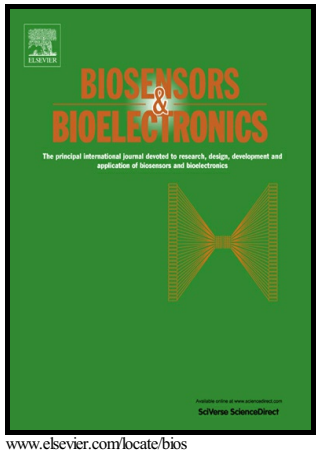

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Printed organo-functionalized graphene for biosensing applications

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Abstract

Graphene is a highly promising material for biosensors due to its excellent physical and chemical properties which facilitate electron transfer between the active locales of enzymes or other biomaterials and a transducer surface. Printing technology has recently emerged as a low-cost and practical method for fabrication of flexible and disposable electronics devices. The

combination of these technologies is promising for the production and commercialization of low cost sensors. In this review, recent developments in organo-functionalized graphene and printed biosensor technologies are comprehensively covered. Firstly, various methods for printing graphene-based fluids on different substrates are discussed. Secondly, different graphene-based ink materials and preparation methods are described. Lastly, biosensing performances of printed or printable graphene-based electrochemical and field effect transistor sensors for some important analytes are elaborated. The reported printed graphene based sensors exhibit promising properties with good reliability suitable for commercial applications. Among most reports, only a few printed graphene-based biosensors including screen-printed oxidase-functionalized graphene biosensor have been demonstrated. The technology is still at early stage but rapidly growing and will earn great attention in the near future due to increasing demand of low-cost and disposable biosensors.

Keywords: Printing technologies, ink preparations, graphene materials, biosensors.

1. Introduction

Graphene is currently one of the most widely studied materials in nearly every field of the natural sciences due to its remarkable physical, chemical and electrical properties including huge specific surface area, mechanical strength, excellent electrical, thermal conductivity, good biochemical compatibility, low hazardous properties and superior electrochemical performances (Geim and Novoselov, 2007; Ratinac et al., 2011; Wei et al., 2010). The properties derived from its two-dimensional, one-atom-thick honeycomb-like lattice structure of sp^2 hybridized carbon atoms make graphene a potential candidate for a wide variety of applications such as supercapacitors, batteries, fuel cells and chemical/biosensors (Fang et al., 2009; Feng et al.,

2011; Yan et al., 2010). Moreover, graphene is frequently considered to be more promising for commercial use than the other nanostructured carbon allotropes, namely one-dimensional nanotubes (1D) and zero-dimensional fullerenes (0D) because it can be produced and processed at low cost (Pumera et al., 2010; Ratinac et al., 2011).

In chemical/biosensor applications, graphene have shown superior performances compared with other carbon based materials including its nearest counterpart, carbon nanotubes (CNTs), because of its double surface area compared to CNTs, higher chemical reactivity due to larger number of edge plane per unit mass and higher electron mobility and conductivity(Pumera et al., 2010; Ratinac et al., 2011). In addition, it can be inexpensively produced from low-cost graphite with no metallic impurity, which would otherwise be found in most CNTs and can introduce anomalous sensing response(Wang, 2005). The successful application of graphene as chemical/biosensors has been mainly driven by advancements in graphene synthesis and fabrication processes as well as graphene's inherent beneficial properties.

Graphene can generally be synthesized and processed based on non-solution and solution-route approaches. Solution-route methods including chemical oxidation/reduction, functionalization of graphene oxide, liquid-phase mechanical exfoliation, electrolytic exfoliation, mechanical exfoliation with intercalation by small molecules, hydro/solvothermal synthesis, total organic synthesis and other solution techniques are generally more suitable for chemical/biosensing than non-solution ones such as mechanical cleavage, chemical vapor deposition (CVD), epitaxial growth via ultra-high vacuum graphitization and other vapor-phase techniques due to its compatibility with biochemical systems and low cost(Dato et al., 2008; Joung et al., 2010; Kim et al., 2009). More importantly, they can be used to produce organo-functionalized graphene and graphene/polymer composites(Kou et al., 2009; Qi et al., 2010) that contain copious structural

defects and diverse functional groups(Chen et al., 2009; Meyer et al., 2008), which are highly advantageous for biochemical sensing reactions as well as biological receptor immobilization(Z. Wang et al., 2009). Graphene has been functionalized with a variety of organic groups such as small to large aromatic/non-aromatic groups and polymeric network(Georgakilas et al., 2012). Moreover, the resulting graphene nanosheet dispersed solutions can directly be used as printable inks or paste that can be deposited by various deposition methods such as spin coating and printing to fabricate chemical/biosensing devices.

Recently, printed electronics has emerged as a promising technology for production of electronic devices such as transistors and sensors on a flexible and disposable substrate due to increasing demand of low cost electronic devices in various consumable products and the advancement of functional printing materials. The use of low-cost high-throughput printing technology will allow low-cost low-performance electronics for applications such as smart sensors for food packaging, smart radio-frequency identification (RFID) for contactless identification, organic light emitting diode (OLED) as smart label, flexible solar cells on vehicle roofs, and so on(Allen et al., 2011). Advanced functional ink materials are the keys to the success of printed electronics. Thus, research in printed electronics has been focusing on innovative ink materials with new and improved properties suitable for various printing processes.

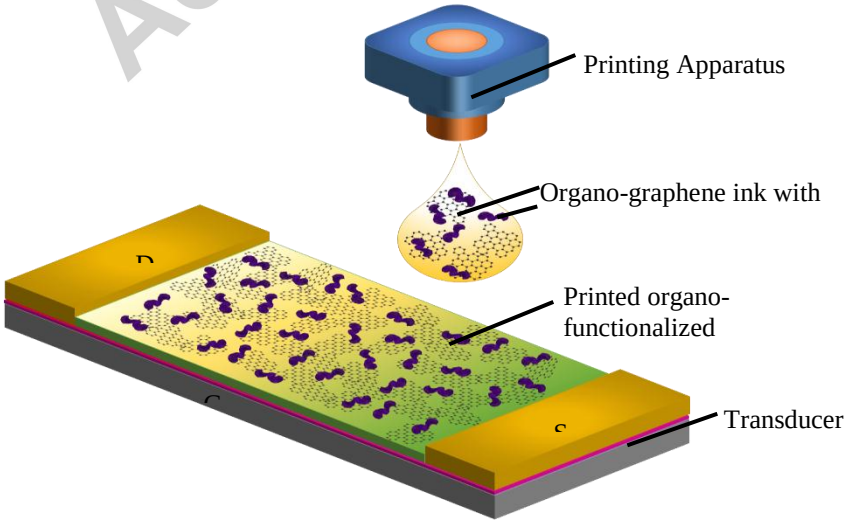


Figure 1. A graphical representation of printed organo-functionalized graphene biosensors.

Graphene-based ink is an innovative functional material that is particularly promising for printed organo-functionalized graphene biosensors. In this platform, organo-functionalized graphene is printed on a transducer as depicted in Figure 1 and functions as an active medium that will enhance the interactions between biological analyte and receptor immobilized on graphene. The charge or mass transfer due to biological interaction will be converted into a form of electrical signal by the underlying transducer. Printed organo-functionalized graphene biosensors may be diversely classified by preparation method, printing technique and transduction platform. Practical preparation methods for organo-functionalized graphene include dispersion of reduced graphene oxide in organic solution, surface functionalization, electrolytic exfoliation and mechanical exfoliation with intercalation of small molecules(Chang and Wu, 2013). The functionalization of graphene, which can be either covalent or non-covalent type, is very crucial for effective immobilization of biological receptors(Sharma et al., 2016). Regarding printing method, organo-functionalized graphene inks may be deposited by screen printing, inkjet printing, aerosol jet printing, nano-imprinting, gravure printing flexography and offset printing. Lastly, organo-functionalized graphene can be applicable to various transduction platforms including electrochemical sensors, field effect transistors (FET) and optical biosensors (Pumera et al., 2010; Zhu et al., 2015).

In this review, we will focus on the development of printed organo-functionalized graphene-based biosensors covering printing technologies for printed electronics and graphene-based devices, graphene ink preparation processes via solution-route approaches and characteristics of graphene-based biosensors. Firstly, printing technology and its application for printed electronics and graphene printing are widely elaborated. Preparation of graphene and graphene ink materials via various solution-processing methods are then explained. Next, biosensing properties of graphene, detection performance of printed and printable graphene-based biosensors are described. More comprehensive details in each part can be referred to other recent review articles devoted to each issue(Allen et al., 2010; Chang et al., 2012; Singh et al., 2011). Lastly, remarks on the present results and the future research direction for printed graphene-based biosensors are discussed.

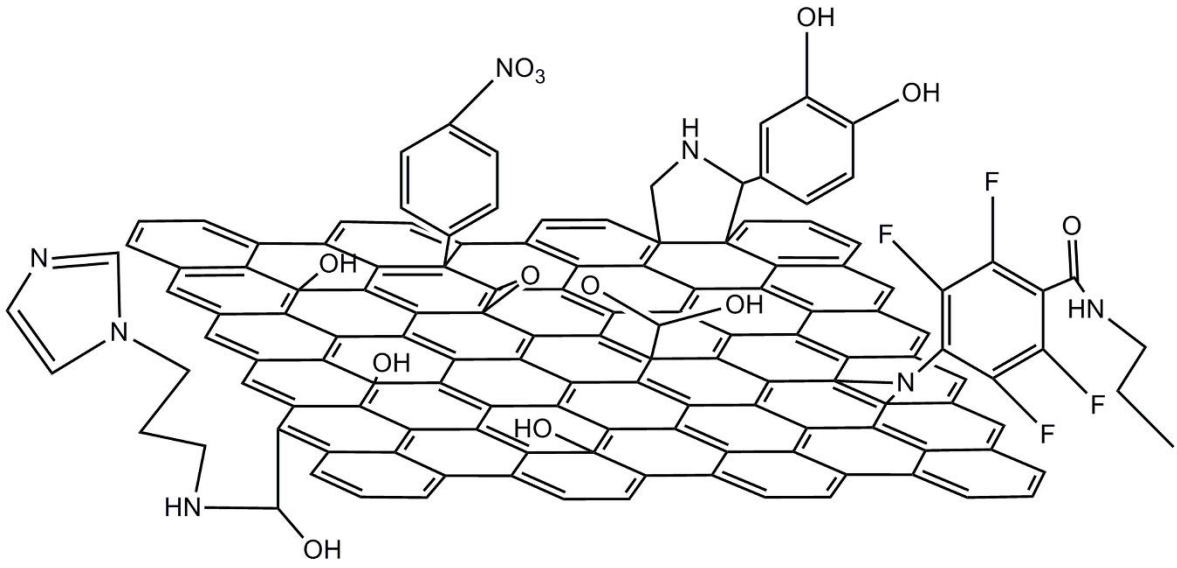
2. Printing technology for printed electronics and graphene-based devices

Printing is the process of depositing material patterns by means of inked-type transfer techniques. Printing technology can be classified into sheet- and roll-to-roll-based schemes. Sheet-based approaches including screen, inkjet, aerosol jet and dot-matrix printing are serial printing processes suitable for low-volume and good-precision work. Roll-based schemes including gravure, offset, flexographic, electrophotographic and laser printing utilize continuous roll-to-roll transfer techniques, which allow rapid mass processing of large areas with fair pattern resolution(Lee et al., 2010). Printing technology is selected based on layer requirements, properties of printed materials as well as economic and technical aspects of printed products. Printed electronics require relatively high pattern resolution, high layer-to-layer registration

accuracy and good thickness control compared to conventional printing applications. Screen, inkjet, aerosol jet, flexographic, gravure and offset printing are among the methods found to be suitable for printed electronics while electrophotography, laser printing and dot-matrix printing are not currently used due to incompatible ink material and unsuitable printing features. Printing methods and materials for printed electronics and graphene-based devices are described with moderate details in the supplementary information, section S1.

3. Graphene: ink preparation and organo-functionalization

A large number of methods for the preparation graphene solutions and dispersions suitable for printing techniques, i.e. inks have recently been developed for printed electronics and other applications. Due to graphene's very low dispersibility in most organic solvents, graphene in solution must be functionalized with organic groups is to increase its processability. This is



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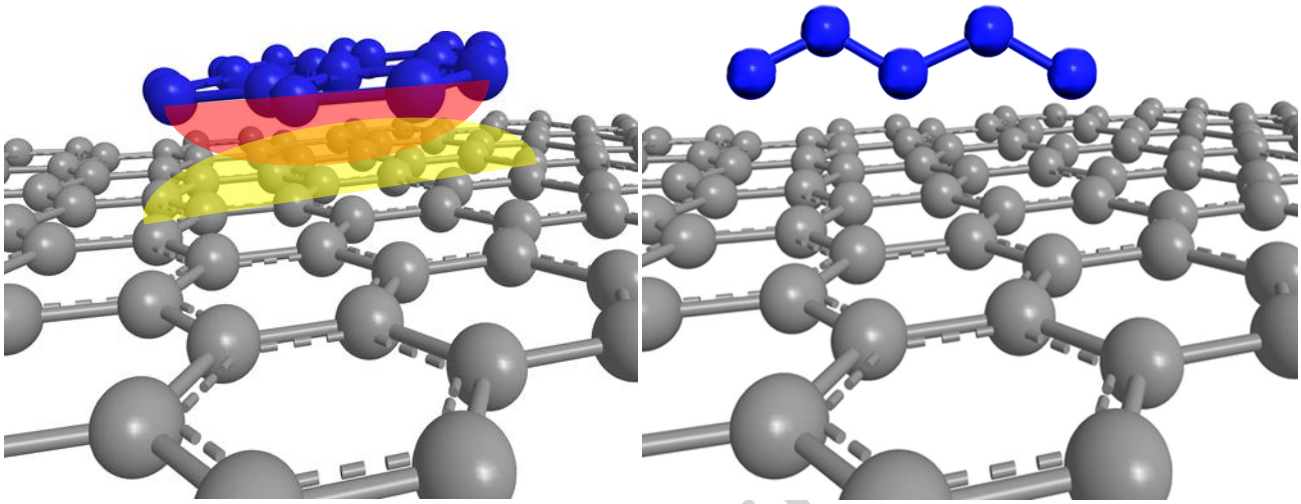
a) Graphene covalently functionalized with various organic groups.



b) Graphene non-covalently functionalized with an aromatic molecule (pyrene) via π-π stacking (left) and a non-aromatic molecule (pentane) via CH-π interaction (right).

Figure 2. Exemplary illustration of covalently and non-covalently functionalized graphene.

Functionalization of graphene can be divided by the nature of the bond between the graphene nanosheets and the attached molecules into two main types, covalent and non-covalent as shown in Figure 2. The first one is covalent functionalization where strong covalent bonds are responsible for the binding between graphene and organic groups such as hydroxyl, imide, benzene-derivative and fluorinate groups as illustrated in Figure 2 (a). This process results in permanent structural alteration or rehybridization of sp^2 carbon atoms into sp or sp^3 configuration, leading to the change of electronic conjugation. It is also considered as an effective mean for substitutional graphene doping with various atoms such as nitrogen and fluorine that may be beneficial for biological interactions and sensing. Covalent functionalization is generally achieved via two distinct routes. Firstly, graphene is oxidized into graphene oxide (GO) and covalent bonds between organic functional groups and the oxygen containing groups

of GO are then subsequently formed. Secondly, covalent bonds between free radicals or dienophiles and sp^2 hybridized carbon atoms of graphene nanosheets are directly created. Covalent functional groups on graphene are particularly useful for molecular attachment of biological receptors such as enzymes, antibody and complementary DNAs, which will specifically bind to target biological species(Sharma et al., 2016). For instance, carboxylic group on graphene can covalently bond to free amino groups of glucose oxidase.

The second type is non-covalent functionalization which primarily involves van der Waals and electrostatic forces leading to physical adsorption of suitable molecules on the graphene nanosheet surface. Physical adsorption can be achieved by mixing of graphene with polymers, adsorption of surfactants and π-π interaction with small aromatic molecules. The noncovalent interactions are thus the main mechanisms responsible for graphene functionalization of various kinds of organic species including aromatic (i.e., benzene and pyrene) compounds, non-aromatic compounds (i.e., pentane), polymers (i.e., polyaniline and polystyrene) as well as biomolecules (i.e., nucleic acids, peptides and lipids). Aromatic molecules such as pyrene will mainly bind to graphene via π-π interactions while non-arromatic compounds typically adsorb on graphene via CH-π interaction (hydrogen bonding) as illustrated in Figure 1 (b)(Georgakilas et al., 2016). For polymer and biomolecules, they can bind on graphene via ionic, van der Waals, CH-π and/or π-π interactions depending on their physical and chemical structures. The graphene-organic configurations may typically enhance biosensing performances in two ways. Firstly, they may allow direct physical or chemical adsorption of target biomolecules, resulting in charge or mass transfer and enhanced sensing response. Alternatively, they can substantially assist the covalent or noncovalent immobilization of biological receptors similar to covalent functional groups. This functionalization approach is more attractive for some applications than the covalent one since

functional groups attached by non-covalent mechanisms do not damage the electronic structure of graphene. However, noncovalent interactions are less stable than covalent ones due to smaller dissociation energy and may thus not be suitable for reusable biosensors. More detailed information on graphene functionalization can be obtained from recent reviews in these topics (Georgakilas et al., 2016; Kuila et al., 2012). Important organo-functionalized graphene ink preparation methods are broadly explained in the supplementary information, section S2.

4. Printable and printed organo-functionalized graphene biosensors

Graphene based biosensors are defined here as sensors that employ graphene/biological receptors as an active sensing material and/or apply it for sensing biological analytes. They may be classified based on working principles into three main groups including electrochemical, FET and other biosensing platforms. Table 1 provides a summary of materials, biological receptors, analytes and minimum reported concentration for graphene-based biosensors. Graphene-based biosensors integrate biological receptors such as enzymes, antibodies and complementary single-stranded DNAs with graphene-based sensing materials.

Table 1. Recent organo-functionalized graphene based biosensors.

Active Material	Bioreceptor	Analytes	Detection method	Minimum reported concentration	Ref.
PDDA[a] capped AuNPs[b]/G/MWCNT[c]	GOD	glucose	CV; amperometry	4.8 µM	(Yu et al., 2014)
G/PANI[d]/AuNPs	GOD	glucose	CV; amperometry	0.6 µM	(Xu et al., 2014)
rGO/AgNPs[e]	GOD	glucose	CV	1.6 mM	(Palanisamy et al., 2014)
G/PVP[f]/PANI	ChOx[g]	cholesterol	amperometry	1 µM	(Ruecha et al., 2014)
G/IL[h]	ChOx; CAT[i]	cholesterol	CV; amperometry	0.05 µM	(Gholivand and Khodadadian, 2014)
G/AAIL[j]	TYR[k]	catechol	amperometry	8 nM	(Lu et al., 2014)
G/Chit[l]	GOD	glucose	amperometry	0.4 µM	(Yang et al., 2012)
G/Nafion	GOD	glucose	CV	0.04 mM	(Hui et al., 2013)
GO/AuNPs/PDA[m]/THin/GO	Anti-AFP	AFP	CV; DPV[o]	0.03 ng/ml	(Peng et al., 2014)
rGO/PPyNT[p]	Non-enzymatic	H_2O_2	FET	100 pM	(Park et al., 2014)
rGO/PNA[q](/PASE[r])	PNA	DNA	FET	100 fM	(Cai et al., 2014)

rGO/hemin; rGO/AgNPs	GOD; anti-CEA	CEA[s]	ELC[t]	0.03 pg/ml	(Jiang et al., 2014)
GO	AuNPs–ssDNA[u]	DNA	SPR	10 fM	(Xue et al., 2014)
rGO(/PBSE[v])	mAb[w]	PSA-ACT[x]	FET	100 fg/ml (~1.1fM)	(Kim et al., 2013)

[a]poly(diallyldimethylammonium chloride);

[b]gold nanoparticles;

[c]multi-walled carbon nano tube;

[d]polyaniline;

[e]silver nanoparticles;

[f]polyvinylpyrrolidone;

[g)]cholesterol oxidase;

[h)]ionic liquid;

[i]catalase;

[j]amino acid ionic liquid;

[k]tyrosinase;

[l]chitosan;

[m] polydopamine;

[n)]thionine;

[o]differential pulse voltammetry;

[p]polypyrrole nanotube;

[q]peptide nucleic acid;

[r]1-Pyrenebutanoic acid succinimidyl (crosslinker molecule);

[s]carcinoembryonic antigen;

[t]electrochemiluminescence;

[u]single-stranded DNA;

[v]1-pyrenebutanoicacid succinimidylester;

[w] PSA monoclonal antibody;

[x]prostatespecificantigen/α1-antichymotrypsin

4.1 Electrochemical biosensor

Graphene-based electrochemical sensors utilized graphene with biological receptors as electrochemical working electrode and detect analytes via electrochemical oxidation or reduction of analytes, which can be measured in various detection modes such as cyclic volatmmetry (CV), chronoamperometry, square wave voltammetry (SWV), electrochemical impedance spectroscopy (EIS) and so on. This class of biosensors can be further divided into two main types including enzyme-based and (b) immuno-based ones. A recent in depth review of electrochemical graphene biosensors was done recently (Lawal, 2015). This review only focuses on printed and printable (i.e. fabricated by solution processing; derived by depositing solution or dispersions of functionalized graphene) organo-functionalized graphene biosensors.

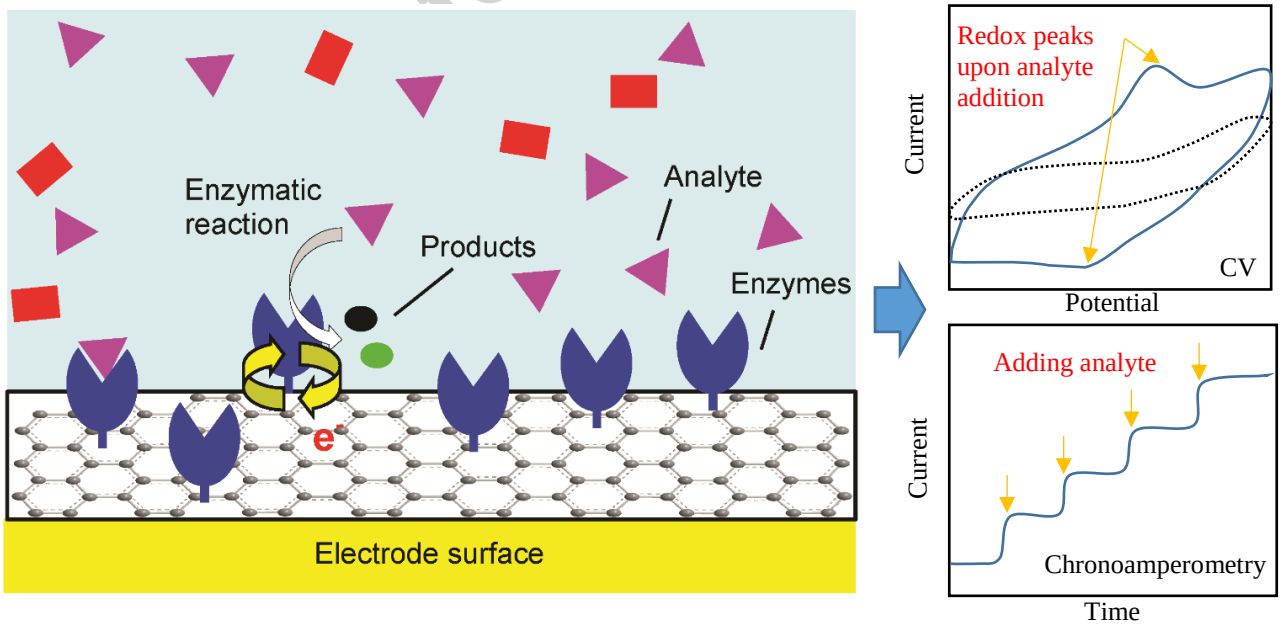


Figure 3. Schematic of graphene based enzymatic biosensor.

4.1.1 Enzyme-based electrochemical biosensor

Enzyme-based electrochemical biosensors employ immobilized enzymes on an electrode surface that selectively react with biological analytes to stimulate electrochemical redox process as schematically represented in Figure 3. One key issue for the construction of a well-functioning biosensor is the efficient immobilization of active biological species in a way that does not impede its biocatalytic functionality, e.g. by not allowing contact to the analyte solution or denaturing the active biomolecules, as well as preventing the species from leaking out over the sensors lifetime. Enzymatic biosensors make use of the bio-recognition by enzymes to target substrates and their bio-catalyzed chemical conversion reactions which can generate electroactive products causing electrochemical responses as CV peaks or current changes as illustrated in Figure 3.

Oxidases (e.g. glucose oxidase (GOD)(Yu et al., 2014), cholesterol oxidase(Ruecha et al., 2014) and tyrosinase(Lu et al., 2014), etc.) are the most widely used class of enzymes that produce hydrogen peroxide (H_2O_2) that can be sensitively detected by electrochemical methods. Various graphene-based electrochemical sensors has been developed to detect H_2O_2. For example, it was recently demonstrated that GO modified glassy carbon (GC) electrodes displayed high H_2O_2 detection sensitivity, wide linear range and lower redox potentials (0.20/0.10 V), which were substantially better than graphite/GC and unmodified GC electrodes(Zhou et al., 2009). Moreover, H_2O_2 sensitivity and selectivity of graphene-oxide based material can be significantly enhanced by mixing graphene oxide and electrocatalytic metal oxide such as MnO_2(Li et al., 2010). Gold nanoparticle/chitosan is another useful functionalization material for enhancing electrocatalytical activity of graphene toward H_2O_2 and O_2(Shan et al., 2010). Additionally, *inkjet-printed* graphene modified carbon paste electrochemical sensors were demonstrated for

H_2O_2, nicotinamide adenine dinucleotide (NAD^+/NADH), ferri/ferro cyanide ($Fe(CN)_6^{3-/4-}$) and Salbutamol detections with enhanced sensitivity(Karuwan et al., 2012; Sriprachuabwong et al., 2012). Also, *inkjet-printed* rGO was directly deposited on a flexible polyethylene substrate for highly sensitive dopamine sensing(Su et al., 2015). Moreover, *screen-printed* graphene/carbon paste electrochemical electrode on PVC substrates was reported for H_2O_2, NAD^+/NADH and $Fe(CN)_6^{3-/4-}$ detections with improved sensitivity(Karuwan et al., 2013).

The promising H_2O_2 sensing performances of graphene-based electrochemical sensors makes them a potential choice for oxidase-based electrochemical biosensing. However, there must also be effective or direct electron transfer between the active center of enzymes and the electrode to achieve an efficient enzyme-based electrochemical sensor. Direct electron transfer can barely occur in typical electrodes since electrons from the electrode cannot approach the deep hydrophobic cavity of the enzyme's active center(Ghindilis et al., 1997) and intermediate electron mediators are needed to assist the electron transfer process, which in turn results in the slower reaction. Thus, direct electron transfer between the electrode and the enzyme active center, which is called direct electrochemistry of enzymes(Armstrong et al., 1988; Léger and Bertrand, 2008) is essential. Nanostructured materials such as CNTs, fullerene, metal nanoparticles and some carbon-metal oxide nanocomposites have been reported to exhibit having efficient electron transfer and direct enzyme electrochemistry properties(Sarma et al., 2009; Yao and Shiu, 2008).

Direct electrochemistry on enzymes particularly GOD of graphene synthesized by various solution processing methods has been expansively studied for glucose detection (J. Wang et al., 2009; Yang et al., 2012). The first step in a GOD catalyzed reaction is reduction of FAD to $FADH_2$ which is then oxidized by O_2 due to its higher reduction potential. After that O_2 is

reduced in H_2O_2 while an electrochemical signal is generated. In general, the direct electrochemistry of GOD on graphene-based electrodes show high repeatability and stability with 95% retention over one-week storage, which are considerably better than most of reported CNTs based electrodes due possibly to its high enzyme loading capacity and high biocompatibility(Cai and Chen, 2004; Deng et al., 2008). For instance, a glucose sensor using direct electrochemistry of glucose oxidase based on nafion-rGO-GOD modified gold electrodes was recently fabricated (Hui et al., 2013). rGO was non-covalently functionalized using GOD solution (1000 U/ml) and coated with a nafion layer as a protective and selective membrane. The biosensor exhibited a high electron transfer rate constant (k_s) of 1.95 s^{-1}, a high CV sensitivity of 21.9 μA mM^{-1} cm^{-2} a wide linear range of 2-14 mM and a low detection limit of 0.04 mM (S/N = 3). In another work, glucose, lactate, xanthine and cholesterol enzymes were covalently immobilized to graphene nanosheets using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)—N-hydroxysuccinimide (NHS) coupling agents and applied on a paper based mircrofluidic system (Labroo and Cui, 2014). The device can provide simultaneous detections of glucose, lactate, xanthine and cholesterol with short measuring times of less than 2 min, low detection limits and wide detection ranges. In another report, rGO functionalized with polyethylenimine-polyvinylpyrrolidone and immobilizated with GOD in ionic liquids demonstrates high glucose-sensing performance with a wide linear range (2 to 14 mM), good sensitivity, high selectivity and high stability(Shan et al., 2009). In addition, rGO co-immobilized with GOD and alcohol dehydrogenase (ADH)(Zhou et al., 2009) displays further improved glucose-sensing performances with high sensitivity, very fast response (response time <9 s) and a low detection limit of 2 μM (S/N = 3), which are superior to many other carbon-based electrochemical electrodes such as CNTs(Lin et al., 2004; Liu and Lin, 2006), highly

ordered mesoporous carbon(Zhou et al., 2008), carbon nanofibers(Wu et al., 2007) and exfoliated graphite nanoplatelets(Lu et al., 2007). Alternatively, rGO functionalized with chitosan and GOD immobilization also exhibits excellent glucose sensitivity, selectivity and long-term stability, which can be attributed to the advantageous effects of chitosan on graphene functionalization and GOD immobilization(Kang et al., 2009). Organo-graphene/metal nanoparticles (NPs) composite is another promising platform for enzyme-based biosensing. For example, highly sensitive glucose biosensor based on GOD/graphene/PtNPs/ chitosan (Wu et al., 2009) has been demonstrated with a low glucose detection limit of 0.6 µM. The results may be attributed to the graphene-metal nanoparticles composite structure, in which nanoparticles act as spacer preventing restacking or agglomeration of graphene sheets, resulting in large effective surface area and conductivity(Shan et al., 2010). Additionally, GOD-catalyzed biosensors based on graphene organo-functionalized with polyaniline (PANI) and gold nanoparticles (AuNP) was developed for glucose sensing (Xu et al., 2014). Graphene was non-covalently functionalized with PANI by standard oxidative polymerization and AuNPs/GOD were successively added by mixing graphene/PANI dispersion with citrate derived Au colloid and GOD before drop casting onto GC electrodes. The composite exhibited a high k_s of ~4.79 s^{-1} (almost twice higher than k_s of graphene (2.68 s^{-1})), a wide linear range of 4.0 µM- 1.12 mM and a low detection limit of 0.6 µM (S/N = 3) (Wu et al., 2010). Recently, GOD-functionalized *screen-printed* graphene biosensors was achieved by adding enzyme stabilizer into graphene paste, demonstrating a full-functional glucose biosensor directly fabricated by printing process(Pepłowski et al., 2015).

Horseradish peroxidase (HRP), an enzyme for H_2O_2, is often used together with oxidase enzyme to further enhance the sensitivity and selectivity sensing performances since the direct reaction with H_2O_2 still requires relatively high working potential while HRP alone has also been used to

develop graphene-based enzymatic H_2O_2 sensors. For example, Zeng et al.(Zeng et al., 2010) used non-covalent self-assembly of sodium dodecyl benzene sulphonate (SDBS) functionalized graphene sheets (GSs) and HRP by electrostatic attraction as means to create sensing material for H_2O_2 detection. Resulting HRP–graphene electrodes display high electrocatalytic activity to H_2O_2 with high sensitivity, wide linear range, low detection limit, fast amperometric response and high stability with little loss of sensitivity after 30 measurements (>90%). Graphene-nanoplatelet H_2O_2 biosensor is directly fabricated from graphite with the aid of 1,3,6,8–pyrenetetrasulfonic acid (TPA)(Dong et al., 2009). Moreover, sulfonated graphene composite with Au nanoparticle/HRP/chitosan synthesized by co-immobilization showed a high H_2O_2 sensitivity, a short response time (<3 s), a wide linear range (5 µM-5.13 mM) and a low detection limit (1.7 µM at S/N = 3)(Zhou et al., 2010). Graphene has also been incorporated with dehydrogenase enzyme that generates NADH as a byproduct. For instance, ADH-immobilized graphene electrode have been demonstrated for ethanol enzymatic sensing, which displays fast ethanol response, high selectivity, wide dynamic range and low detection limit compared with ADH-immobilized graphite and ADH-immobilized GC electrodes(Zhou et al., 2009). The observed performance may be attributed to excellent NADH catalytic activity of graphene and effective transport of ethanol/NADH products through graphene composite(Chen et al., 2008).

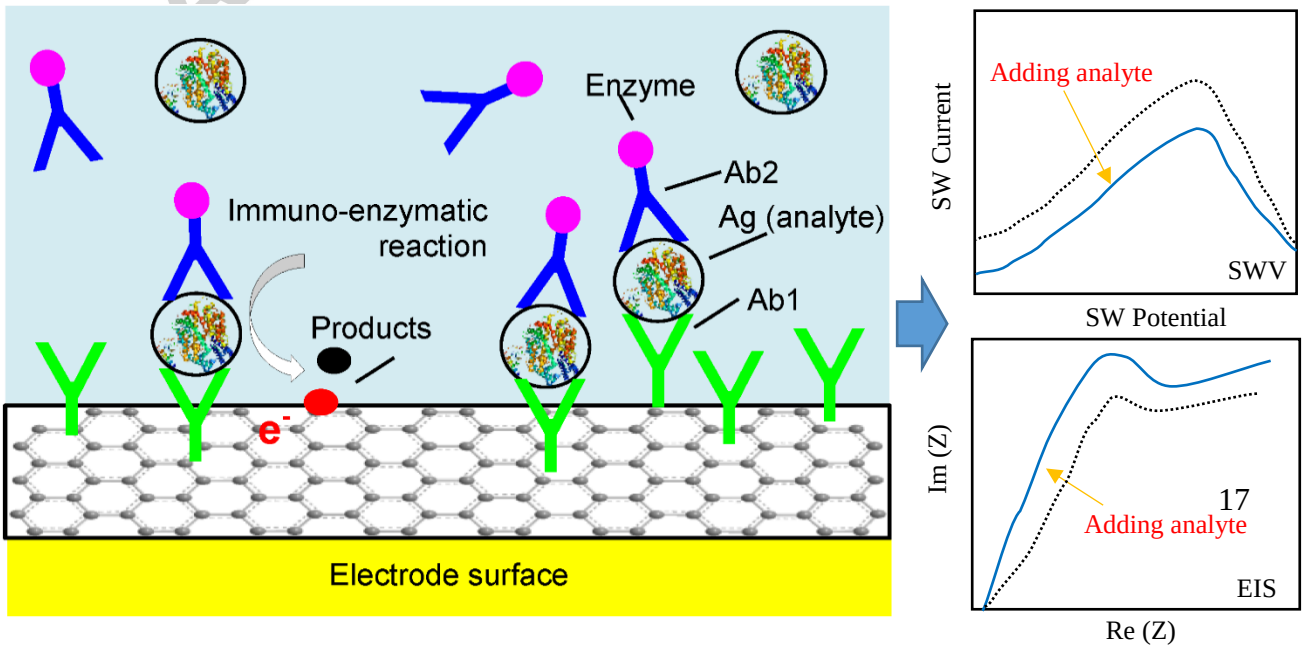


Figure 4. Schematic of graphene-based immunosensor.

4.1.2 Immuno-based electrochemical biosensors

Immuno-based electochemical biosensors typically utilize enzyme-conjugated antibodies (Ab) that will bind with antigen (Ag), resulting in the change of electrochemical activity of the enzyme and generating electrochemical response. SWV and EIS modes as illustrated in Figure 4 are more popularly used than CV or chronoamperometry for electrochemical immunosensors due to higher sensitivity(Cai et al., 2011; Peng et al., 2014). The most commonly used scheme for electrochemical immunosensors is a sandwich structure of a primary antibody (Ab1) immobilized on the electrode surface and enzyme-labeled secondary antibody (Ab2) attached to an antigen (Ag) that will specifically binds to the Ab1 on the other side another in the immuno reaction as graphically depicted in Figure 4(Cui et al., 2007). The effectiveness of such biosensors relies mainly on the antibody immobilization capacity, enzyme activity and electrode characteristics such as specific surface areas and electron transfer coefficients.

To achieve high performance electrochemical immunosensors, different antibody immobilization methods have been investigated on graphene surfaces. For instance, effective immobilization of enzymes and antibodies of various bio-systems such as human immunoglobulin G (HIgG)(Wang et al., 2010) and the cancer biomarker, alpha–fetoprotein (AFP)(Su et al., 2010), on graphene surfaces was achieved with the addition of nanoparticles of metals such as gold, yielding highly sensitive electrochemical immunosensors. In an extension of this method, chitosan–enwrapped graphene and multi–Au nanoparticles were used as a core and a shell for the conjugation of

HRP–anti–carcinoembryonic antigen (CEA), which was used as Ab2 for electrochemical immunosensors that displayed high CEA detection sensitivity, wide dynamic range of 0.05–350 ng/ml and a low CEA detection limit of 0.01 ng/mL (Zhong et al., 2010).

Alternatively, an ultrasensitive AFP immunosensor was achieved by the effective immobilization of Ab1-AFP with multi-enzyme labeled carbon nanospheres (CNSs) on functionalized graphene coated screen–printed carbon electrodes. The method provided signal amplification via multiple binding events of HRP–AB2-CNSs on graphene surface, resulting in a wide linear dynamic range of 0.05-6 ng/ml and a very low detection limit of 0.02 ng/ml(Du et al., 2010). Also, ionic liquid, mesoporous silicas SBA–15 and HRP labeled antibodies was immobilized on graphene-modified electrodes to fabricate an ultrasensitive electrochemical immunosensor for detection of breast cancer susceptibility gene (BRCA1), achieving a wide working range from 0.01 to 15 ng/mL and an ultra-low BRCA1 detection limit of 4.86 pg/mL (Cai et al., 2011). Similarly, ultrasensitive detection of phosphoylated p53 at Ser392 (phospho–p53392) was attained based on multienzyme amplification using graphene oxide (GO) with bioconjugates of HRP and p53392 antibody (p53392-Ab2) as a nanolabel that would be captured onto the electrode surface via a cascaded immuno-reaction, causing the decrease of enzymatic oxidization of thionine due to the electrocatalytically amplified H_2O_2 product. Likewise, graphene was non-covalently functionalized via π–π interaction with 1–pyrenebutanoic acid organic molecules, which were covalently conjugated with Ab1 of a prostate–specific antigen via succinimidyl ester bonding while Ab2 conjugated with HRP was covalently bound to the amino group of thionine mediator, which would be physically adsorbed on graphene and generate highly effective immunosensing with regeneration ability due to strong binding of Ab1 on graphene(Yang et al., 2010).

A label-free electrochemical immunosensors is favorite for practical applications since enzyme labeling involves complicated assay procedure. A label–free electrochemical immunosensor for AFP detection was recently developed by immobilizing anti–AFP on GCE modified with graphene and thionine, achieving high AFP detection sensitivity, dynamic range (0.05-2.00 ng/mL) and an ultra-low detection limit (5.77 pg/mL, S/N = 3)(Wei et al., 2010). Another label-free electrochemical AFP immunosensor based on multi-functional gold nanoparticles–polydopamine–thionine–graphene oxide (Au–PDA–THi–GO) nanocomposite film was developed by reacting GO, THi, $HAuCl_4$ and dopamine in PBS, drop-coating on GC electrodes and incubating in Ab, demonstrating a wide linear range of 0.1-150.0 ng/ml and a low detection limit of 0.03 ng/ml (S/N = 3) (Peng et al., 2014).

4.2 Graphene-based field effect transistor biosensor

Graphene FET biosensors include a graphene layer as part of FET structure which is functionalized and holds immobilized biological receptors at both microbial and molecular scales (e.g. microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids). The selective binding between bioreceptor and target biomolecules results in charge transfer to and from the graphene layer and leads to a change of conduction current of graphene channel (I_{DS}) with a bottom gate control as illustrated in Figure 5. In addition, electrochemical coupling can be optionally applied via a liquid gate electrode whose transfer characteristic (I_{DS} vs V_{g1}) is shifted upon analyte addition as depicted in Figure 5 (Kim et al., 2013). Graphene FET biosensors have been extensively developed for detection of a wide variety of chemical and biological targets including bacteria, DNA, protein/DNA mixture and other anti-body-specific biomolecules(Mao et al., 2010; Mohanty and Berry, 2008; Ohno et al., 2011, 2010). Recently, a non-enzymatic H_2O_2 FET biosensors based on polypyrrole-nanotube embedded rGO was fabricated by mixing

GO and PPy nanotubes followed by hydrazine reduction (Park et al., 2014). The graphene FET biosensors exhibited a very low detection limit of 100 pM and very short response time of <1 s. In addition, the GO-based bacterial and DNA sensor utilizing a p-type graphene FET immobilized with bacterial antibody was demonstrated to be ultra-sensitive with single bacterium detection capability (Mohanty and Berry, 2008). For DNA sensing, single-stranded DNA was tethered on graphene p-type FET and then hybridized with its complementary target DNA strand, causing a significant increase of the surface hole density up to 5.61×10^{12} cm^{-2}, which enabled a label-free, reversible and highly sensitive DNA detection. In another development, a polarity-specific polyelectrolyte molecular transistor, in which DNA/protein mixture is immobilized on rGO transistor was devised to simultaneously detect DNA and protein from dynamic cellular secretion with good specificity(Mao et al., 2010). The conductivity/mobility of the rGO transistor was increased upon DNA hybridization due to the electron transfer (electronic n-type doping) from charged amine group of DNA to rGO while protein binding caused the change in the opposite direction. An rGO-based FET biosensor was demonstrated for ultrasensitive label-free detection of DNA via peptide nucleic acid (PNA)-DNA hybridization, achieving a detection limit as low as 100 fM and ablility to distinguish non-complementary DNA with one-base mismatch (Cai et al., 2014).

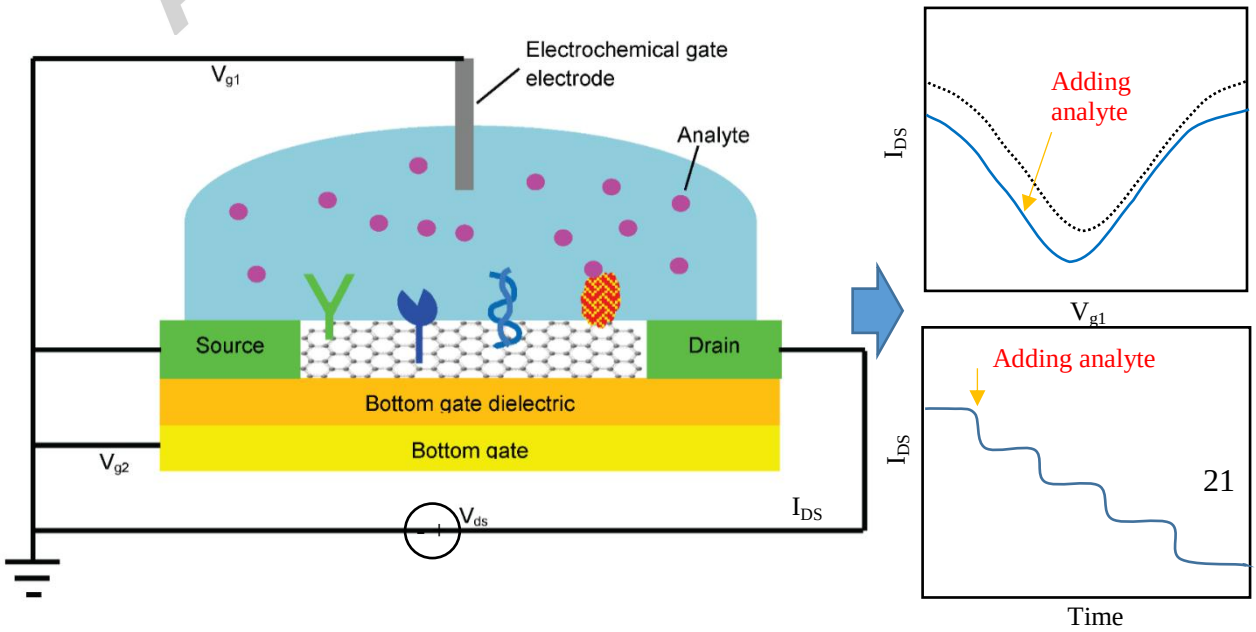


Figure 5. Schematic of graphene-based FET biosensor.

Graphene-based immunosensors has also been developed by immobilization of specific antibodies on graphene FETs. For example, a highly sensitive and selective FET biosensor using Au-NPs-antibody conjugates decorated with GO sheets was reported for detection of a pathogen rotavirus(Mao et al., 2010). As another instance, a graphene FET was modified with aptamer for sensitive and selective detection of immunoglobulin E(Ohno et al., 2011, 2010). In addition, an rGO FET biosensor for label-free ultrasensitive detection of a prostate cancer biomarker, namely prostate specific antigen/α1-antichymotrypsin(PSA-ACT) complex, offered a wide working range and an extremely low detection limit of 100 fg/ml (~1.1 fM) (Kim et al., 2013).

4.3 Other graphene based biosensors

Biosensors based on other platforms such as photoluminescence (PL), electrochemiluminescence (ECL), fluorescence resonant energy transfer (FRET) and surface plasmon resonance (SPR) could be a future alternative for printed organo-functionalized graphene biosensing technology. For instance, GO was used as a binding medium for labeled protein and DNA detections with PL, achieving very low minimum detection range on the order of nM(Lu et al., 2009). In another technology, CdTe/rGO composite was employed in an amplified ECL of QDs platform for glutathione detection(Y. Wang et al., 2009). In addition, hemin-rGO and AgNPs-rGO were employed to enhance ECL of luminol-based immunosensor for detection of CEA. The employed material system could load large amounts of primary antibody (Ab1) and Hemin-rGO could

significantly enhance the electron transfer rate and amplify the ECL signal of luminol in the presence of H_2O_2, yielding good analytical properties with a wide CEA detection range of 0.1 pg/ml-160ng/mL and a very low detection limit of 0.03 pg/ml (S/N = 3) (Jiang et al., 2014). In another platform, direct DNA/GO binding interaction was monitored by surface plasmon resonance for ultra-sensitive detection of single-stranded DNA (ssDNA). The method offered a wide linear range of 10 fM - 1μM, a very low detection limit of 10 fM and a high stability under repeated regeneration (Xue et al., 2014). Moreover, GO *printed* on microporous membranes for the FRET detection of peptides, protein, and DNA(Mei and Zhang, 2012).

5. Discussions

A rapid growth in the use of graphene-based materials for biosensing has been witnessed over the past few years. Majority of graphene-based biosensor research work have been focusing on innovating graphene synthesis/functionalization and enhancing immobilization capacity of bioreceptors including enzymes, antibodies and complimentary DNAs using novel organic-based materials including specific organic molecules, copolymers, organic/polymer complexes and organic composites. In particular, the combinations of organic materials particularly polymer with inorganic nanostructures such as metal nanoparticles, carbon nanotubes and metal oxide nanoparticles are shown to be highly promising routes to functionalize graphene and enhance the biosensing performances. Morover, organic nanostructures (i.e., polymeric nanofibres, nanosphere, etc.) and its composites with other nanostructured materials should be promising routes to advanced organo-graphene functionalization and biological immobilization for biosensing. Regarding functionalization strategies, noncovalent methods are more commonly employed than covalent means due to superior electronic/biosensing properties and wide

diversity of applicable materials. Nevertheless, covalent functionalization is more stable and necessary for some purposes such as doping and permanent immobilization of bioreceptors.

Concerning transduction platform, organo-functionalized graphene are most widely employed in enzymatic and immunological electrochemical sensing methods due to graphene's excellent electro-biochemical properties and ease of sensor fabrication while organic-graphene FET structures for biosensing are still relatively less studied due to fabrication complexity as well as graphene's metallic nature, which is not ideally optimal for FET operations. Other transduction mechanisms such as photoluminescence, FRET and SPR may be promising alternative biosensing platforms for organo-functionalized graphene materials and research in this area is rapidly growing. Organo-functionalized graphene electrochemical and FET biosensors have been widely demonstrated to exhibit remarkable sensitivities and specificities towards a number of important analytes such as glucose, cholesterol, Hb, H_2O_2, UA, AA, DA, DNAs, proteins and so on. The biosensing performances of these graphene-based biosensors are generally superior to many other carbon-based electrochemical electrodes such as CNTs, highly ordered mesoporous carbon and carbon nanofibers. In comparison with other emerging 2D or layered semiconducting materials including MoS_2, WS_2 and BN that possess similarly large specific surface area but distinct electronic and optical properties, organo-functionalized graphene when employed as enzymatic and electrochemical immunosensors still hold better sensitivity performances due to graphene's superior electrical conductivity, electron transfer rate and direct electrochemistry. However, electrochemical sensors based on other 2D structures such as MoS_2 have demonstrated to offer particular advantages in term of selectivity towards particular analytes such as DA for non-enzymatic biosensing(Wu et al., 2012). Moreover, other 2D layered structures tend to perform better as FET and optical biosensors due to their direct band gap semiconducting

properties that permit full functional FET operation and provide useful optical characteristics for PL and FRET detection platforms (Valappil et al., 2015).

To date, most solution derived graphene biosensors are still fabricated by non-printed deposition or modification methods, which suffer from poor control of deposition materials and inability to directly form electrode patterns leading to poor reproducibility when produced in large scale. Thus, graphene-based biosensing materials should be fabricated by printing technology to realize printed organo-functionalized graphene biosensors that can be reproducibly manufactured in large-scale for commercialization. To develop printed organo-functionalized graphene sensors, various production methods of organo-functionalized graphene ink have been proposed based on non-covalent and covalent functionalizations together with solution-route graphene syntheses including oxidation/reduction/dispersion, surface functionalization, electrolytic exfoliation, mechanical exfoliation with ultrasonication and small-molecule intercalation. Graphene-based inks have recently been established for various printing methods including screen, inkjet, aerosol-jet, gravure and flexographic printings. However, so far only few screen-printed and inkjet-printed organo-graphene electrochemical sensors have been demonstrated for biosensing applications. In particular, there is still only one fully printed organo-functionalized graphene biosensor with immobilized bioreceptor, which is GOD-immobilized screen-printed graphene-paste glucose biosensor (Pepłowski et al., 2015).

6. Concluding remarks, Challenges and Future trends

Conclusively, the development of printed organo-functionalized graphene biosensors for low-cost and disposable applications is still at a very early stage and there are some key challenges to be overcome. Firstly, there must be adaptation of presently available printing technology for efficient printing of bioreceptor-immobilized organo-functionalized graphene ink. The

component of printing instruments such as printing head, ink holder, ink transfer device and substrate holder must be biocompatible, which means that they should have minimal interactions with the inks so that the materials will not be degraded or denatured. In addition, printing mechanism may need to be adjusted to comply with the physical and chemical properties of organo-graphene inks or vice versa. Secondly, organo-graphene inks with superior sensing performance but low-cost, good stability and high processablility should be further developed. In this regard, the research in advanced graphene functionalization with novel organic/inorganic nanostructures particularly 2D layered materials and 3D nanonetworks for high-performance biosensors has been very active and continues to grow. While much research is focusing towards the high-performance aspect, more attention should be paid to stability problems arising from aggregation and degradation. Stabilization of nanostructures and organic/biological components in the ink will thus be another important task to be done. Lastly, the fabrication process of organo-functionalized graphene biosensors from material synthesis to final transducer production must be scalable, transferable, reproducible and economical. Complicated preparation procedure as well as high cost organic/inorganic precursors and biological receptors will inhibit their use in commercial applications. Thus, the research in organo-functionalized graphene biosensors should also consider the practicality of involved materials, method and instrumentation. Based on various consideration, organo-functionalized graphene biosensors based on roll-to-roll printing processes (i.e. gravure and flexographic printing) are proposed as promising future choices for practical printed graphene biosensors since roll-to-roll processes can offer high throughput and high reproducibility at a moderate cost. This will involve innovative organo-functionalized graphene-based ink for roll-based printing techniques and process optimization to achieve practical and high-performance printed graphene-based biosensors.

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Highlights

- Organo-functionalized graphene and printed biosensor technologies are comprehensively reviewed.
- Various methods for printing graphene-based fluids on different substrates are largely discussed.
- Different graphene-based ink materials and preparation methods are succinctly described.
- Biosensing performances of printed graphene-based sensors are compared and elaborated.
- Potentials and research direction of printed organo-graphene-based biosensors are suggested.