity of proteases [134,135].

4.2. LDI-MS-based assay platform

In the LDI-MS-based assay system, labels on the enzyme substrate are not required for measuring the amount of enzyme substrate/product, which is one of the most desired advantages of the MS-based assay system. The label-free assays are preferred because the chemical modification of substrate during labeling can unexpectedly alter its affinity with the corresponding enzyme. For label-free enzyme activity assay, GO was employed as matrix to analyze substrates and products of enzyme, detecting molecular weight changes before and after enzyme reactions. Liu *et al.* suggested the highly efficient acetylcholinesterase (AChE) assay platform by utilizing GO and functionalized magnetic carbonaceous (MC) microspheres [136]. The substrate and product were easily separated from AChE immobilized onto the MC microspheres and analyzed by LDI-MS using GO as matrix. In addition, Lee *et al.* reported the phospholipase assay platform using the GO/MWNT LDI films on which the hydrophobic phospholipid and fatty acid, substrate, and product of phospholipase, respectively, could be easily ionized and desorbed by laser irradiation (Fig. 7b) [137]. Conventionally, phospholipase activity is hard to quantitatively measure without any labels such as radioisotope or fluorophore. This GO-based sensor enabled quantitative and

sensitive measurement of phospholipase activity, exhibiting technical simplicity and compatibility with an array format and feasibility for multiple enzymatic assays based on mass spectrometric analysis without requiring any labels.

4.3. Electrochemical assay platform

In 2009, Shan *et al.* reported an electrochemical assay platform for glucose oxidase (GOD) activity using polyvinylpyrrolidone-protected graphene that showed good dispersibility in water and electrochemical reduction toward O_2 and H_2O_2 [138]. These two properties enabled its linear response to glucose concentration up to 14 mM by direct electron transfer of GOD. On the other hand, Li *et al.* developed a new method for DNA methyltransferase (MTase) activity assay in which GO was used to load multiple reporter molecules to the end of DNA probe [139]. Upon the enzymatic reaction with MTase followed by endonuclease, reporter molecules (thionine) conjugated to methylated DNA maintained electrochemical signal, whereas reporters conjugated to non-methylated DNA was cleaved and released from electrode. This system could determine DNA methylation at the site of CpG and discriminate the single-base mismatched sequence in the target DNA sequence with high sensitivity. For another example, Wu *et al.* constructed a label-free electrochemiluminescence sensor for telomerase detection based on a cationic porphyrin functionalized chemically converted graphene (TAPP/CCG)-modified glassy carbon electrode (Fig. 7c) [140]. The

elongated DNA by telomerase induced the adsorption of positively charged signal reporter, $Ru(bpy)_3^{2+}$ onto electrode surface through electrostatic interaction. In this sensor, graphene served as an electrode possessing excellent electrical conductivity as well as a substrate interacting with the elongated DNA produced by telomerase.

5. Biosensing in cell/tissue

5.1. FRET-based imaging

Because GO is highly biocompatible and size-controllable into tens of nanometer [141–143], many studies were performed to deliver the GO/probe complex into living cells and, consequently, apply the GO-based sensing system in live cell imaging. In 2010, Wang *et al.* demonstrated an ATP *in situ* imaging system by designing the dye-labeled DNA aptamer/nanosized GO complex that become internalized into living cells [144]. The authors demonstrated that GO could play multiple roles as an efficient DNA cargo for cellular delivery, excellent protector of genes against nuclease mediated degradation and fluorescence quencher for biosensing platform. For another approach, Wang *et al.* developed the GO sheet covalently conjugated with fluorescent dye-labeled caspase-3 substrate peptide as a fluorescent substrate for caspase-3 activity assay in cells [145]. The peptide conjugated GO sheet was utilized to monitor the activity of caspase-3 and to obtain the images representing its activity in live cells. Another strategy

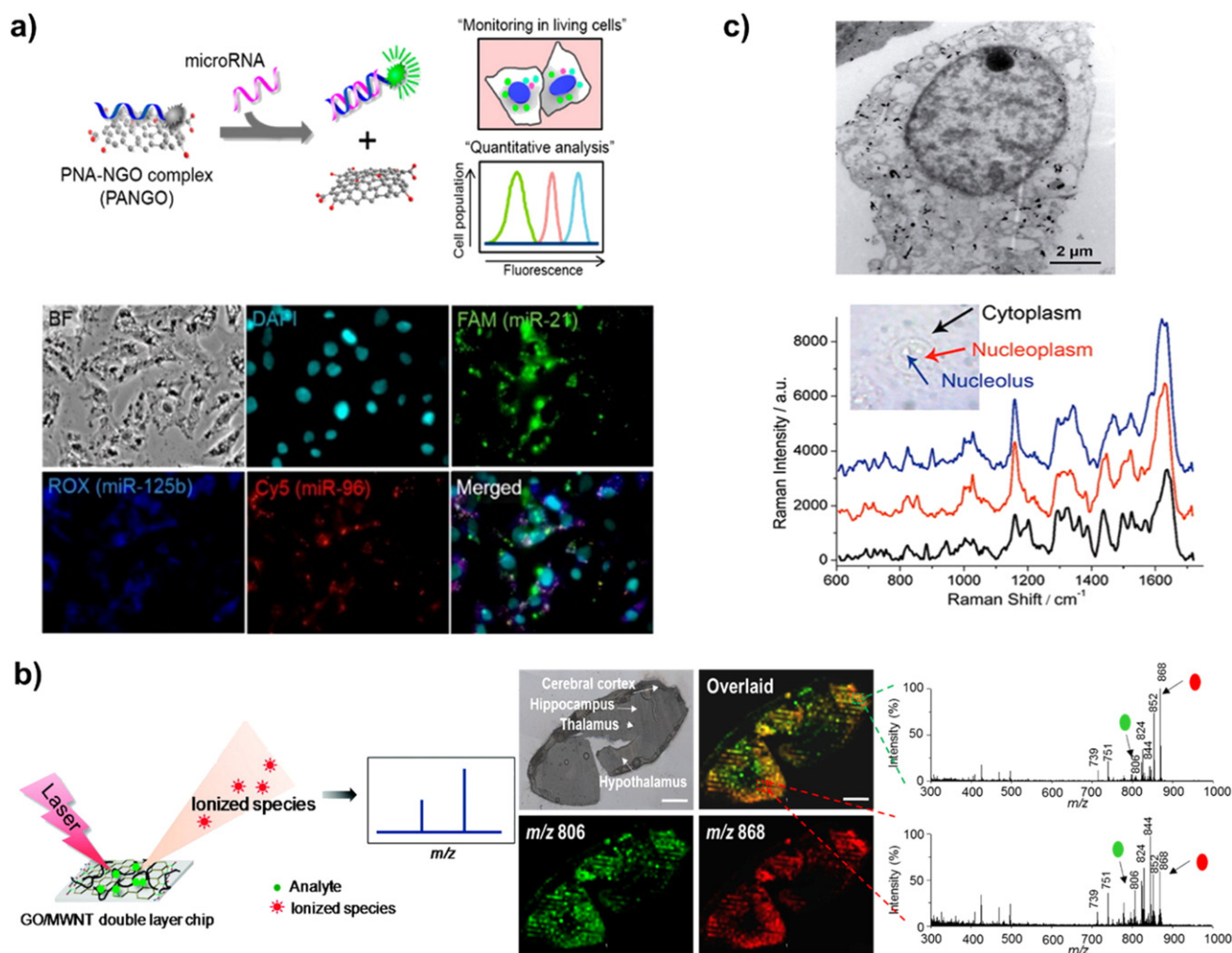


Fig. 8. (a) PANGO-based multiplex miRNA imaging in cells. (b) GO/MWNT-based tissue imaging using LDI-MS. (c) SERS-based bio-imaging through GO-AuNP hybrid nanostructure. Adapted and reproduced with permission from (a) Ryoo *et al.* [146], Copyright 2013, American Chemical Society, (b) Kim *et al.* [75], Copyright 2011, American Chemical Society, and (c) Liu *et al.* [150], Copyright 2012, American Chemical Society.

reported by Ryoo *et al.* demonstrated a multiplexed microRNA imaging system using PNA probe/nanosized GO complex, named as PANGO (PNA + nGO) by the authors. The high loading capacity of PNA on GO and strong interaction between them enabled quantitative monitoring of multiple microRNAs in living cells with high sequence specificity and low background signal. (Fig. 8a) [146]. In addition, many examples have been reported for analyzing other biomolecules in living cells, for example, messenger RNA detection using DNAzyme/GO [147] and multiple nucleotide sensing using multiple aptamer/GO complex [148,149].

5.2. LDI-MS-based imaging

The GO/MWNT LDI film, developed by Kim *et al.*, was also applied for mouse brain tissue imaging with high sensitivity and low background signal (Fig. 8b) [76]. LDI-MS-based imaging using GO/MWNT film revealed the reliable lipid distribution in mouse brain tissue of which thin section was just placed on the film without requiring additional matrix treatment. More recently, Huang *et al.* reported multivalent aptamer/AuNP modified GO for imaging of breast tumor tissue based on LDI-MS [74]. The authors exploited aptamer as a targeting probe for protein mucin 1 overexpressed on MCF-7 cells and GO as a template for the formation of gold cluster and matrix for absorbing the laser energy.

5.3. SERS-based imaging

Liu *et al.* reported GO–AuNP hybrid materials for SERS-based cell mapping in which gold nanostructures were intracellularly synthesized (Fig. 8c) [150]. Quite homogeneous intracellular distribution of the hybrid particles allowed to distinguish each biochemical component of the specific subcellular regions corresponding to the cytoplasm, nucleoplasm, and nucleus by acquiring distinct SERS spectra. Also, along with the enhancement of the SERS signals of biomolecules in cells, GO also act as a Raman probe in the SERS-based live cell imaging. Liu *et al.* demonstrated highly reproducible procedure to image live cells using AuNP decorated GO which showed strong characteristic G band in Raman spectra [151]. By using this platform, the authors revealed that the uptake of Au–GO hybrid was an energy dependent process and most likely occurs via an endocytosis pathway of cellular internalization.

6. Applications of GO-based biosensors in high-throughput screening

As one of the practical applications of GO-based sensing platform, helicase assay platform served as a tool for high-throughput screening in antiviral drug discovery. Jang *et al.* reported multiplexed version of the established GO-based helicase assay system using SARS coronavirus (SCV) helicase and hepatitis C virus (HCV) helicase and its successful application in drug discovery through chemical library screening [152]. By utilizing the multiplexed GO-based helicase activity assay, the 10,000 small molecules from a chemical library were screened in multi-well plates to find out new compounds that inhibit helicase activity effectively. Out of the 10,000 small molecules, 26 specific inhibitors of SCV helicase and 22 of HCV helicase were found, and 24 compounds were found to inhibit both helicases (>50% inhibition). The discovered HCV helicase inhibitors successfully blocked HCV gene replications in human liver cells (human hepatocarcinoma cell line, Huh-7) carrying HCV replicon RNA, which proved the practical applicability of GO-based sensing system in potent drug discovery.

Liu *et al.* established a high-throughput methodology for inhibitor screening acetylcholinesterase (AChE) using GO-based LDI-MS analysis [136]. By combining the magnetic microspheres for AChE immobilization and GO as matrix, this platform presented simple, cost effective, and efficient performance, which are important characteristics to be utilized for large-scale inhibitor screening. These excellent performance of

GO in practical applications demonstrates that the GO-based sensors have a great potential to be convenient and useful tool in pharmaceutical research.

7. Challenges and future perspective

There is no doubt that GO has been one of the most attractive and potent materials in biomedical fields for a decade. A number of fundamental and applied studies of GO have been intensively performed, which allowed the development of novel bioanalytical systems. However, most of them remain in its infancy partly due to its relatively short history and thus, there are several challenges in order to practically implement the bioanalytical platform technologies based on GO in biomedical applications.

The first challenge is the reproducibility in sensing performance, especially in use of different batches of GOs. The properties of GO are significantly different from each other between prepared batches, which causes the difference in sensing performance between sensors. Thus, to achieve the uniform and reliable analysis results, we need to utilize controllably synthesized GO with consistent size and oxidation degree and establish the valuable systems based on mechanistic understanding of interactions between biomolecules and the well-characterized GO. Second, it is necessary to block the nonspecific adsorption of various biomolecules on GO surface for sensing of the target with GO-based sensors especially in clinical samples such as body fluids or blood. Recently, strategies such as surface modification of the GO with biocompatible polymers and utilization of more advanced sensing probes were introduced in some biosensors to lower the background signals and thus, to raise the feasibility in practical applications. The efficient control of the interaction happening on the GO surface enables the biosensors to detect the biomarkers with high specificity and accuracy. Lastly, the GO-based sensors aim to be miniaturized and fabricated into small sized devices. This improvement will lead to higher sensitivity/stability of the sensors as well as faster response time, resulting in the advances in the practical healthcare and diagnosis by commercialization. Accordingly, there still remains important and exciting areas to explore before the GO-based platforms are utilized as routine analytical tools.

8. Conclusion

Since 2004, when graphene came into the spotlight by Andre Geim and Konstantin Novoselov, graphene and its derivatives have been fabricated by various methods and harnessed in diverse applications. GO, a water-dispersible derivative, has been considered to have infinite potential for the development of novel biological systems, due to (1) the unique properties such as large surface area, fluorescence-quenching capability, and high energy transfer efficiency originated from graphene structure; (2) biocompatibility, facile chemical modification, and water dispersability, generated from chemical oxidation; and (3) facile and large-scale synthesis with low cost. In particular, there has been a lot of attempt to develop biomolecule sensing systems by using GO and its derivatives in various ways, including FRET-based fluorescence sensors, LDI-based sensors, electrochemical sensors, and SERS-based sensors. The sensors enabled quantitative detection of biologically important molecules ranging from small molecules, ions to large biomolecules with improved sensing performance compared to conventional methods. Although most of the sensor systems still remained in stage of proof-of-concept due to its relatively short history, they have shown great promise for practical and commercial applications with further development in the near future. In addition, we think that further advances in chemistry of GO and its derivatives will promote faster establishment of the related biosensors in routine implementation with more robust performance in various biomedical applications.

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