Review

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Emerging Approaches for Graphene Oxide Biosensor

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Introduction

Graphene is a 2D material having honeycomb-like structure composed by single sp^2 carbon layer, which was first isolated from graphite in 2004.[1] It has drawn much attention from various research fields due to its unique physical/chemical properties such as high planar surface area, superior mechanical strength, thermal/electrical conductivity and optical property.[2] The intrinsic properties of graphene have driven many researchers to apply graphene to various research fields, including energy storage, sensor, transparent electrode, etc.[3-8]

Meanwhile, graphene oxide (GO) has also received attentions for its various biomedical applications including biosensors.[9-19] GO is literally the oxidized derivative of graphene,[19-20] possessing both the graphene-like 2D carbon sheet structure and various oxygen functional groups such as epoxy, carboxyl, carbonyl, hydroxyl groups. These functional groups may be placed at either the basal plane or edge of the sheet structure.[21-23] GO could be synthesized by oxidizing graphite with strong acid. There have been various synthesis protocols established for GO. In 1859, Brodie first reported a methodology for synthesis of graphene oxide from graphite by treating potassium chlorate and fuming nitric acid.[24] Staudenmaier[25] and Hofmann[26] also suggested the modified synthetic method of GO by changing acid compounds. One of the most common preparation methods for GO was proposed by Hummers in 1957,[27] also known as the Hummers' method. By using sulfuric acid, sodium nitrate and potassium permanganate, this method required shorter time, lower reaction temperature than other previous methods. Recently, various modified Hummers' methods to synthesize GO were reported.[28-30]

The coexistence of 2D single layer sp^2 carbon sheet structure and oxygen functional groups in GO endows its useful physicochemical properties including amphiphilicity,[31] stability in aqueous solutions,[32] strong adsorption of certain molecules onto GO plane through π- π stacking[33] and hydrogen bonding,[34] and facile surface modifications.[35-41] GO may also serve as a fluorescence quencher, involving the adsorption process of dye molecule on GO plane, followed by fluorescence resonance energy transfer (FRET) to result in quenching of the fluorescence signal.[42] GO can absorb laser light energy and transfer the energy to other molecules on its surface.[43] Moreover, GO not only enhances Raman signal by chemical enhancement mechanism on its surface,[44] but also has peroxidase-like catalytic activity.[45] In addition, GO has electroactive property which enable reversible electrical reduction and oxidation.[46] Also, GO is precursor of reduced GO (rGO) prepared by treating GO with reducing agent,[47-49] which shows better conductivity than GO.[50]

Due to its favorable intrinsic properties, GO has been harnessed for the development of various types of biosensors including electrochemical, optical (fluorescence, colorimetric and Raman), and mass analysis. Yet, strategies only based on the basic properties of GO have been restricted to the detection

of simple targets such as nucleic acids and small molecules. Recent studies have given priority to adaptation of biochemical properties of other functional materials along with GO to overcome restricted applications. As a result, research on GO biosensor has been explosively expanding in recent five years (Figure 1).

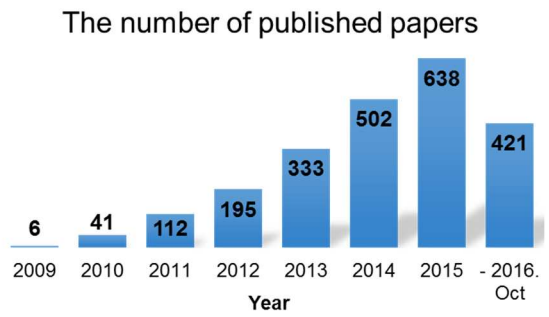

Figure 1. The expansion of research field in GO based biosensors since 2009. The result is derived from web of science by keyword searching "graphene oxide" and "biosensor or biosensing".

In this review, we define GO biosensor as the system that could detect biological targets including small molecule, nucleic acid, peptide, protein and even cell, aided by GO. In GO biosensor, GO may serve as a material for dynamic interaction with probe and/or for transduction of specific response towards target molecule into signal. This transduction process is achieved by fluorescence, laser desorption/ionization, Raman scattering, electrochemical reaction, catalytic oxidation, etc. Based on the definition, we will discuss state-of-the-art strategies of GO based biosensors developed in recent years.

Fluorescence biosensor

Background Principles and sensing strategies. One of the basic principles widely used in fluorescence signal-based biosensor is FRET. FRET involves energy transfer between two molecules named as donor and acceptor. When the donor molecule with excited electronic state approaches acceptor in sufficiently short distance, energy transfer from the donor can be induced towards the acceptor. Since energy transfer efficiency is inversely proportional to the 10^6 of the distance between donor and acceptor, FRET is extremely sensitive to even small changes in distance.[51] In general FRET-based fluorescence signaling biosensors, probe for the detection usually consists of fluorescent molecule, which acts as a donor. Quencher, which is an acceptor of the energy transfer, receives the energy from the probe and induces the fluorescence extinction.[52] In this manner, a highly qualified quencher is required for a successful detection via fluorescence signal-based biosensing technique.

GO possesses several advantages to serve as a fluorescence quencher. One of the most notable features of GO is amphiphilicity, which allows adsorption of various biomolecules on its planar surface. GO possesses strong binding affinity towards either hydrophobic biomolecules with aromatic rings via π-π stacking, or those with hydrophilic properties by hydrogen bonding. If the adsorbed molecules are conjugated with fluorescent dye, GO is capable of efficient energy transfer to result in quenching of the fluorescence with minimal background signal.[42] Such features are the clear evidences that GO would be employed as a highly valuable quencher as well as adsorption mediator, with the high potency to support development of various fluorescence-based sensing strategies.

One of the most well-known strategies incorporating GO is to make use of natural nucleic acid probe conjugated with fluorescent dye and GO as a quencher, to induce relative target-dependent change in fluorescence signal by specific target-probe interaction.[53] Taking advantage of this concept, various approaches have been introduced to derive the novel sensing strategies. One approach was to alter the organic dye with non-organic dyes such as inorganic quantum dot to enhance the fluorescence signal and stability.[54] For the detection of various biomolecules ranging from nucleic acid to protein, a nucleic acid probe called aptamer was designed to have high affinity towards the target.[55] There also have been several efforts to omit covalent conjugation-based labeling process by either production of non-covalent labeling probe via nucleation of nanocluster on specific DNA sequence or separated addition of independent fluorescent molecule in the sensing system.[56,57] In the meantime, various strategies have been introduced to alter natural nucleic acid probe with other natural biomolecules or artificially modified mimetics of nucleic acid.[58] For example, peptide nucleic acid (PNA) had been considered as an alternative probe for GO-based biosensor. PNA is a synthetic, biomimetic nucleic acid with pseudo-peptide backbone with special features of high affinity with natural nucleic acid with enhanced stability, as well as high thermal/chemical stability and resistance against nuclease. For the detection of targets with severely trace amount, there also have been various amplification strategies on the fluorescence signal incorporating GO. Procedures can be operated either with or without aids of enzyme.[59,60]

Recently, GO-biosensors based on fluorescence have been demonstrated in various analytical research areas, including not only simple detection[55] or functional activity analysis of biomolecules of interest,[61,62] but also advanced high-throughput screening for drug discovery.[63]

Aptamer probe. Several drawbacks have been reported on conventional aptasensors based on fluorescence signaling, including labor-intensive process of dual-label modifications of aptamer with fluorophore and quencher, and risk of poor sensitivity due to weakened binding affinity between the probe and the target.[64] Various trials have been made on development of novel aptasensor combined with GO as effective quencher, and applied to wide range of detection.

Numerous modification strategies have been presented to modify aptamers to possess specific functions. In the field of biomolecular sensing, on-chip FRET GO aptasensor, which enables quantitative evaluation with enhanced sensitivity, was developed.[65] This system adopted a double-stranded DNA spacer between GO and aptamer to achieve stable analysis. In 2016, another GO-based aptasensor was reported by Apiwat *et al*. for diagnosis and monitoring of glycated albumin in diabetes mellitus.[66] The system involves modified DNA aptamers with a specific hairpin motif, which enables conjugation with a marker for diabetes mellitus. With precise sensing ability of the system, the limit of detection (LOD) was calculated to be 50 μg/ml. Along with such investigations, advanced studies have been done to configure optimized condition to maximize sensing quality have also been studied. For example, methods for precise control of the GO-based aptasensor were reported by Zhang *et al.*.[67] They introduced the size-dependent

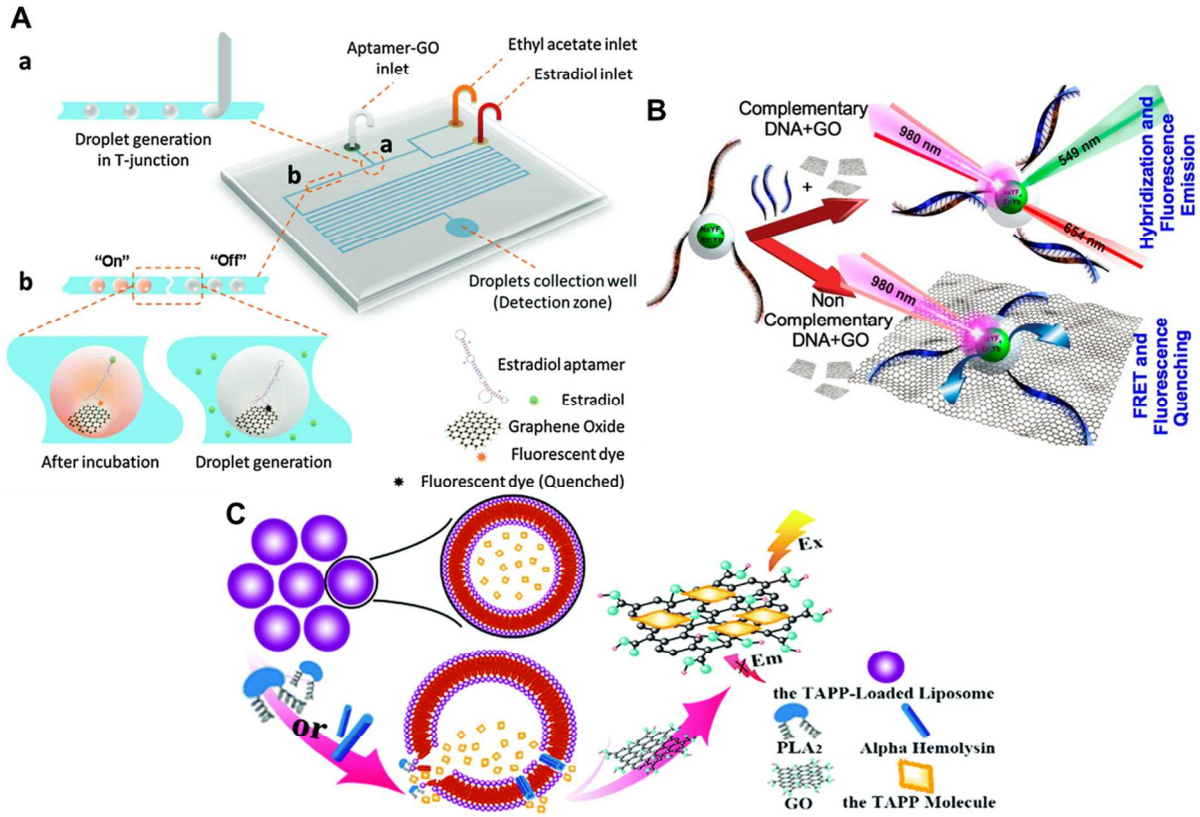

Figure 2. Representative sensing strategies on fluorescence biosensors (A) Scheme for the detection of molecules with low solubility based on microfluidic droplets and aptamer-functionalized GO. Reproduced from Dou, M.; García, J. M. Jr.; Zhan, S.; Li, X. Chem. Commun. 2016, 52, 3470−3473 (ref 70), with permission of The Royal Society of Chemistry. (B) Scheme for DNA sensor with combination of upconversion nanoparticles, as non-organic dye label, conjugated probe and GO. Reproduced from Alonso-Cristobal, P.; Vilela, P.; El-Sagheer, A.; Lopez-Cabarcos, E.; Brown, T.; Muskens, O. L.; Rubio-Retama, J.; Kanaras, A. G. ACS Appl. Mater. Interfaces 2015, 7, 12422−12429 (ref 78). Copyright 2015 American Chemical Society. (C) Scheme for the detection of the activity of the membrane pore-forming proteins (Phospholipase A2 (PLA2) and Alpha hemolysin). Reproduced from Liu, Z.; Long, T.; Wu, S.; Li, C. Analyst 2015, 140, 5495−5500 (ref 82), with permission of The Royal Society of Chemistry

programming of the conventional aptasensor based on GO for detection of heavy metals, by controlling nanometer-size effect on GO which would influence interaction between GO and aptamer probe.

There also have been various trials made to develop aptasensors in small device format. For example, Zhang *et al*. developed a paper-based microfluidic device that enables simultaneous, multiplex detection of various chemical contaminants in food.[68] This system made best use of the paper substrate to fabricate microfluidic device, involving efficient physical adsorption of apta-GO sensor into the substrate. Another paper-based atpasensor was reported by Li *et al*., involving the adsorption of GO, integrated with dye-conjugated aptamer, on the paper chip fabricated with glass fiber.[69] Moreover, in 2015, aptasensor-based small device for detection of molecules with low solubility was reported by Dou *et al*., by combination of high surface-area-to-volume ratio of microfluidic droplets and aptamer-functionalized GO (Figure 2A).[70] The intrafacial nano-biosensing system showed precise and highly sensitivity one-step detection of 17β-estradiol, with LOD as low as 0.07 pM. Researches on aptasensor have been not only focused on the molecular-scale detection, but also expanded to application on cellular-level detection. Meanwhile, Nellore *et al*. presented an aptasensor based on aptamer-conjugated GO membrane to efficiently capture and identify multiple types of circulating tumor cells.[71] Based on porous GO conjugated with dye-modified aptamers, the system showed accurate performance on capturing circulating tumor cells from the blood sample infected with multiple types of cancer cells.

Non-organic dye-label. Traditional fluorescent dyes have shown several limitations such as high cost and low photostability.[72] To overcome these drawbacks, some researchers have put much effort to derive alternative approaches, including carbon dot (CD) and upconversion nanoparticle. Due to its unique properties including ease of synthesis and functionalization and high photostability, CD has been considered as a potent, alternative labeling dye.[73-75] In 2014, Cui *et al*. reported a simple fluorescence biosensor based on oligodeoxyribonucleotide labeled with CDs and GO for detection of heavy metal.[76] The system showed high sensitivity and selectivity towards mercury ion (Hg^{2+}), with considerably low LOD calculated as 2.6 nM, which is comparable to concentration range of 5~200 nM estimated in other systems. There also have been other strategies to make use of upconversion nanoparticles, which allows absorption of low-energy photons followed by emission of fluorescence corresponding to shorter wavelength than the excitation wavelength.[77] In 2015, Alonso-Cristobal *et al*. presented a highly sensitive biosensor for detection of DNA with combination of upconversion nanoparticles and GO

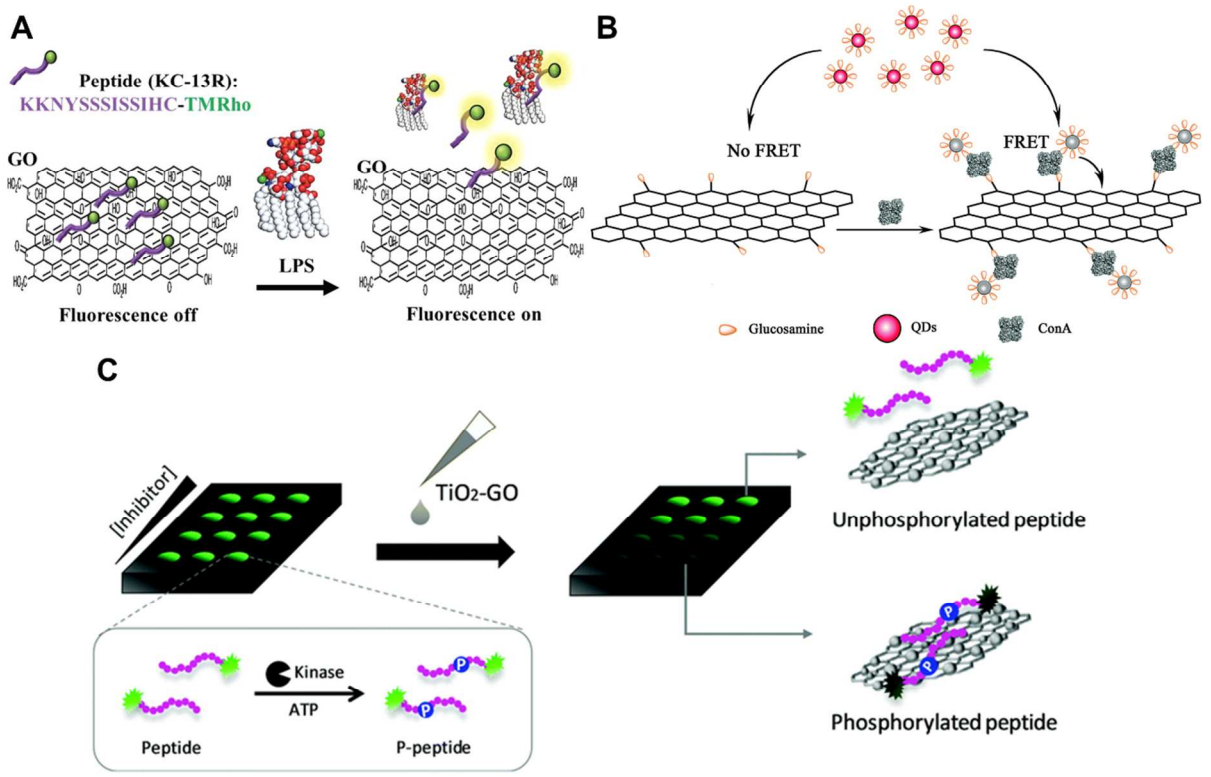

Figure 3. Scheme for GO-based biosensors incorporating non-nucleic acid probe. (A) Representative GO-based biosensing platform incorporating peptide probe for detecting lipopolysaccharide (LPS). Reproduced from Lim, S. K.; Chen, P.; Lee, F. L.; Moochhala, S.; Liedberg, B. Anal. Chem. 2015, 87, 9408−9412 (ref 88). Copyright 2015 American Chemical Society. (B) Glucosamine probe for protein detection. Reproduced from Li, Y.; Shi, F.; Cai, N.; Su, X. New J. Chem. 2015, 39, 6092−6098 (ref 89), with permission of The Royal Society of Chemistry. (C) Scheme for high throughput enzyme inhibitor screening via enzyme activity assay platform incorporating GO and peptide probe. Reproduced from Lee, J.; Park, I. S.; Park, G.; Cho, K.; Park, H. S.; Min, D.-H. Chem. Commun. 2016, 52, 12112−12115 (ref 96), with permission of The Royal Society of Chemistry.

(Figure 2B).[78] The sensor system involved DNA-functionalized upconversion nanoparticles, which would be localized at distal position of the GO in the presence of the target DNA strand and show fluorescence. The system showed highly sensitive and specific sensing performance, with LOD of 5 pM.

Non-covalent label. Along with efforts to alter traditional labeling methods, diverse strategies have been investigated to design probes without covalent labeling. One common approach is to utilize optical properties of metallic nanoclusters, especially fluorescence emission.[79] For example, silver nanocluster has been widely applied to the development of nucleic acid probes due to its intrinsic fluorescence property with high quantum yield and photostability.[80] A notable evolution of this probe was introduced by He *et al*., which is a microRNA (miRNA) sensor derived from application of Y-shaped probe and GO.[81] Synthesized by sodium borohydride reduction with silver nitrate and a cytosine-rich molecular beacon, the arms of Y-shaped DNA template sufficiently captured silver ions to form the overall nanocluster-like structure. Then the single stranded loop structure of the probe enabled the efficient adsorption onto the RGO surface, which would be selectively detached in the presence of the target in high specificity. In this way, Y-shaped probe showed enhanced fluorescent signal than conventional DNA-templated silver nanoclusters. Applications of materials with specific structures which endowed self-fluorescence property have been under consideration. For example, in 2015, Liu *et al*. applied liposome encapsulating porphyrin as a probe to detect membrane pore-forming activity of the target enzyme (Figure 2C).[82] As the liposomal membrane is disturbed by enzymatic activity of the target, porphyrin molecule is leaked out to reach GO and its fluorescence is quenched by GO. Another application was reported by Li *et al*., which involved aptasensors for the detection of thrombin by utilizing interaction between a chiral ruthenium complex (OMO) and GO.[83] With a pair of aptamers, the system allowed direct readout of the target thrombin recognition from diluted bovine serum sample with high sensitivity and selectivity.

Non-nucleic acid probe. Although nucleic acid has been one of the most common probes for biosensor, there have been some limitations reported such as ease of degradation by nuclease, which is critical drawback to be applied in advanced analytical research with clinical samples. Numerous approaches on fluorescence biosensors based on non-nucleic acid probe was introduced, mainly for the detection of functional biomolecules or metal ions. For detection of biomolecules, strategies often involved alteration of nucleic acid probes with peptides.[84-87] For example, Lim *et al*. demonstrated application of peptide-GO-based biosensor for detection of LPS, which is a toxic inflammatory stimulator (endotoxin) (Figure 3A).[88] Peptide-assembled GO system sufficiently captured LPS in high specificity, followed by release of peptide-LPS complex from GO to induce fluorescence recovery. The system showed high detection performance with LOD of 130 pM. Along with peptide probes which have been major alteration of nucleic acid

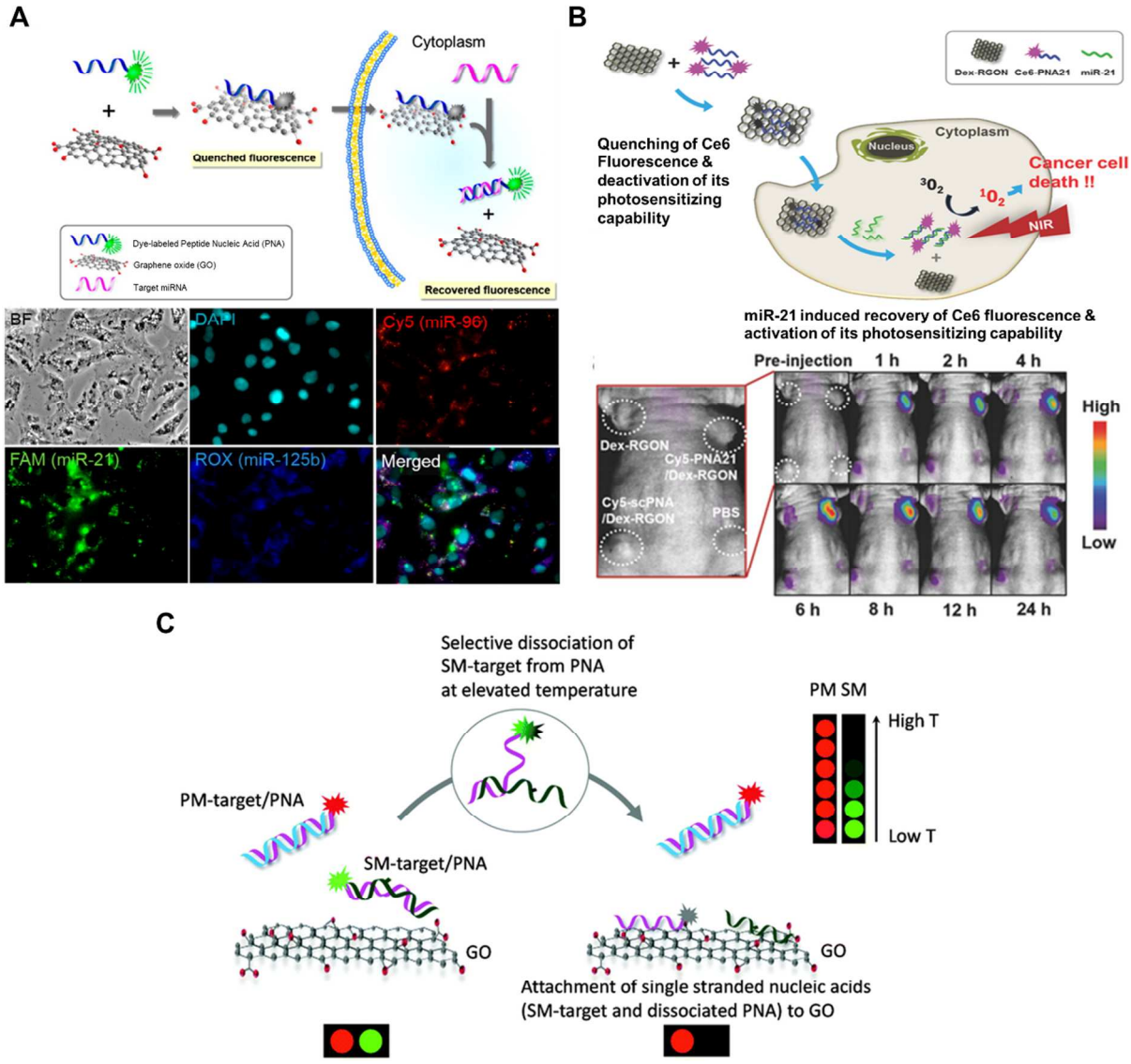

Figure 4. Strategies based on artificial nucleic acid probes for fluorescence biosensors (A) Scheme for PNA-GO (PANGO) biosensor platform detecting miRNA in live cells. Reproduced from Ryoo, S. R.; Lee, J.; Yeo, J.; Na, H. K.; Kim, Y. K.; Jang, H.; Lee, J. H.; Han, S. W.; Lee, Y.; Kim, V. N.; Min, D.-H. ACS Nano 2013, 7, 5882−5891 (ref 58). Copyright 2013 American Chemical Society. (B) Application of PANGO for *in vivo* imaging of miRNA and photodynamic therapy. Reproduced from MicroRNA-Responsive Drug Release System for Selective Fluorescence Imaging and Photodynamic Therapy In Vivo, Lee, J. S.; Kim, S.; Na, H. K.; Min, D. H. Adv. Healthc. Mater, Vol. 5, Issue 18 (ref 97). Copyright 2016 Wiley (C) PNA probe-based detection of single-base mismatch of target miRNA. Reproduced from Lee, J.; Park, G.; Min, D.-H. Chem. Commun. 2015, 51, 14597−14600 (ref 99), with permission of The Royal Society of Chemistry.

probes in biomolecule detection, recent approaches also involved other bio-molecular probes as well. Li *et al*. demonstrated GO-based biosensor for detection of concanavalin A involving quantum dot (QD)-conjugated glucosamine as a probe (Figure 3B).[89]

In case of metal ion detection, strategies have been under investigation by applying various organic or inorganic probes to alter nucleic acid probes.[90-93] In addition, some biosensors employed molecular recognition of natural biomolecules. For example, in 2015, Yuan *et al*. came up with a strategy to apply intrinsic features of calcium ion receptor calmodulin (CaM) for the detection of biological calcium ion (Ca^{2+}).[94] With cationic polymer-conjugated GO hybrid probe, the system enabled a quantitative detection and analysis of Ca^{2+} by specific conformational changes of CaM. Studies reported by Tao *et al*. is also a notable example of fluorescent nanodot-GO-based biosensor applied to identification of triple-negative breast cancer cells.[95] By utilizing six luminescent nanodot-GO complexes as probe, the system demonstrated highly sensitive, precise detection of the human epidermal growth factor receptor-2 positive, target cells at accuracy of 98%. Recently, such strategies have broaden its range of application to the detection of biomolecular activities as well. Enzyme assay platform introduced by Lee *et al*., which enabled a quantitative analysis of protein kinase activity, was also proven to be applied to multiplex, high throughput inhibitor screening (Figure 3C).[96] The system utilized TiO_2-coated GO to enhance specific affinity toward phosphate group, which enabled quantitative detection

Amplification method	Target		Linear dynamic range	Limit of detection	Reference
DNase I-mediated cyclic signal amplification	Bacteria	*S. paratyphi A*	1×10^2 - 1×10^{11} cells/ mL	1×10^2 cells/ mL	Yan *et al.*[110]
T7 exonuclease assisted cyclic signal amplification	Enzyme activity	DNA methyltrans-ferase	N/A	0.1 U/mL	Ma *et al*.[109]
Isothermal exponential am-plification	Nucleic aicd	microRNA	10 fM - 10 pM	3 fM	Li *et al*.[105]
Polymerase based cyclic signal amplification	Nucleic aicd	RNA	10.0 pM - 1.0 nM	10 pM	Wu *et al*.[107]
Rolling circle amplification	Nucleic aicd	microRNA	1 fM - 50 pM	0.4 pM	Hong *et al.*[112]
rGO-assisted rolling circle amplification	Nucleic aicd	microRNA	10 fM - 100 pM	10.3 fM	Zhu *et al*.[111]
Nicking enzyme signal am-plification	Protein	Thrombin	2 fM– 200 nM	1 fM	Huang *et al*.[113]
Exonuclease III-induced signal amplification	Protein	α-fetoprotein	25 - 150 pg/ml	18 pg/ml	Liu *et al.*[108]
Nicking endonuclease-assisted signal amplification	Protein	$VEGF_{165}$	5–200 pM	1 pM	Li *et al.*[114]
Nicking enzyme signal am-plification	Small molecule	Adenosine	4 pM – 10 μM	2.0 pM	Huang *et al.*[113]
Exonuclease III as a signal amplifying	Small molecule	Adenosine	10 μM – 0.7 mM	3.1 μM	Xing *et al*.[106]
Nicking endonuclease-assisted signal amplification	Small molecule	ATP	0.01–1 μM	4 nM	Li *et al.*[114]
RNA-cleaving chimera DNAzyme recycling ampli-fication	Inorganic molecule	UO_2^{2+}	0.29 – 30 nM	86 pM	Li *et al.*[117]

Table 1. Summary of enzyme mediated signal enhancement strategies

of phosphorylation via the enzyme activity in a considerably short time with high accuracy.

Artificial nucleic acid probe. Recent approaches on non-nucleic acid also employed artificial nucleic acid. In 2013, for example, Ryoo *et al*. developed a novel biosensor detecting specific miRNA targets in live cells by use of combination of PNA probe and GO, so called 'PANGO' system (Figure 4A).[58] PANGO successfully demonstrated simultaneous detection and monitoring of the intracellular expression level of the target miRNA without additional treatment on live cells. With high sequence specificity by PNA probe and sufficient loading capacity of GO, the system showed reliable performance with LOD of ~1 pM. The unique properties of the system derived various researches on further applications. According to recent studies reported Lee *et al*., PANGO could be successfully applied to *in vivo* theranostics system to achieve simultaneous sensing of cancer cells and activation of photosensitizer in response to oncogenic miRNA (Figure 4B).[97] The system utilized photosensitizer(PS)-conjugated PNA probe and dextran-modified rGO nanoparticle (Dex-RGON) to demonstrate selective, simultaneous miRNA-responsive photodynamic therapy.

Along with such investigations, efforts have been made to enhance quality of PNA-incorporating biosensor. For example, Lee *et al*. suggested a simple strategy to realize practical applications of PNA-based biosensor by optimization of the condition with BSA additive to reduce background signals.[98] It was reported that addition of BSA was sufficient to reduce unfavorable adsorption of PNA-DNA duplex on GO surface while facilitating efficient adsorption of ssPNA, resulting in enhancement on sensitivity. With regard to this development, FRET-based sensing system incorporating PNA and GO was often applied to distinguish single base difference within nuclide acid analytes. In 2015, Lee *et al*. broadened the range of the application to the temperature-sensitive sensor to detect a single mismatch of nucleic acid, based on the relative affinity change between PNA probe-target double strand nucleic acid and GO (Figure 4C).[99] As an effective scavenger for adsorption of ssPNA, GO successfully facilitated the precise discrimination of target by single nucleotide level.

Signal enhancement by enzyme reaction. For the detection of trace amounts of analytes, it has not been long until thorough investigation was made on various strategies to enhance the signal intensity. Various signal enhancement strategies were presented with aids of enzymatic properties, including polymerase chain reaction (PCR), ligation chain reaction (LCR) and rolling circle amplification (RCA).[100-102] In spite of their high performance on detection of trace analytes, alternative methods have been suggested to overcome the intrinsic limitations, such as laboring process and high risk to contamination. Cyclic enzymatic signal amplification method (CEAM) is one representative alternative proposed by Ju *et al*.[59] With target recycling process mediated by exonuclease III, CEAM enables the enhancement on fluorescent signal during detection of the target DNA. Due to these properties,

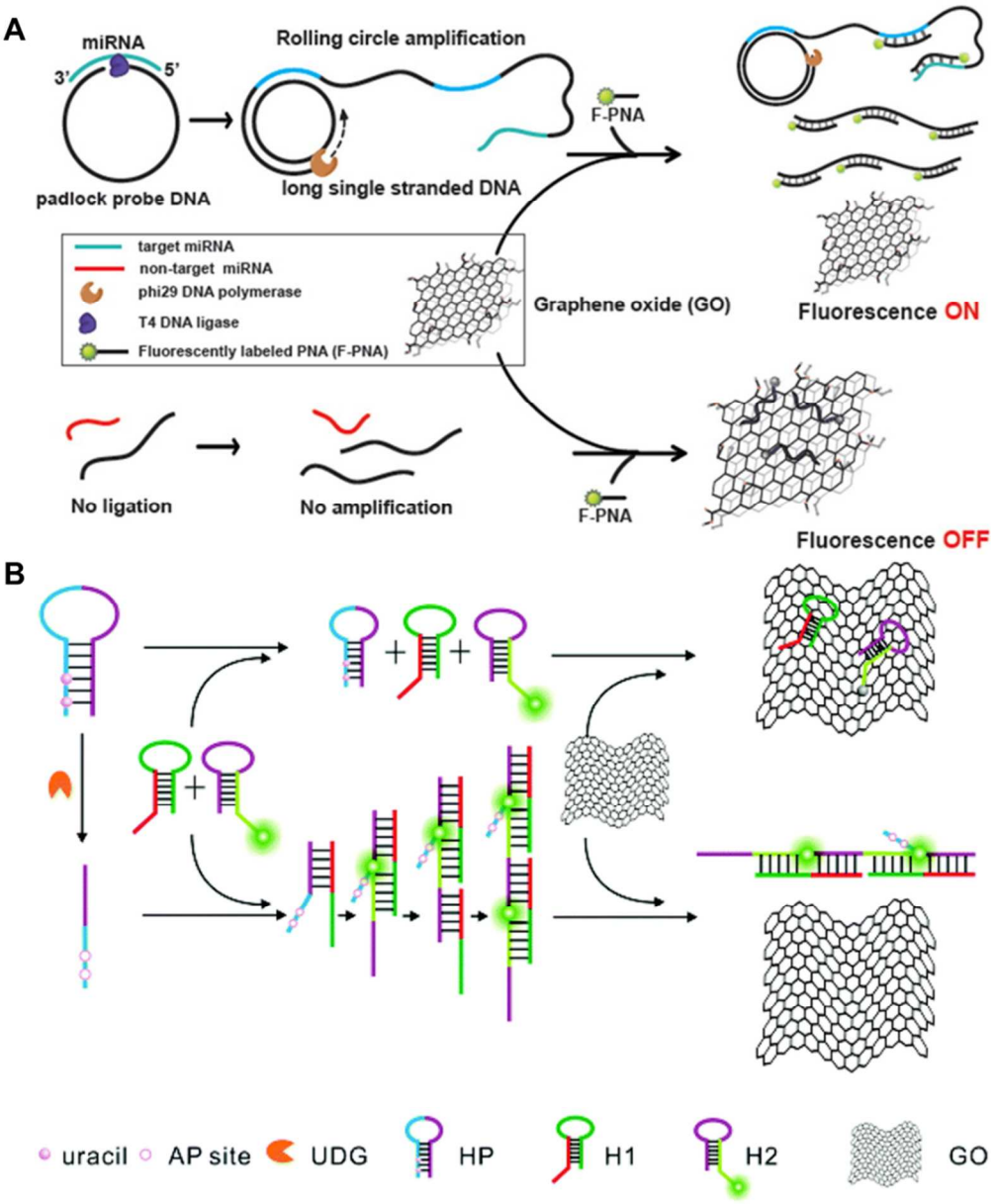

Figure 5. Signal enhancement strategies on fluorescence biosensor. (A) GO-based biosensor with enzyme assisted signal amplification for sensitive miRNA detection. Reproduced from Hong, C.; Baek, A.; Hah, S. S.; Jung, W.; Kim, D. E. Anal. Chem. 2016, 7, 2999−3003 (ref 112). Copyright 2016 American Chemical Society. (B) GO-based biosensor with hybridization chain reaction (HCR) for base excision repair enzyme activity detection. Reproduced from Xi, Q.; Li, J. J.; Du, W. F.; Yu, R. Q.; Jiang, J. H. Analyst 2016, 141, 96−9 (ref 122), with permission of The Royal Society of Chemistr

Cui *et al*. applied this system in combination of GO to multiplex miRNA analysis.[103]

Recent approaches also focused on derivation of the improved methods for the traditional enzyme-mediated process. RCA is one of the most commonly studied strategies for such purpose, which is the isothermal enzymatic amplification method involving polymerization around circular templates. This amplification process is often combined with target recycling via nuclease activity, to induce exponential amplification.[104] Various strategies applied RCA based approaches to demonstrate the enhanced sensitivity of the biosensor for the detection of biomolecules. Moreover, some of them enabled the analysis of functional activity of biomolecules as well.[105-110] In 2015, Zhu *et al*. presented a single nucleotide polymorphism (SNP)-detectable biosensor based on RCA assisted by GO.[111] Furthermore, in 2016, Hong *et al*. reported a novel fluorometric, GO-based miRNA sensor with combination of RCA (Figure 5A).[112] With the target-specific RCA reaction along with use of padlock probe DNA, the sensor system demonstrated highly sensitive, quantitative analysis on miRNA with LOD of 0.4 pM. Several attempts have been made to improve methodological strategies involving RCA, by adopting a specific nicking endonuclease. Due to its intrinsic properties, nicking endonuclease is free from side effects which may occur by other restriction enzymes, including degradation of single strand DNA probes. Thus, target-recycling signal amplification process aided by nicking endonuclease have shown great suitability to be applied to aptasensor, with

Amplification method	Target		Linear dynamic range	Limit of detection	Reference
Hybridization chain reaction	Enzyme activity	Uracil-DNA glycosylase	0.0001 - 100 U/ mL	0.00006 U/ mL	Xi *et al.*[122]
Hybridization chain reaction	Inorganic molecule	Hg^{2+}	0 - 1.0 nM	0.3 nM	Huang *et al.*[118]
Toehold-mediated non-enzymatic amplification	Nucleic acid	microRNA	10 pM - 100 nM	10 pM	Huang *et al.*[119]
Hybridization chain reaction	Nucleic acid	microRNA	0 - 200 pM	0.18 pM	Li *et al.*[120]
Hybridization chain reaction	Nucleic acid	RNA	5 pM - 10 nM	2.7 pM	Shi *et al.*[121]
Hybridization chain reaction with non-covalent label	Nucleic acid	microRNA	1 pM - 1 nM	1 pM	Zhu *et al.*[123]

Table 2. Summary of non-enzymatic signal enhancement strategies

enhanced aptamer stability followed by low background false signal.[113] In 2015, Li *et al*. applied nicking endonuclease-assisted signal amplification strategy to develop a GO-based aptasensor.[114] With split molecular beacon, the system demonstrated efficient quantitative analysis of vascular endothelial growth factor (VEGF) and ATP in biological samples, with LOD of 1 pM and 4 nM, respectively.

While various strategies involved traditional protein enzymes, recent studies have focused on utilization of a special type of enzyme consisting of DNA, known as DNAzyme. Discovered by Breaker and Joyce in 1994, the functions of this unique enzyme was proven as recognition of the target sequence on the substrate nucleic acid and catalysis of hydrolysis reaction.[115] One representative study was reported by Zhao *et al*., which included the first development of GO-DNAzyme based biosensor. [116] With an organic dye-labeled DNAzyme-substrate hybrid, the system demonstrated precise detection of metal ion Pb^{2+} with an amplified fluorescence signal. Since then, strategies have emerged to combine GO with not only original DNAzyme but also its derivatives. In 2015, RNA-cleaving chimera DNAzyme (RCDzyme)-based, target-recycling signal amplification strategy was applied to the development of GO-based sensor without requiring covalent label for ultrasensitive detection of uranyl.[117] Containing a guanine (G)-rich sequence to replace the partial sequence in a uranyl-specific DNAzyme, RCDzyme acts not only as the target recognition element with DNAzyme activity, but also as the primer of signal amplification. In the presence of uranyl, substrate strands in RCDzyme are cleaved to be hybridized with another substrate strands, followed by repetitive cleavage cycles by binding uranyl. G-quadruplexes, made from split guanine-rich oligonucleotide fragments, then binds to N-methyl-soroporphyrin IX (NMM), resulting in amplified detection signal for the target. With combination of GO to absorb free ssDNA and NMM, the system demonstrated sensitive detection of uranyl by LOD of 86 pM.

Signal enhancement without enzyme reaction. Although enzymatic signal amplification strategies have been widely applied to analytical research, several drawbacks such as temperature, pH dependence of enzyme activity are still needed to be solved. Signal amplification strategies without enzymatic process have emerged in purpose to overcome these obstacles. Some of them often involved simultaneous polymerization-like process of probe molecules, known as hybridization chain reaction (HCR). In 2014, Huang *et al*. reported a fluorescence biosensor for detection of heavy metal Hg^{2+}, by combining GO-based quenching system with signal amplification system by HCR.[118] The sensor system adopted a helper DNA along with two hairpin probes. Based on the fact that Hg^{2+} has high affinity towards thymine-thymine (T-T) base pairs in nucleic acid, resulting in formation of T-Hg^{2+}-T structures, the system successfully demonstrated sensitive detection with amplified signal by HCR occurred among three DNAs in the presence of Hg^{2+}. The system also showed high selectivity of the target against other divalent metal ions. Recently HCR has been widely adopted to not only simple *in vitro* analysis[119] but also analytical trials to detect nucleic acids such as mRNA or miRNA within living cells.[120,121]

Combination of GO-based quenching system and HCR-based non-enzymatic signal amplification has also been under investigation for application on functional analysis of biomolecule. In 2016, Xi *et al*. applied this combined system to develop a fluorescent biosensor for the detection of a base excision repair enzyme activity (Figure 5B).[122] In 2014, Zhu *et al*. reported a GO-based fluorescence biosensor utilizing non-covalent label combined with isothermal enzyme-free amplification for the detection of miRNA from clinical samples[123]

Laser desorption/ionization mass spectrometry biosensor

Background principle and sensing strategies. GO shows a high affinity towards target molecules via π- π stacking and hydrogen bonding. When irradiated with laser in wavelength of 337 or 355 nm, GO is apt to absorb its energy, and enable ionization/desorption of analytes on its surface via efficient energy transfer. Due to this property, GO could act as an efficient laser desorption/ionization mass spectrometry (LDI/MS) matrix especially for the detection of analyte molecules with low molecular weight by reducing the interference peaks originated from organic matrix at low *m/z* region.[43]

For construction of LDI/MS biosensor, several GO-based strategies have been followed in common. First strategy is to fabricate hybrid composite based on GO. These composites have been evolved to show better properties than that of GO itself, with both enhanced efficiency of energy absorption from laser and reduction of fragmentation followed by decrease in interference. Therefore, GO hybrid composite matrix shows much lower signal interference at low *m/z* region.[124,125] Second strategy is to modify the surface of GO for target-specific binding. In this strategy, modification of GO with target specific probe would increase the selectivity and sensitivity of LDI/MS biosensor.[126,127]

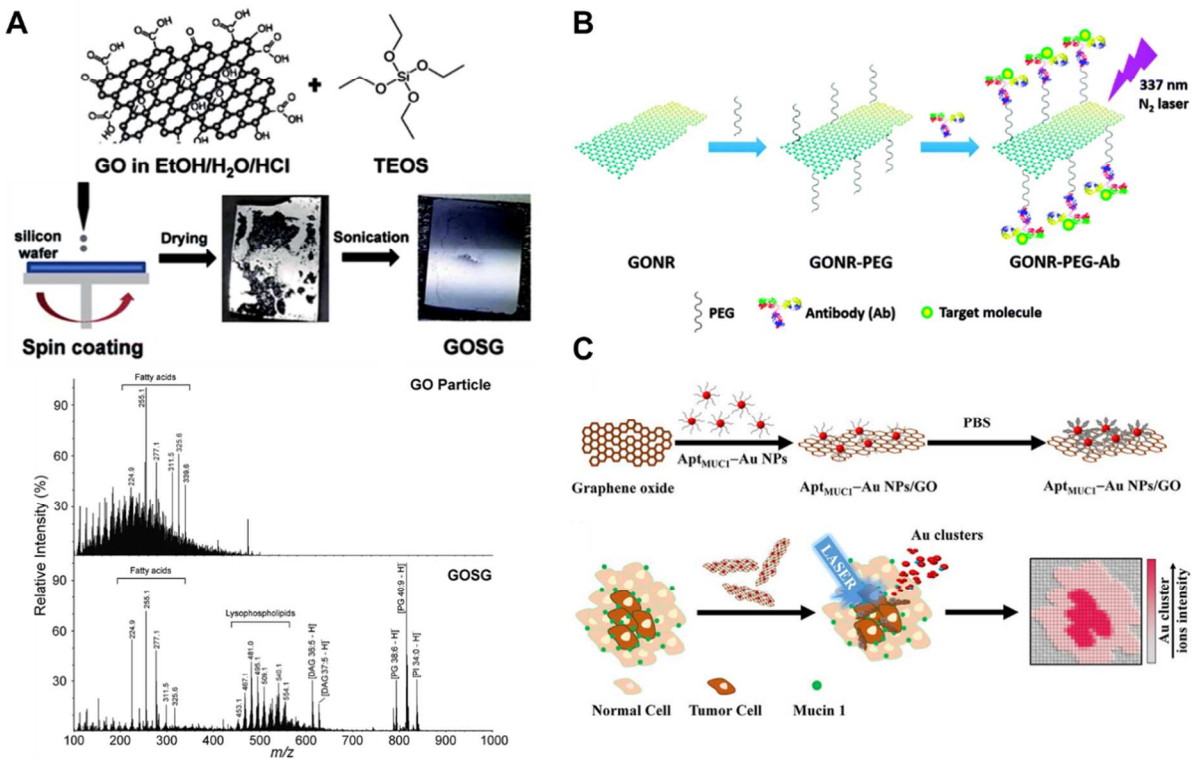

Figure 6. Different sensing strategies on LDI/MS biosensors (A) Scheme for preparation of GO embedded sol-gel (GOSG) film and compared MS spectrum of *Chlorella vulgaris*' metabolite fingerprint between GO particle and GOSG. Reproduced from Lee, G.; Bae, S. E.; Huh, S.; Cha, S. RSC Adv. 2015, 5, 56455−56459 (ref 130), with permission of The Royal Society of Chemistry. (B) Scheme for synthesis of GONR-PEG-Ab and its use as a LDI/MS probe for specific detection in complex media Reproduced from Wang, J.; Cheng, M.; Zhang, Z.; Guo. L.; Liu, Q.; Jiang, G. Chem. Commun. 2015, 51, 4619−4622 (ref 133), with permission of The Royal Society of Chemistry. (C) Scheme for tumor tissue imaging by using surface modified GO with aptamer-conjugated AuNPs (AptMUC1-AuNPs). Reprinted with permission from Huang, R. C.; Chiu, W. J.; Lai, I. P. J.; Huang, C. C. Multivalent Aptamer/Gold Nanoparticle-Modified Graphene Oxide for Mass Spectrometry-Based Tumor Tissue Imaging. Sci. Rep. 5, 10292. (ref 136) Copyright 2015 Nature Publishing Group.

Based on these strategies, various biosensors have been reported in a range of research area, including small molecule detection,[127] DNA sensing,[128] enzyme activity assay[124] and tissue imaging.[125]

Hybrid composite. The studies for making hybrid composite started by combining GO with other carbon materials due to applicability of carbon-based materials as alternative matrices. In 2010, Lee *et al*. first reported GO/carbon nanotube (GO/CNT) hybrid films as matrix which showed efficient energy absorption and transfer.[124] Based on GO/CNT hybrid films, small molecules produced by phospholipase could be detected by LDI/MS. Another hybrid composite combined with multiwalled carbon nanotube (MWCNT) was made by Kim *et al*.[125] The synergistic effect of GO/MWCNT films enabled MS imaging of tissue by enhancing surface area for analyte adsorption and increasing LDI efficiency. However, high energy of laser might induce background signal originated from fragmentation of carbon nanomaterials. Therefore, it is important to minimize background signals from carbon materials by enhancing the efficiency of energy absorption and LDI process. In this matter, sol-gel films which include LDI matrix effectively decrease background signals.[129] Because the porosity of a sol-gel structure provide an effective contact between matrix and analyte, sol-gel film effectively transfer laser energy to analytes. Moreover, numerous advantages exist when graphene materials are incorporated with porous, silica-based sol-gel film (GO embedded sol-gel, GOSG).[130] Similar to graphene coated porous silica, GOSG also shows reduced background signal at the low *m/z* region by high ionization efficiency. This concept was applied as a MS substrate for detecting metabolite fingerprinting by Lee *et al*. (Figure 6A).

Compared to GO which showed only fatty acid peaks with relatively high background from *Chlorella vulgaris*, GOSG clearly showed metabolite fingerprint peaks from *Chlorella vulgaris* including fatty acid, phospholipids and diacylglycerides. As another approach to increase the efficiency of laser energy transfer, Hong *et al*. reported AgNP/rGO based nanoporous hybrid film.[131] With its porosity from layer-by-layer electrostatic self-assembly, the nanoporous hybrid film could enhance the ionization efficiency and reduce fragment-induced signal interference. In this way, the hybrid film showed very clear fatty acid peaks without interference at the low *m/z* region compared to its single layer. For similar purpose, Abdelhamid *et al*. combined sinapinic acid and GO (SA@GO) as hybrid matrix.[132] This hybrid matrix inhibits aggregation of GO and crystal growth of SA, resulting in increased ionization efficiency of analyte. It was reported that SA@GO offered improvement in signal intensity (2-5 folds) and high sensitivity (1-10 fmol) for pathogenic bacteria proteins having high molecular weight.

Surface modification with target probe. For detection of biomolecules, GO may act as a mediator of energy transfer as well as selective capturer of the targets by covalent modification with target specific probes. Based on this strategy, Gulbakan *et al*. first showed aptamer-conjugated GO platform for selective analyte enrichment and MS analysis in 2010.[127] In 2015, Wang *et al*. showed antibody functionalized graphene oxide nanoribbon (GONR) for detection of chloramphenicol (CAP) in human serum and river water (Figure 6B).[133] In comparison with aptamer, antibody has some advantages such as numerical diversity and a good binding affinity even in complex media. To avoid functionalization with expensive large molecules including aptamer and antibody, there were some recent efforts on selective detection by modifying GO with chemical functional groups. For example, using boric acid-functionalized GO, Zhang *et al*. reported selective enrichment and analysis of small molecules with vicinal diol in 2015.[134] In this case, GO, which provides pi-conjugated system for laser absorption and energy transfer, was functionalized with 4-vinylhenylboronic acid (VPBA) to selectively capture vicinal diol compounds (catechol, adenosine, cytidine and guanosine). GO-VPBA showed 50-fold lower LOD (1.34, 1.59, 1.95, 0.63 pmol/ml) against those compounds than using GO as matrix. In addition, small molecules with vicinal diols could be selectively detected in urine sample. Also, Zheng *et al*. showed that GO modified with hydroxyethyl methacrylate was capable of detection for trace dopamine.[135]

Not only covalent modification of GO with target probe but also non-covalent modification is possible to give GO target specificity. In 2015, Huang *et al*. established tumor tissue MS imaging based on multivalent aptamer/gold nanoparticle-modified GO (Figure 6C).[136] Mucin1 (MUC1) is cancer biomarker overexpressed in most adenocarcinomas. The system involved MUC1-binding aptamer conjugated AuNPs that were immobilized on GO (Apt_{MUC1}-AuNP/GO) via π- π interactions between aptamer and GO. The sensor system with Apt_{MUC1}-AuNP/GO demonstrated effective detection of target cancer cells with MS signals generated by Au cluster by LDI process.

Raman biosensor

Background principle and sensing strategies. As GO itself is a Raman active reporter molecule, GO shows specific Raman peaks such as G band (~1585 cm^{-1}) and D band (~1345 cm^{-1}).[137] Moreover, fluorescence from fluorescent Raman reporter adsorbed on GO becomes quenched through resonance energy transfer mechanism, enabling reduction of fluorescence interference from Raman reporter signal.[138] GO enhances Raman signal from adsorbed Raman reporter via electronic coupling called chemical enhancement mechanism between GO and Raman reporter.[44,139] These properties make GO a good Surface-Enhanced Raman Scattering (SERS) substrate. Therefore, Raman activity and SERS effect from GO would promote the development of Raman biosensor based on GO.

First strategy is to apply GO as Raman active reporter molecule with application of Raman signal from GO as a label for cellular imaging in theranostic platform.[140-142] Second strategy is to use GO as SERS substrate. Most of studies in this strategy aim to enhance Raman signal of analyte.[143-145] Third strategy is to utilize GO as a 2D scaffold for SERS active nanoparticle to make SERS hot-spot.[146,147] In addition, the facile modification of GO surface with target-specific probes enables the development of target specific Raman biosensor.[148]

To date, there have been numerous reports regarding GO based Raman biosensor for bacteria detection[144] and cellular imaging.[140]

GO as Raman active reporter molecule. As a Raman reporter, GO is usually incorporated with metal nanoparticles to enhance Raman signal from GO by SERS. Au nanoparticle, which is one of the SERS active noble metals, has been commonly combined with GO for cellular imaging agent.[141,142] One critical drawback of this hybrid composite materials was that the synthetic processes are complicated. In 2015, Kim *et al*. reported a facile preparation method for Au-GO hybrid structures. In this paper, application of GO as both the reducing and stabilizing agent for Au was also investigated. They showed simple one-pot synthesis of Au@graphene oxide nanocolloid (Au@GON) particle which can be used as cellular imaging agent.[149]

Another advance in GO as a Raman reporter was conducted by Yim *et al*. in 2015. They developed GO-encoded Ag nanoshells (GONS) with single-particle detection sensitivity for cancer cell imaging (Figure 7A).[150] The structure consists of Ag nanoshell encapsulated with 4, 7, 10-trioxa-1, 13-tridecanediamine (TEG)-functionalized GO by electrostatic interaction, which enhances Raman signal intensity from GO. The intense Raman signal from GONS enabled high sensitivity even at a single particle level in complex cellular system. Further application of GONS as a bioimaging agent was demonstrated on live cancer cells.

GO as SERS substrate. GO has intrinsic SERS effect by chemical enhancement. To enhance such effect, various strategies have been came up with to derive synthetic methods for hybrid composite with noble metals. For example, in 2012, Kim *et al*. reported enhanced SERS effect by GO/polyallylamine-AgNP/polyallyamine-rGO flims.[143] These hybrid films showed highly reproducible, enhanced SERS signals with GO functioning as protecting agent from oxidation and additional SERS enhancer. In biomolecule sensing application, Fan *et al*. showed that of metal nanoparticle attached GO could enable detection bacteria with ultrasensitivity as low as 10 CFU/mL.[144]

Recently, there was advance in GO hybrid structure for label-free detection with enhanced sensitivity by enriching target analyte on hybrid composite. Demeritte *et al*. established GO based biosensor platform for selective separation and label-free detection of Alzheimer's disease(AD) biomarkers from whole blood (Figure 7B).[151] In this platform, GO was used to separate and enrich targets by conjugating antibody into GO and to amplify Raman signals from targets by synergetic enhancement effect with Au shell. This platform could capture 98% AD biomarkers from whole blood and detect the Raman fingerprint of AD biomarkers even at 100 fg/mL without interference of any non-specific proteins.

GO as 2D scaffold for SERS hot-spot. Unlike previous strategies, GO could also serve as 2D scaffold for assembly of metal nanoparticles to generate stable SERS hot-spot. Saha *et al*. showed that highly sensitive SERS signals could be produced by Ag@Au attached GO via stabilization of SERS hot-spots.[152] Moreover, incorporation of magnetic separation process may enable not only sensitive but also rapid detection of biological analytes. For example, Liu *et al*. demonstrated simple, rapid and highly efficient SERS platform to detect pesticide residues from fruit peels by adopting surface magnetic solid phase extraction technique based on Fe_3O_4@GO@AgNP

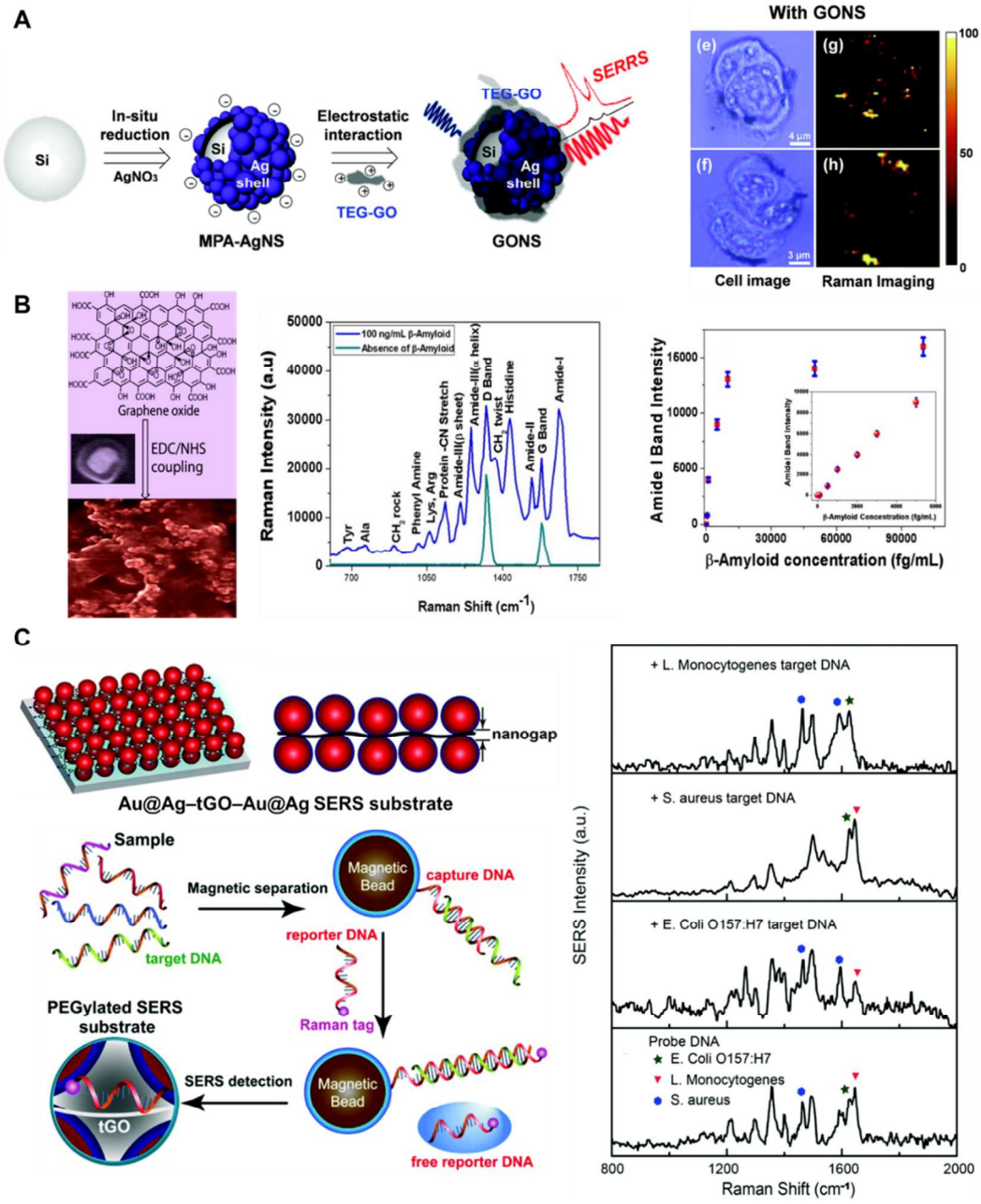

Figure 7. Strategies on Raman biosensors (A) Cancer cell imaging by using GO's Raman signals from GO-encoded Ag nanoshells (GONS). Reproduced from Yim, D.; Kang, H.; Jeon, S. J.; Kim, H. I.; Yang, J. K.; Kang, T. W.; Lee, S.; Choo, J.; Lee, Y. S.; Kim, J. W.; Kim, J. H. Analyst 2015, 140, 3362−3367 (ref 150), with permission of The Royal Society of Chemistry. (B) Label-free detection of Alzhimer's Disease biomarkers based on GO's SERS effect. Reproduced from Demeritte, T.; Nellore, B. P.; Kanchanapally, R.; Sinha, S. S.; Pramanik, A.; Chavva, S. R.; Ray, P. C. ACS Appl. Mater. Interfaces 2015, 7, 13693−13700 (ref 151). Copyright 2015 American Chemical Society. (C) Scheme for multiplexed DNA detection on graphene- nanosheet-gapped plasmonic nanoparticle arrays. Reproduced from Duan, B.; Zhou, J.; Fang, Z.; Wang, C.; Wang, X.; Hemond, H. F.; Chan-Park, M. B.; Duan, H. Nanoscale 2015, 7, 12606−12613 (ref 154), with permission of The Royal Society of Chemistry

,[153] The system showed precise detection of thiram and thiabendazole with aid of GO to enrich analyte and generate SERS hot-spot of AgNP on its surface, showing LOD of 0.48 and 40 ng/cm^2, respectively. In addition, magnetic property from Fe_3O_4 could simplify the whole process.

Another advance in this strategy was shown for the development of SERS based multiplexed DNA detection platform (Figure 7C).[154] Duan *et al.* prepared a trilayered Au@Ag-thiolated GO-Au@Ag for sensitive detection. GO anchor Raman active molecule conjugated DNA reporters into nanogap hot-spots for ultrasensitive detection. Target DNA could be separated by partial hybridization of magnetic probe followed by elimination of DNA reporter from nanogap hot-spots on trilayered nanocomposite, followed by signal disappearance of the reporter.

Electrochemical biosensor

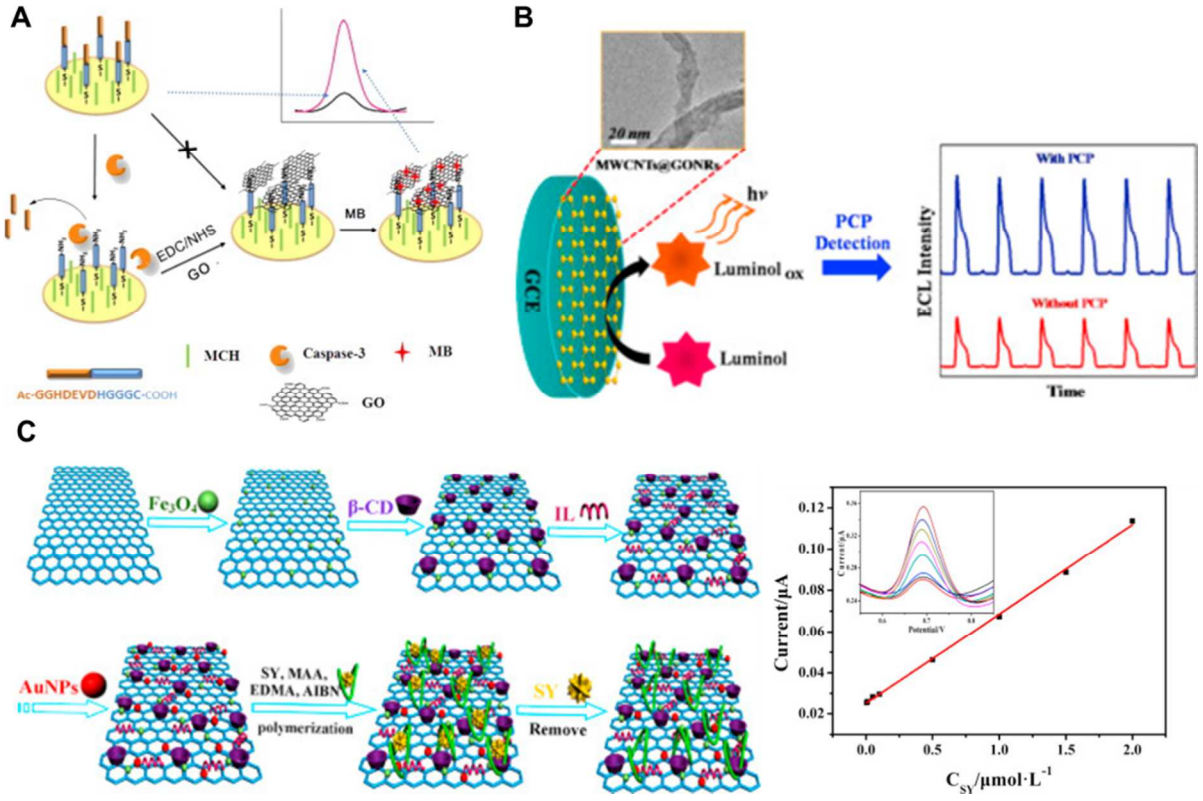

Figure 8. Electrochemical biosensors based on different sensing strategies (A) a scheme of Caspase-3 activity assay platform by enriching methylene blue (MB) on GO. Reprinted from Biosens. Bioelectron., Vol. 68, Chen, H.; Zhang, J.; Gao, Y.; Liu, S.; Koh, K.; Zhu, X.; Yin, Y. Sensitive cell apoptosis assay based on caspase-3 activity detection with graphene oxide-assisted electrochemical signal amplification, pp. 777−782 (ref 165). Copyright 2015, with permission from Elsevier. (B) Scheme of ECL sensor for pentachlorophenol (PCP) detection based on MWCNT@GONR modified electrode. Reprinted from Talanta, Vol. 134, Liu, Q.; Huan, J.; Fei, A.; Mao, H.; Wang, K. "Signal on" electrochemiluminescence pentachlorophenol sensor based on luminol-MWCNTs@graphene oxide nanoribbons system, pp. 448−452 (ref 166). Copyright 2015, with permission from Elsevier. (C) scheme for preparation of selective imprinted electrochemical sensor for sunset yellow(SY) and the linear relationship between the peak current and the concentration of SY. Reprinted from Talanta, Vol. 147, Li, J.; Wang, X.; Duan, H.; Wang, Y.; Bu, Y.; Luo, C. Based on magnetic graphene oxide highly sensitive and selective imprinted sensor for determination of sunset yellow, pp. 169−176 (ref 169). Copyright 2016, with permission from Elsevier.

Background principle and sensing strategies. GO has electroactive property like reversible electrical oxidation and reduction.[46] Meanwhile, rGO shows low charge transfer resistance and good electrochemical properties similar tographene.[50] In this manner, various researches have been conducted with application of GO/rGO to electrochemical biosensor.

Many GO/rGO based electrochemical biosensors involve enzyme reaction,[155,156] and hybrid nanocomposite with conductive materials,[157,158] to enhance electrochemical signal for high sensitivity. Moreover, biosensors can be modified with target specific probes[159,160] to give target specificity.

Till now, based on these strategies, GO based electrochemical biosensors have been developed for enzyme activity[161,162] and DNA[163], protein[160] and cancer cell detection[159]

Enrichment of electrochemically active molecules. In the development of electrochemical biosensor, GO has been often applied as electrochemically active indicator. When GO is attached to electrodes, the signal is generated by electrochemical reaction. Such phenomena was applied to detect biomolecules[163,164] Meanwhile, in 2015, Chen *et al.* utilized GO as electroactive molecule enricher rather than indicator to establish a sensitive cell apoptosis assay platform (Figure 8A).[165] The system introduced N-terminal acetylated peptide substrate, which was immobilized to gold electrode. In the presence of caspase-3 from apoptotic cells, active amine terminal of substrate was released by hydrolysis and then conjugated with GO. Signal could be amplified by absorption of methylene blue (MB), electrochemically active molecule, via π - π interaction with GO. This sensor system demonstrated detection of cell apoptosis with human pulmonary carcinoma, with low LOD of 0.06 pg/mL.

Hybrid composite. To improve electrochemical performance of GO/rGO based electrochemical biosensors, making hybrid composite with conductive materials was proposed.[165] Recently, Liu *et al.* showed that amplified electrochemiluminescence (ECL) sensor could sensitively detect pentachlorophenol (PCP) by incorporating GONR with MWCNT (Figure 7B). [166] Luminol is one of the most commonly used ECL luminophore. In this system, luminol oxidation current could be enhanced for ~5.3 fold by significant electrocatalytic activity of MWCNTs@GONRs. The calculated LOD of this sensor was 0.7 pg/mL, with the ECL signal amplification linear to the concentration of PCP.

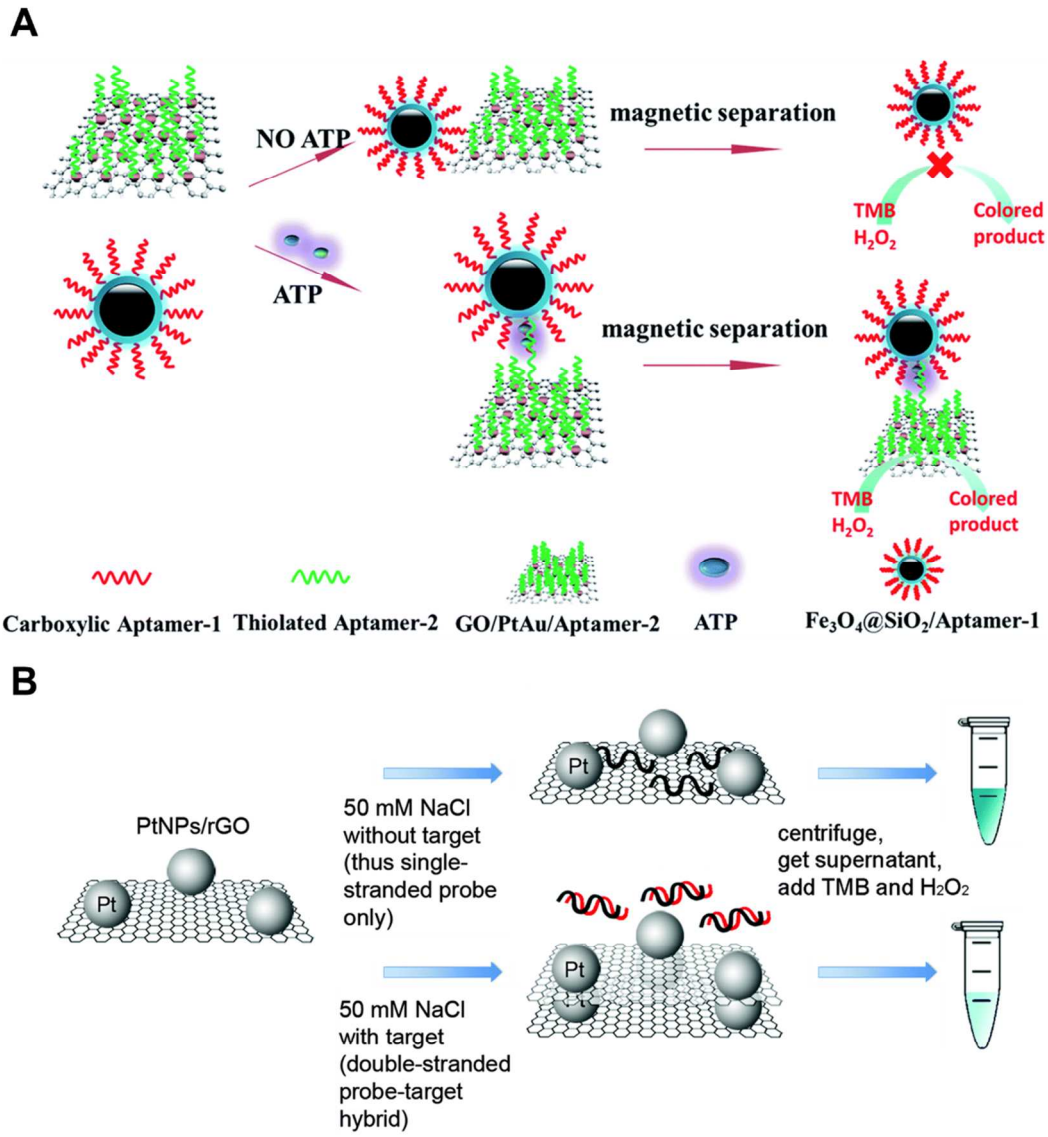

Figure 9. Colorimetric biosensors based on surface modification with target probe (A) Scheme for colorimetric detection of ATP utilizing aptamer modified hybrid composite (GO/PtAu/Aptamer-2) and aptamer-conjugated magnetic nanoparticles (Fe_3O_4@SiO_2/Aptamer-1). Reproduced from Zhang, S.; Wang, K.; Li, J.; Lia, Z.; Sun, T. RSC Adv. 2015, 5, 75746−75752 (ref 176), with permission of The Royal Society of Chemistry. (B) Scheme for sequence-specific DNA colorimetric sensor by non-covalent modification of DNA on PtNPs/rGO. Reproduced from Chau, L. Y.; He, Q.; Qin, A.; Yip, S. P.; Lee, T. J. Mater. Chem. B 2016, 4, 4076−4083 (ref 177), with permission of The Royal Society of Chemistry.

Unlike previous studies with conductive carbon material, electrochemical biosensor based on combination of inorganic nanoparticle and rGO was reported by Benvidi *et al*. [167] Based on ssDNA/AuNP/rGO modified glassy carbon electrode, the system showed high selectivity towards target DNA for breast cancer detection. Also, the hybrid material consisted of chitosan, fishbone-shaped Fe_2O_3 and rGO which showed superior electrocatalytic activity was developed for detection of gallic acid with high sensitivity. [168]

Modification with specific probe. In 2015, Li *et al*. reported a molecular imprinted, highly sensitive and selective sensor for Sunset yellow detection (Figure 8C). [169] Molecular imprinting method adopted in this electrochemical sensor system enhanced target specificity, and eventually diminished the disadvantages shown in conventional systems such as slow binding time, weak electrical conductivity. The system demonstrated fast re-binding dynamic for target analyte detection with a wide linear range from 5.0×10^{-9} to 2.0×10^{-6} M and a detection limit of 2.0×10^{-9} M, showing the possibility for application to spiked drink sample. Another common strategy involved conjugation of antibody on conductive material, also known as electrochemical immunosensor. In 2015, Zhang *et al*. advanced electrochemical immunosensor toward a double signal electrochemical immunosensor for reliable detection. [170] In this biosensor platform, the direct oxidation current from antibody conjugated AgNP/carbon nanocomposite and the indirect reduction current of hydroquinone in the presence of IgG were monitored via GO/AuNP/Antibody modified electrode. The proposed immunosensor showed the successful application to human IgG detection in real samples, with LOD

Analysis types	Applications							
	Detection				Imaging		Enzyme assay	High Throughput Screening
	Small molecule	Nucleic acid	Protein & Peptide	Cell	In vitro	In vivo		
Fluorescence sensor	Yuan *et al.*[94] Zhang *et al.*[67] Zhang *et al.*[68] Cui *et al.*[76] Zhang *et al.*[90] He *et al.*[93] Li *et al.*[117] Huang *et al.*[118] Dou *et al.*[70] Lim *et al.*[88] Basiruddin *et al.*[91] Xing *et al.*[106] Li *et al.*[114]	Lee *et al.*[99] Lee *et al.*[98] Alonso-Cristobal *et al.*[78] He *et al.*[81] Li *et al.*[105] Wu *et al.*[107] Zhu *et al.*[111] Hong *et al.*[112] Huang *et al.*[119] Zhu *et al.*[123] Li *et al.*[69]	Li *et al.*[89] Sun *et al.*[84] Liu *et al.*[86] Sun *et al.*[87] Ueno *et al.*[65] Apiwat *et al.*[66] Li *et al.*[83] Shirai *et al.*[85] Liu *et al.*[108] Huang *et al.*[113]	Nellore *et al.*[71] Tao *et al.*[95] Yan *et al.*[110]	Li *et al.*[120] Shi *et al.*[121]	Lee *et al.*[97]	Lee *et al.*[96] Liu *et al.*[82] Xi *et al.*[122]	Lee *et al.*[96]
Raman sensor	Liu *et al.*[153]	Duan *et al.*[154]	Demeritte *et al.*[151]		Kim *et al.*[149] Yim *et al.*[150]			
LDI/MS sensor	Zhang *et al.*[134] Hong et al[131] Lee *et al.*[130] Zheng *et al.*[135] Wang *et al.*[133]			Abdelhamid *et al.*[131]	Huang *et al.*[135]	Not applicable		
Electrochemical sensor	Liu *et al.*[166] Gao *et al.*[168] Li *et al.*[169]	Benvidi *et al.*[167]	Zhang *et al.*[170] Azahar *et al.*[171]		Not applicable	Not applicable	Chen *et al.*[165]	
Colorimetric sensor	Zhang *et al.*[176]	Chau *et al.*[177]		Zhang *et al.*[175]		Not applicable		

Table 3. The applications of GO biosensor on analysis types

of 0.001 ng/mL. Along with these demonstrations, Ali *et al*. reported anti-apolipoprotein B 100 functionalized nanocomposite consisted of mesoporous few-layer rGO and nickel oxide for highly sensitive detection of low density lipoprotein molecules (LOD : 0.07 mg/dL).[171]

Colorimetric biosensor

Background principle and sensing strategies. GO has an intrinsic peroxidase-like activity with higher stability compared to the natural enzyme.[45] In presence of H_2O_2 and TMB which is substrate for peroxidase, GO produces a blue color from oxidized TMB which follows ping-pong mechanism like HRP.

In recent reports, GO based colorimetric biosensors were based on not only intrinsic peroxidase-like activity but also its large surface area. The GO provides surface for assembly of peroxidase-like materials to increase stability and catalytic activity.[172,173] In addition, GO was modified with probe to establish target specific colorimetric biosensor.[174]

Therefore, various GO based colorimetric biosensors have been developed for the detection of biomarker,[174] DNA[172] and cell.[173]

Modification of hybrid composite with target probe. GO based colorimetric biosensors basically consist of nanohybrid composite with peroxidase like nanoparticles to increase the sensitivity.[175] Recent developments on selective biosensor were focused to expand the applications of colorimetric biosensor. In 2015, Zhang *et al*. developed an ultrasensitive and selective colorimetric biosensor for ATP detection based on target separation and superior catalytic activity of GO/PtAuNP (Figure 9A).[176] ATP binds to both aptamer modified GO/PtAuNP and aptamer linked magnetic beads. The binding of ATP makes the complex of GO/PtAuNP and magnetic beads. After magnetic separation of the complex, reaction of the complex with H_2O_2 and TMB produces blue color. The LOD is 0.2 nM, the lowest value among other ATP colorimetric sensors. In addition, the color change was clearly discriminated with naked eyes at 50 nM level. Meanwhile, Chau *et al*. reported Pt/rGO based colorimetric biosensor for sequence

specific detection of DNA (Figure 9B).[177] DNA probes which are adsorbed on Pt/rGO stabilized nanocomposite in salt buffer. When target DNA hybridized with probe on Pt/rGO, the nanocomposite forms aggregates due to salt-induced aggregation. After centrifugation, the blue color was produced by remained nanocomposite. This biosensor showed that 3-base-mismatched sequence could be distinguished.

Perspective and future direction of GO biosensor

Due to the unique properties of GO, various types of biosensor have been developed. Each type of biosensors has been thoroughly investigated based on various strategies to overcome disadvantages of previous methods and improve its performances. In fluorescence biosensor, recent studies have been focused on the expansion of detection spectrum and the signal amplification. LDI/MS biosensors aimed to increase the efficiency of energy transfer and gain target specificity by hybrid composite and functionalization with probes. Raman biosensors have been investigated on purpose of simplifying the analytic process and enhancing Raman signal by magnetic separation and adaptation of hybrid nanocomposite. Lastly, electrochemical and colorimetric biosensors have been developed with major goals on enhancement of their sensitivity and selectivity.

Summarized applications on each sensing type in recent two years are listed in Table 3. As shown on the table, fluorescence based biosensors have been studied within a range of applications from small molecule detection to *in vivo* imaging. In other analysis types, on the other hand, the applications have been focused on simple direct detection of target molecule. The development of novel GO-based biosensors for specific application in the future will require decision of appropriate analysis type, along with proper sensing strategy.

Yet several problems remain with regard to development of GO biosensor. One critical drawback to be solved is the physicochemical inhomogeneity of GO due to high variation among batches. Lack of homogeneity may result in restriction to deeper mechanistic studies and reduce reproducibility in sensing performance. To acquire physically and chemically uniform GO, there have been many studies conducted to optimize synthetic conditions including alternative carbon source, [178,179] ultrasonication time[180] and oxidation path control, [181-184] pH-assisted sedimentation, [185] mild thermal annealing. [186] Along with them, thorough study of GO is required to significantly control size, degree of oxidation and location of functional groups. Moreover, mass production of GO in high homogeneity with low cost will also be in demand to apply GO biosensor to clinical research and commercialization. Another problem is complicated process of surface modification. Various types of GO-based biosensors except fluorescence sensor adopted covalent surface modification to improve target specificity and sensitivity. Surface modification involves complicated and delicate process, resulting in a lack of reproducibility of the proposed sensors. To solve this problem, thorough studies are required, for satisfactory controls on surface chemistry such as location selective functionalization on GO surface. In addition, it is important to precisely understand fundamental principles on interaction between GO and probes including nucleic acid and peptide in order to improve specificity and sensitivity and expand application of fluorescence biosensors. It is reported that recovery efficiency of quenched probe is affected by affinity between GO and fluorescent probes, which is dependent to environmental factors (temperature, salt concentration, etc.) and intrinsic factors (length and sequence of probes, oxidation degree of GO, etc.).[187-190] The affinity variation towards GO may cause several drawbacks such as unreliable sensitivity to limit further application. Therefore, it is highly required to put efforts on probe design to have uniform affinity on GO regardless of those factors.

Conclusion

In conclusion, the fascinating properties of GO allow it to be utilized into broad research area. It is no doubt that GO is one potent candidate for biosensing platforms to be routinely implemented in biomedicine. Based on its unique properties, there have been numerous efforts put on development and improvement of GO biosensors with various strategies. In the future, it is anticipated that deep mechanistic studies on GO biosensors will be investigated, followed by expansion of their application to commercialization.

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The manuscript was written through contributions of all authors.

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Jungho Kim is Ph.D candidate in the Department of Chemistry at the Seoul National University. He received a B.S. in Chemistry from Konkuk University in 2014. His work in the Dal-Hee Min group focuses on the development of biosensor based on carbon nanomaterials.

Se-Jin Park studied at the College of Natural Science, Sungkyunkwan University, in South Korea where she received a B.Sc. in Chemistry and Biology. She is currently a Ph.D candidate in the laboratory of Dal-Hee Min in Chemistry at the Seoul National University. Her research interest includes various biological applications of functional nanomaterials.

Dal-Hee Min is a Professor in the Department of Chemistry at the Seoul National University, South Korea. She received degrees in Chemistry from Seoul National University (B.S. & M.S.) and the University of Chicago (Ph.D.) and completed her postdoctoral training at MIT. She started her independent academic career at KAIST (Korea Advanced Institute of Science and Technology, Korea) as an Assistant Professor in Oct. 2007 and moved to Seoul National University in 2011. Prof. Min's research focuses on developing bioanalytical platform technologies based on various nanomaterials including graphene for diagnostics, biosensing, drug discovery and drug delivery system. Especially, her group actively explores diverse biological applications of graphene derivatives and reported many seminal works on biological applications of graphene including 'graphene-based enzyme activity assay', 'behaviors of human cells cultured on graphene surface' and 'microRNA sensor based on graphene'. She won many awards including KCS-Wiley Young Chemist Award (KCS-Wiley) and Korea L'Oreal UNESCO Fellowship Award for Women in Science. Prof. Min serves as an associate editor for J Mater Chem B (RSC, UK) and as an editorial board member of 2D Materials (IOP, UK) since 2013. She also founded a company 'Lemonex Inc.' in 2014, specialized in biomedical applications of nano/micro materials.

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