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# Clinical Detection of Neurodegenerative Blood Biomarkers using Graphene Immunosensor

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**Abstract**

Accurate detection of blood biomarkers related to neurodegenerative diseases could provide a shortcut to identifying early stage patients before the onset of symptoms. The specificity, selectivity and operational requirements of the current technologies, however, preclude their use in the primary clinical setting for early detection. Graphene, an emerging 2D nanomaterial, is a promising candidate for biosensing which has the potential to meet the performance requirements and enable cost-effective, portable and rapid diagnosis. In this review, we compare graphene-based immunosensing technologies with conventional enzyme-linked immunosorbent assays and cutting-edge single molecule array techniques for the detection of blood-based neurodegenerative biomarkers. We cover the progress in electrical, electrochemical and optical graphene-based immunosensors and outline the barriers that slow or prevent the adoption of this emerging technology in primary clinical settings. We also highlight the possible solutions to overcome these barriers with an outlook on the future of the promising, graphene immunosensor technology.

**Keywords**

Graphene, clinical detection, neurodegenerative disease, protein biomarkers, immunosensor

# 1. Introduction

## 1.1 A Need for Blood Immunoassay in Primary Settings

Neurodegenerative disease presents a broad category of clinically and pathophysiologically heterogeneous, complex brain diseases, which cause gradual long-term cognitive deficits, which continue to worsen with time, causing memory and functional loss in daily living. These affect over 50 million people worldwide, with 63% of them living in low- and middle-income countries [1]. Therefore, diagnosis and clinical monitoring of the disease and how it progresses is an urgent need for the global population.

To date, great progress has been made in the discovery of neurodegeneration biomarkers, which include clinical neurological biomarkers, imaging biomarkers and biochemical markers. Main candidates under each category have been comprehensively summarised by Abreu's group [2]. Currently, clinical neurological and neuropsychological examination forms a significant part of accurate, but late-stage diagnosis to individuals displaying symptoms. However, due to the complex nature of these diseases and the expertise difference between practitioners, the patients continue to receive inadequate assessment and incorrect referrals from primary care visits. Long waiting times and unnecessary referrals cause critical delays for patients before being adequately assessed by specialists [3, 4]. To this end, an accurate and reliable biomarker-based diagnostic technology would be helpful for primary practitioners in referring patients to the most suitable specialty care. This will ensure that at-risk individuals will be identified at the earliest stage in their primary care, and this will reduce the overall clinic and medical system burden by decreasing unnecessary diagnostic procedures [5, 6]. PET (positron-emission tomography), MRI (magnetic resonance imaging) and CSF (cerebrospinal fluid) biomarker analysis have already presented many advances in improving diagnosis accuracy. However, the time and expense of performing PET, MRI and the invasive lumbar puncture procedure of CSF analysis limit their accessibility and availability as frontline diagnostic tools, especially for patients in lower-income and developing countries [7, 8].

With the above restrictions, diagnosis through detection of blood-based biomarker has gathered attention and is considered as a fast and reliable primary patient screening process. Firstly, the acquisition of blood samples and handling infrastructures have been well-established in most countries as routine clinical checks in primary care settings, which requires no further training or professional experience [9]. Frontline availability will allow

patients to be identified at their earliest stages and monitored at repeated intervals as their disease progresses. Then since there are no definitive biomarkers [10], the large number of potential candidates in blood samples provides the possibility of simultaneous multi-biomarker tests beyond the widely studied ones for Alzheimer's disease (AD) (such as tau and amyloid), which will offer a full molecular spectrum to enhance the diagnosis accuracy [11]. This makes blood biomarker-based diagnosis an ideal first step towards multitiered diagnosis, to be placed adjunct to high cost, complicated processing in order to meet accessibility requirements for the broad population [12], as shown in Fig. 1.

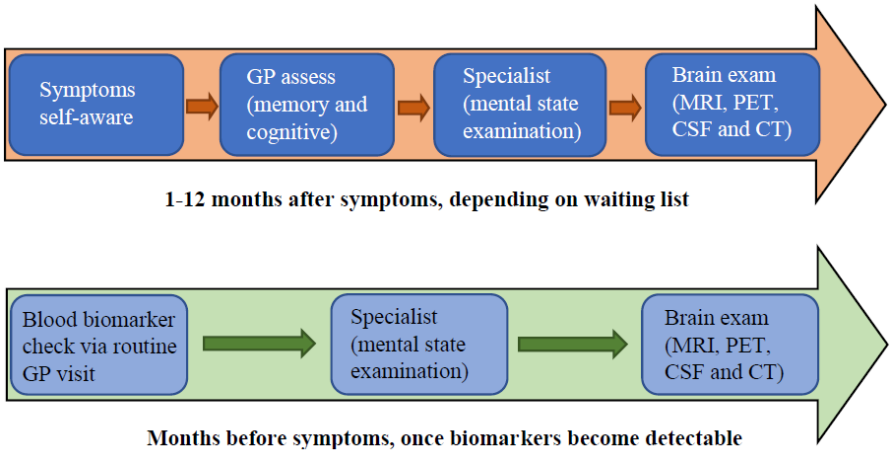


Figure 1. Workflows for conventional neurodegenerative diagnosis with onsite symptoms (red) and proposed blood biomarker-based early diagnosis (green).

In most countries, the available method for the detection of blood-based neurodegenerative biomarkers is the enzyme linked immunosorbent assay (ELISA), shown in Fig. 2 (a). ELISA detects the presence of certain protein biomarkers using pre-immobilised capture antigens, detection (primary) antibody and secondary antibody, which are normally linked with enzymes or tags for signal generation [13]. This quantitative detection requires large volumes of dilute reaction products and enzyme labels to generate detectable signals for conventional plate readers. Although ELISA presents a sensitivity down to $10^{-1}$ pg/mL, it fails in the detection of many biomarkers at clinical concentrations, for examples, interleukin (IL)-2 and IL-17A [14, 15], and for the simultaneous detection of multiple biomarkers.

In 2010, single molecule array (SIMOA) technology was developed to detect the protein biomarker at the $10^{1}$ fg/mL level [16]. SIMOA relies on the high efficiency binding between half a million antibody-modified magnetic beads and the low-concentration protein molecules suspended in 0.1 mL diluted solution. After the beads are loaded into an array of femtoliter-sized wells, the protein concentration is determined by digitally counting the beads, where the

fluorescent signal is proportional to the total number of beads on the array. The high binding efficiency, low background signal from the assay and the way to measure signal presence or absence (rather than integrating) offer a $10^3$-fold higher sensitivity and a single molecule Limit-of-Detection (LOD), as shown in Fig. 2 (b). This technology enables quantitative detection of neurodegenerative biomarkers that were impossible to achieve using conventional ELISA.

However, both conventional ELISA and cutting-edge SIMOA technology require complex fluorescence-labelling processes, demanding laser excitation, sophisticated emission capture instrumentation and highly skilled persons to operate and maintain the system. These limitations preclude their adoption in a primary clinical setting. Therefore, the development of a cost-effective, portable, reliable and rapid method for the point-of-care (PoC) detection of blood neurodegenerative biomarkers in primary care settings remains a challenge.

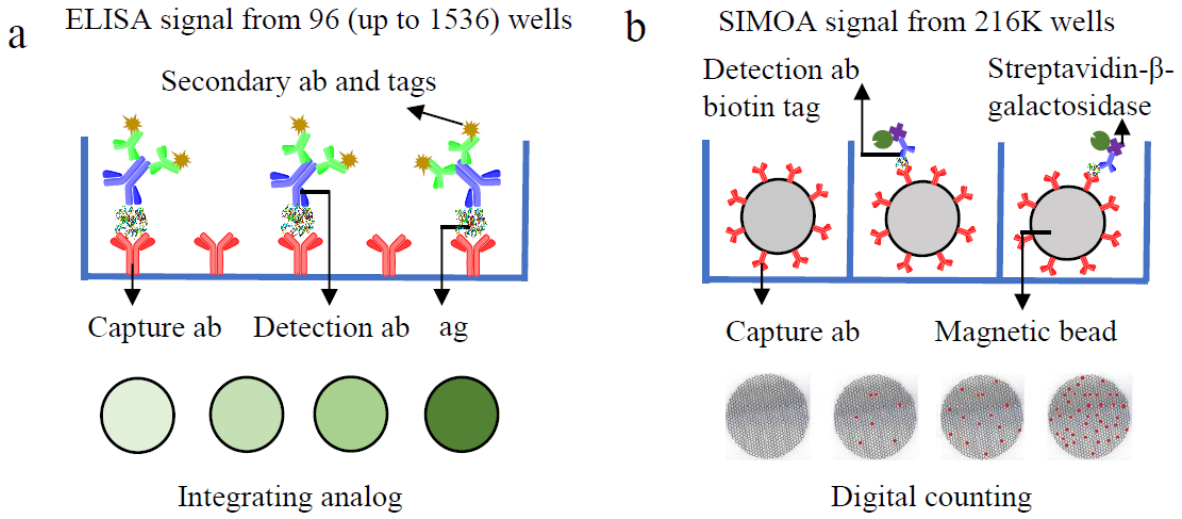


Figure 2. Comparison of (a) conventional sandwich indirect ELISA method and (b) cutting-edge SIMOA technology for the detection of protein biomarkers.

### 1.2 Wonder Materials and Reality

Graphene, as a "wonder material", has drawn a lot of attention in the scientific community since its discovery in 2004 [17]. Pristine graphene is a single-atomic layer 2D structure consisting of $sp^2$- hybridised carbon atoms. In each lattice unit, carbon atoms connect the adjacent three carbon atoms via $\delta$ electrons having a bond length of 0.142 nm. The delocalised $\pi$ bond is formed by the non-bonded electrons in $2p_z$ orbits, which are perpendicular to the graphene plane and interact with those from other adjacent carbon atoms [18]. This unique molecular structure empowers graphene with outstanding physical and chemical properties over other nanomaterials, such as large surface-to-volume ratio, excellent

electrical conductivity, mechanical strength, broad and tuneable absorption, chemical inertness as well as ease of biological functionalisation [19, 20]. These features make graphene and its derivatives great candidates for the development of the next generation of biosensors, which can potentially yield extreme sensitivity, reproducibility and reliability from devices fabricated using high throughput technologies.

Over the past decade, graphene-based biosensors have been widely reported for the detection of different biomarkers, including ions [21], nucleic acids [22], proteins [23] and even cells [24]. Based on the sensing mechanisms employed, graphene biosensors have been mainly categorised into electrical [25], electrochemical [26], optical [27] and acoustic devices [15]. Many improvements in sensitivity and selectivity have been made using different forms of graphene (chemical vapour deposition (CVD) graphene, graphene oxide (GO), reduced graphene oxide (rGO) and quantum dots [28]) and labelled (tagged) detection (florescence dye, magnetic tag and enzyme [29]). In addition, the production cost of raw graphene materials is continuously decreasing [30], and the fabrication procedures of biosensor devices which can be integrated within current semiconductor fabrication processes is able to bring the cost down further for large-scale applications and implementations. These advances have further strengthened the case for applying and developing commercial graphene biosensors. To date, thousands of articles have been published; however, only a few of these are focused on neurodegenerative biomarker detection, and none of them has demonstrated the capability to replace the current technologies in clinical settings.

This review aims to provide an overview of the progression in three types of graphene-based immunosensors for the detection of blood neurodegenerative biomarkers, unveil the technical challenges each technology faces, and summarise the critical strategies to promote translating technologies into clinical settings. In the end, we share our vision for the future development trends in graphene-based immunosensors for the diagnosis of neurodegenerative diseases.

## 2. Graphene Field-effect Transistor Immunosensor

### 2.1 Sensing Mechanism

A graphene field-effect transistor (GFET) is one of the most basic forms of graphene electronic sensor. In a biosensor FET (BioFET), a graphene channel acts as a transducer directly connected to source and drain electrodes, with an additional top or bottom electrode for providing a gate potential. For GFET immunosensors, antibodies which act as bio-active receptors are immobilised onto graphene sheets to bind with the target antigens, as shown in

Fig. 3 (a). Detection is based on GFET transconductance changes in response to the extent of antibody-antigen conjugation, which is linked to the concentration of the target antigen within a solution. The transport behaviour of the graphene channel changes due to the charge (positive or negative) of the antigens immobilised near the graphene surface. This change in transconductance can be picked up by a DC measurement of the transistor's I-V characteristics. As the Fermi level of graphene changes during this process, and owing to its bipolar transport characteristics, the detection of the antigen biomarker can also be observed as a shift in the Dirac point (point of minimal conductance) in I-$V_g$ measurement [28, 31, 32].

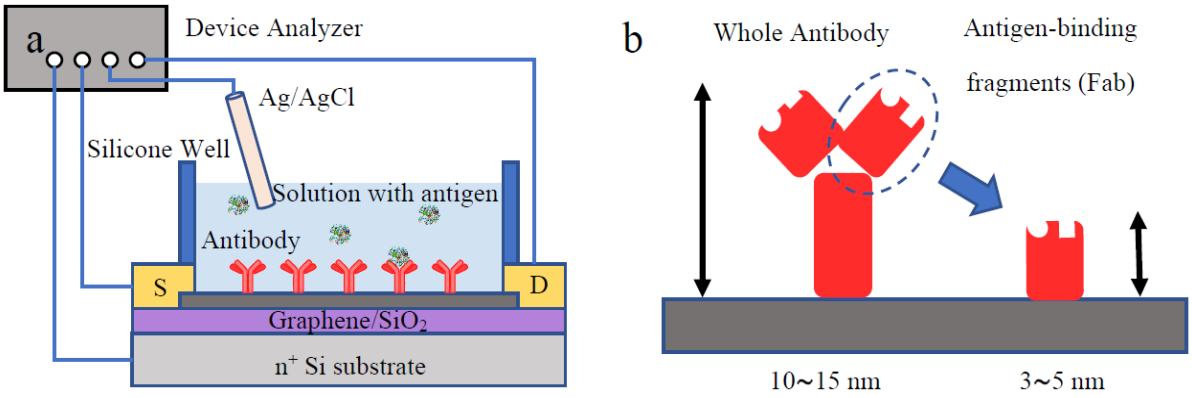


Figure 3. (a) Schematic of a GFET immunosensor set-up. Graphene serves as the transducer, functionalised with antibodies as receptors for antigen detection. Au contacts act as source and drain for monitoring the transconductance of the functionalised channel. In some cases, the gate potential may be applied on the back via the doped Si substrate. (b) Schematic of a whole antibody and an antigen-binding fragment (Fab). The size of these bioreceptors dictates how far the immobilised antigens will be away from the sensor surface, influencing the detector's performance.

### 2.2 Device Optimisation

Selectivity is one of the most important considerations in GFET immunosensors. As discussed, graphene is a material with theoretically infinite surface to volume ratio due to its atomic thinness, and thus is responsive to all kinds of molecules in proximity to the surface. In a graphene immunosensor, selectivity towards an antigen is achieved by functionalising the graphene sheet with complementary antibodies. To further ensure selectivity and minimise the influence from other molecules, passivation of non-selective binding sites is often carried by applying optimal blocking agents to the graphene after antibody functionalisation [33].

Unlike typical FETs which operate in the atmosphere, GFET immunosensors operate in a

fluidic environment. As such, an electrical double layer is expected to form at the graphene-electrolyte interface, and is an important factor affecting sensing performance [34, 35]. It is important to consider the Debye length, which is a measure of the electrical double layer, beyond which charges are heavily screened and have minimal impact on the GFET's conductance. Therefore, detection of a biomarker requires immobilising the target molecules as close as possible to the graphene surface, preferably within one Debye length. The Debye length $\lambda_D$ of a given solution is proportional to the inverse square root of its ionic strength [36]. This provides a route to enhance sensitivity, by using a buffer solution with low ionic strength [37]. Stern et al. demonstrated this effect in their study on streptavidin detection with a silicon nanowire functionalised with biotin molecules. The introduction of 10 nM of streptavidin into a 1 x phosphate buffered saline (PBS) solution ($\lambda_D$ ~ 0.7 nm) yielded no response from the biosensor, as the receptor's length exceeded the Debye length and most of the charge was screened. In 0.1 x PBS ($\lambda_D$ ~ 2.3 nm), the charge of streptavidin was partially screened. In 0.01 x PBS ($\lambda_D$ ~ 7.3 nm), the same introduction of 10 nM of streptavidin induced a large drain current response [35]. Typical sizes of an antibody and antigen are about 10 - 15 nm and 5 - 10 nm respectively, which explains why detection signals can be substantially influenced by the buffer solution [38, 39]. A similar approach of using diluted buffer solution has been used in the detection of viruses, which are significantly larger in size compared to proteins and require large Debye length. For example, Patolsky et al. demonstrated real-time sensing of single influenza A virus as it binds and unbinds with the receptors on a nanowire sensor using PBS containing 1 μM of KCl which has ionic strength lower than 0.01 x PBS (1 x PBS contains 2.73 mM KCl) [40]. Similarly, by using a diluted 0.01 x PBS, Chen et al. has successfully detected the Ebola virus with a rGO FET biosensor [41].

Clinical samples are often high in salinity and rich in chemical composition. Therefore, using a buffer solution with low ionic strength for sensing would require additional treatments such as dilution or desalination. Besides the additional processing steps, these sensors also need to have very high sensitivity (or low LOD) to be able to detect signals of the diluted biomarkers. Desalination may also lead to unintended filtering of the target biomolecules. To address this challenge while ensuring that the target molecules are immobilised within one Debye length, researchers have proposed the use of shorter bio-receptors for capturing the biomarkers. Ohno et al. proposed using smaller receptors such as aptamers (3-4 nm) and Fab to achieve antigen capture, and have demonstrated a LOD as low as 10 pM with GFET [23, 42], as

shown in Fig. 3 (b). Similarly, Osaka et al. conducted a systematic study by comparing BioFETs functionalised with different sized bioreceptors as well as blocking agents [43]. Their results showed that BioFETs functionalised with smaller-sized Fab receptors showed a 100 times improvement compared with their counterparts with whole antibodies in their detection of α-fetoprotein samples, reaching a LOD of 10 ng/mL. Another finding from this study was that sensors using a smaller blocking agent, ethanol amine, outperformed counterparts which used bovine serum albumin (BSA) as blocking agents by over a factor of 3 in sensitivity. This is likely due to the larger size of BSA proteins, which may cause more obstruction and hinder the antibody-antigen reaction during sensing [43]. These findings highlight once again the importance of the molecular size of bioactive agents during the detection of biomarkers.

Aside from considerations of Debye length relative to size of bioreceptors, the control of antibody orientation could also serve as a route for sensing performance optimisation. Loss of bioactivity of antibodies after immobilisation on surfaces is well recorded and commonly attributed to the denaturation of the protein's three-dimensional structure as well as spatial obstruction of antigen-binding sites [44]. Therefore, the orientation of the immobilised antibody plays a significant role in maintaining high antigen recognition for sensing applications. Prior to covalent binding, antibodies undergo physisorption at the surface, with an orientation depending on the pH of the solution, isoelectric point of the antibody and surface charge. With careful optimisation of these conditions, control of antibody orientation may be achieved [45]. On the other hand, researchers have found that surfaces covered with intermediate proteins such as protein A or G, which carry binding sites specific to the Fc part of the antibody, are conducive to immobilised antibodies with a "tail-on" binding. In such orientation, antibodies are shown to have stronger affinity towards antigens compared to their randomly orientated counterparts [46, 47]. Lo et al. have demonstrated such an improvement of BioFETs by engineering the orientation of bioactive receptors. In their study on targeting carcinoembryonic antigen (CEA) proteins, a CEA-binding Fab with hexahistidine [$(his)_6$] tag is immobilised onto the surface of Ni-coated carbon nanotube walls. Uniform orientation was achieved by engineering the position of the $(his)_6$ tags and utilising their binding with the densely-packed Ni nanoparticles. FETs functionalised with uniformly oriented Fab showed a conductance change between 50-100% when CEA protein solutions ranging from 1 to 100 ng/mL were introduced, whilst control devices functionalised with randomly oriented Fab showed no observable response. The result was explained by analysing the protein crystal

structure of the CEA-binding Fab, which predicts a 10% probability of antigen reaction when the Fab is randomly oriented on the transducer's surface. By engineering the $(his)_6$ tag onto the Fab, exposure of all the possible binding sites to the CEA antigen is enabled with controlled orientation, thus enhancing the interaction between bioreceptors and antigen [48]. This result points towards a potential route for improving BioFET performance by control of bioactive receptors' orientation.

### 2.3 Application in Neurodegenerative Diseases

To date, several works have been reported on the use of GFETs for detecting biomarkers of neurodegenerative disease, namely amyloid-β peptides [49-51] and IL-6 proteins [52].

These studies commonly chose GO as the transducer material, due to ease of fabrication, high surface area and sensitivity. GO forms a homogenous aqueous solution, owing to its abundance of polar moieties, which are hydrophilic in nature. These functional groups also promote effective surface modifications by binding with linker groups and antibodies. The drawback however is that GO is an insulator, and thus an additional reduction process is usually performed to obtain rGO, which is conductive and suitable for BioFETs. In 2012, Kurkina et al. demonstrated a liquid-gated rGO FET for detection of amyloid-β peptides in solution. To achieve high specificity, amyloid-β antibodies were immobilised in a two-step procedure onto rGO. Staphylococcus aureus protein A (SpA) were first bonded to the rGO surface via carbodiimide coupling. As SpA proteins have high binding specificity towards the Fc fragments of antibodies, this step is designed to ensure a uniform orientation of anti-amyloid-β antibodies subsequently immobilised onto rGO. The detection range of such sensors was reported to be 1 fM to 100 pM, which is about an order of magnitude improvement on commercial ELISA [49].

Work towards scalability and quality control of rGO sensors has followed. In 2014, Huang et al. developed a dry-etching technique for reliable control of both pattern formation and layer thickness of rGO sensor arrays on a 4-inch wafer. A low deviation of resistance of the sensors within a range of ±10% was achieved. The sensors showed a linear response between 100 fg/mL to 100 pg/mL and achieved a LOD of ~100 fg/mL in amyloid-β 40 peptides detection. Discrimination tests were performed on the plasma samples collected from transgenic (TG) mice with human amyloid-β peptide and wild type mice as the control group. Sensor resistance change of around 5% was observed in response to the TG sample, and a p-value of 0.0452 was obtained in distinguishing the two groups of samples [50].

In a clinical study reported in 2017, Chae et al. pushed for further improvements in sensitivity of rGO sensors toward amyloid-β peptides by treating the devices with $O_2$ plasma. The enhanced surface functionality of the treated sensors showed a 3-fold improvement in the slope of the electrical response versus analyte concentration curve (on logarithmic scale) compared to the untreated counterparts. Plasma samples from 15 AD patients and 15 normal controls were collected and treated to obtain clinical samples containing neural-derived exosomal amyloid-β peptides for testing. By monitoring the resistance change of the rGO-FET, successful identification of samples of AD patients was achieved with statistical significance ($p<0.001$) [51].

Other than amyloid-β peptides, researchers have also investigated the detection of IL-6 proteins, a biomarker that could indicate neuroinflammation. For targeting IL-6 proteins, Huang et al have used a GO FET made using a self-assembly method [52]. A linear dynamic range from 4.7 to 18.8 pg/mL was observed with the LOD of 1.53 pg/mL. IL-6 protein levels in sedentary middle-age populations are around 10 pg/mL and may increase to higher levels if the inflammatory response is triggered. The sensing range of the developed GFET is thus in range with clinical levels.

### 2.4 Barriers to GFET in Clinical Blood Biomarker Detection

GFETs have the potential of providing label-free, highly sensitive, real-time, point-of-care detection of biomarkers. However, there are some limitations and challenges which are mainly specific to GFETs towards application in clinical settings.

Signals from BioFETs rely first and foremost on the charge carried by target molecules. Depending on the isoelectric point of the target antigen and the pH value of the test solution, there are cases where the target molecules carry little or no charge. For detection in such cases, a GFET will be categorically unfit as a sensing device.

As successful detection using BioFETs also depends on charge-carrying molecules having sufficient influence on the transducer's transport behaviour, considerations on the Debye screening as well as the receptors' size and orientation as previously mentioned requires thorough consideration. These factors will vary from case to case, therefore application-relevant tests are necessary to validate the efficacy of the sensors. To date, most studies on GFETs reported are with experiments in standard buffer solutions with only the target biomolecule or a few other biomolecules as controls. However, the bioliquid in which measurements are taken may differ in viscosity and ionic strength from the ideal buffers.

Also, bioliquids such as whole blood or serum are known to be complex matrices that contain a large variety of biological components, including salt, many of which could interfere and lead to false readings during sensing [31]. It is therefore critical to test these sensors in clinically relevant conditions to obtain a more accurate understanding of the devices, both in terms of sensitivity and selectivity.

## 3. Graphene-Based Electrochemical Immunosensor

### 3.1 Electrochemical Sensing Mechanism

Electrochemical immunosensors are normally used for the detection of the electroactive protein molecules or the protein biomarkers in an electroactive system. This type of immunosensor transforms the chemical binding event into a digital signal via potentiometry, voltammetry (cyclic (CV), differential pulse (DPV) and square wave voltammetry (SWV)), amperometry, or electrochemical impedance spectroscopy (EIS). Potentiometry analyses the open-circuit potential changes between the working and reference electrodes without current flow. Amperometry and voltammetry both require a third counter electrode to set the desired working electrode potential independent of the potential drop across the solution. Amperometry measures the redox current from the electroactive molecules at a constant or stepped potential over time, whilst voltammetry gauges the current during a sweeping potential in linear, cyclic, or combined with pulses manner (e.g. DPV or SWV). In EIS, sinusoidal potentials over a sweeping frequency is applied. The corresponding resistance and capacitance of the system can be calculated to study the surface properties of the working electrodes. Detailed waveforms, response signals and analysis methods have been comprehensively presented by Dincer et al [53]. In immunosensor configurations, while the antigen binds to the corresponding antibody-functionalised working electrode, its surface electroactivity will decrease or increase depending on the charge condition of the antigens. This will either prevent or promote electron transport between the electrode and the electrolyte, resulting in changes in redox current peaks and impedance (Fig. 4). In more advanced sandwich immunosensors, the capture antibody is pre-immobilised onto the surface of the electrodes, followed by antigen detection. The primary antibody (known as the detection antibody), which is labelled with enzyme or tags for signal enhancement, is then added onto the electrode surface and reacts with an antigen to form the antibody-antigen-antibody complex. During any electrochemical process, response to reactants mainly depends on heterogeneous electron transfer kinetics and available surface area. The electrons predominantly transport through the crystal defects and edges on the electrode surface [54].

Therefore, using graphene nanosheets or rGO as the electrode materials in electrochemical immunosensors will provide more edges and defects for fast electron transportation [55], which in turn leads to improved sensitivities and LODs for detection.

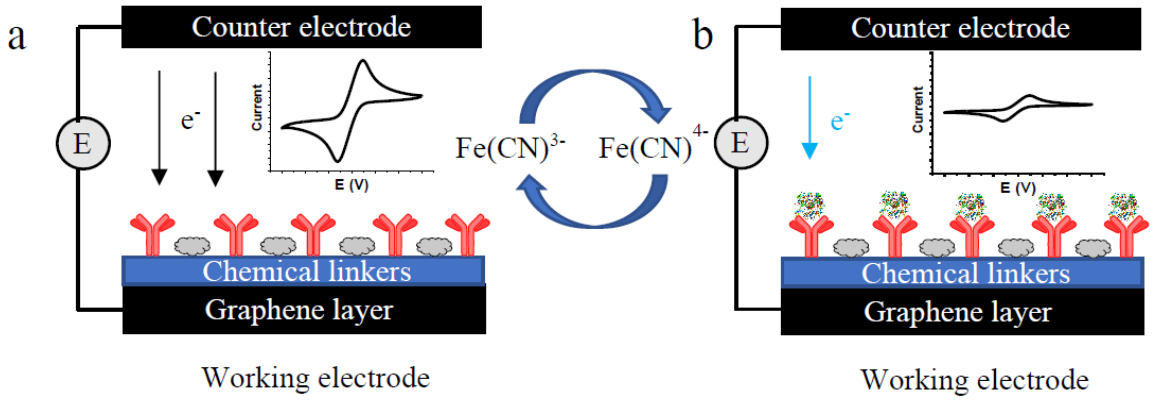


Figure 4. Working principle of a graphene cyclic voltammetry immunosensor. (a) Before the antibodies bind to the target antigen, the sensing electrode presents a baseline electron exchange rate reflected by the intensities of redox peaks. (b) While the antigen protein binds to the antibody functionalised electrode, it leads to a change in the intensities of redox peaks, which can be used for quantitative detection of biomarkers.

### 3.2 Application in Neurodegenerative Diagnosis

Graphene electrochemical immunosensors are the most commonly used devices for the detection of blood neurodegenerative biomarkers, due to their high sensitivity, selectivity and ease of use. Depending on their configuration and complexity, they can be categorised into non-signal amplification and signal amplified sensing platforms. The basic non-signal amplification immunosensor normally consist of a graphene nanomaterial modified electrode, a linker material layer for antibody immobilisation, and the corresponding antibody layer. Under this category, Xu et al first reported α-synuclein/GO/cystamine modified gold electrodes as an impedance sensor for the detection of preclinical Parkinson's disease biomarkers. The sensor presented a linear response from 5 to 1000 pM and a 1.2 pM LOD in the non-faradaic detection of α-synuclein autoantibodies in artificial serum, with only 30 µl sample for each shot [56]. Ye et al recently demonstrated a method to precisely control and maintain the separation of rGO layers to form a monolayer rGO modified electrode for the determination of Tau-441 protein [57]. Using square wave voltammetry, the sensor achieved a linear response to Tau-441 from 0.08 pM to 80 pM with a LOD of 75 fM, which provides adequate sensitivity to clinically distinguish the health controls from mild cognitive impairment (MCI) and AD patients [58].

To enhance the sensitivity and LOD of immunosensors, dopant, nanostructure and nanoparticle can be used together with different graphene modified electrodes for signal enhancement. Ma's group developed a reusable magnetic nitrogen-doped graphene immunosensor for the detection of Aβ42 [59]. The GO was reacted with EDA to provide nitrogen doping for higher electron exchange rate, then decorated by $Fe_3O_4$ nanoparticles and functionalised by anti-Aβ42. This complex was assembled onto the electrode surface with the magnetic field turned on and disassembled by turning off the magnetic field. This sensor presented a linear detection range from 5 pg/mL to 800 pg/mL with LOD of 5pg/mL and a reusability of 50 times. Raw GO has also been used as an electrode material, for example, Li's group produced a sandwich structure immunosensor on GO electrode for the detection of IL-6 proteins [60]. The GO electrode was functionalised with IL-6 capture antibodies and the signal was enhanced using the porous anti-IL-6/ferrocene/$CaCO_3$/polyelectrolyte nanocomplex, leading to a LOD of 1 pg/mL. Another group assembled both $Co_9S_8$ and Pd nanoparticles onto GO electrodes to achieve a wide linear range from 0.1 pg/mL to 50 ng/mL and an extreme LOD of 41.4 fg/mL for Aβ detection [61]. Furthermore, a graphene nanoribbon (GNR)-modified electrode was used for the simultaneous detection of interleukin-6 (IL-6) and matrix metallopeptidase-9 (MMP-9) [62]. GNR was produced from GO and subjected to a chemical reduction in $NH_4OH$ to regain its electroactivity. The GNR modified electrode was functionalised by anti-IL-6 and anti-MMP-9 mixture, while the anti-IL-6/PS@PDA/$Cd^{2+}$ and anti-MMP-9/PS@PDA/AgNP were used as corresponding signal enhancers.

To further improve the sensing performance, researchers started to employ multiple signal enhancers for the functionalisation of both graphene electrodes and detection antibodies. Derkus's group developed a low-cost screen-printed electrode, of which the surface was successively modified with GO, trimethylolpropane tris[poly(propyleneglycol)] dendrimer and two types of antibodies for the simultaneous detection of Myelin Basic Protein (MBP) and Tau proteins [63]. The pPG/CdS/anti-MBP and pPG/PbS/anti-Tau nanocomplex were synthesised as signal enhancers, which lead to LODs of 0.3 nM and 0.15 nM for the detection of MBP and Tau in human serum. In addition, Gao et al used AuNP and $AuCu_xO$@m-$CeO_2$ nanoparticles for the functionalisation of rGO modified electrodes and the detection antibody respectively, leading to a LOD of 100 fg/mL for Aβ detection [64]. This is 2 orders of magnitude more sensitive than immunosensors developed without graphene [65]. Although more complicated nanostructures can be integrated into the device configuration for better

performance [66], the above methods already provided superior sensitivity compared to other immunosensors for the detection of the same biomarkers (shown in Table 1), which would be adequate for biomarker detection in clinical samples. It is also worth noting that the more complex the sensor is, the less reproducibility/stability there will be. Therefore, a balance needs to be achieved between the sensitivity of the detection and the complexity of the device.

Table 1. Leading non-graphene electrochemical immunosensors for the detection of neurodegenerative biomarkers.

| **Target** | **Configuration** | **Techniques** | LOD |
|---|---|---|---|
| tau-441 | Oriented antibodies | CV and EIS | 0.03 pM [67] |
| T-tau | T-tau antibody | DPV | 1000 pg/mL [68] |
| Aβ1-40/1-42 | Antibody to N-terminus of Aβ peptide | CV | 10 pM [69] |
| APOE4 | Au nanostructure, anti-APOE4, HRP-anti APOE4 | CV | 0.3 ng/mL [70] |
| Aβ1-42 | Anti-Aβ1-42, gold electrode | CV | 0.1 ng/mL [71] |
| Aβ1-42 | Anti-Aβ1-42 on AuNPs array | EIS | 1 pg/mL [72] |
| Total Aβ | SA-ALP, TCEP and anti- Aβ1-16 | CV | 5 pM [65] |
| Total Aβ | anti-mAβ on AuNP | EIS | 0.57 nM [73] |
| Aβ oligomer | Antibody on carbon, aptamer-AuNP-thionine label | DPV | 100 pM [74] |
| IL-1b, IL-10 | Anti-IL-1b, anti-IL-10 on Au | EIS | 0.7 pg/mL, 0.3pg/mL[75] |
| TNF-α | Poly(3-thiophene acetic acid)/anti-TNF on ITO | CV, EIS | 3.7fg/mL [76] |
| IL-12 | 16-mercaptohexadecanoic acid/anti-IL-12 on gold | EIS | 3.5pg/mL [77] |
| IFNγ | HRP/ab2/AuNP label, ab1/AuNP on ITO | DPV, EIS | 48fg/mL [78] |
| α-Synuclein | Ab1/AuNPs on $TiO_2$NPs, ab2/AuNP/glucose oxidase | I-V | 34pg/mL [79] |
| Anti-MBP | MBP/TiO2/gelatin on Pt | CV, EIS | 0.1495 ng/mL [80] |

### 3.3 Barriers to Electrochemical Sensors in Clinical Blood Biomarker Detection

Electrochemical immunosensors are considered to be the most promising technique for blood biomarker detection among the three types of sensors discussed in this review. In addition to the noteworthy points for GFET, currently the main drawback is that the reproducibility falls short of practical device standards. The unsatisfying reproducibility comes from many aspects, but mainly from quality of raw graphene materials and the device fabrication procedures required in electrochemical immunosensors [81].

As the essential materials for the working electrode surface, graphene nanosheets, GO and rGO are generally produced through the exfoliation of graphite in solution [82]. The layers of graphite are separated by subjecting them to a sonication or shearing process, which results in a wide range of thickness, sheet size, randomly distributed defects and impurities. Depending

on the specific oxidants, the amounts of hydroxyl and epoxide groups on the layer plane, and the number of carbonyl and carboxyl groups at the edges of GO sheets are significantly different, resulting in differences in electron transport resistance [83], which is particularly impactful for the performance of electrochemical immunosensors. Centrifuging at certain speeds, dissolving in specific solvents, or making them into the thin films helps to obtain nanosheets with narrower distribution and may wash out some differences between individual nanosheets [84]. However, the difference still leads to errors for the detection of biomarkers at the femtogram level. Furthermore, commercial suppliers and research labs aim to achieve individual goals using their own recipes for higher yield, fewer defects or oxygen moieties [85]. The structural features and the surface cleanness of nanosheets are highly sensitive to the change of production parameters, leading to difficulties in reproducing the devices from raw materials and quality control over multiple batches.

The process of electrode fabrication could also have significant influence on reproducibility and stability. For example, for GO, a metastable material, its molecular stability changes when dispersed in a solvent with different ionic strength, pH value, or presence of organics, and even sunlight [86]. This may lead to the degradation of the functional moieties on the GO surface or reaction with the solvent components. Dispersion stability, another critical parameter, heavily depends on the surface oxidation polarity and the face-to-face interaction in certain solvents [87]. These factors could make Van der Waals and π–π binding predominate, in turn contributing to the restacking and aggregation over the electrode fabrication; therefore, redispersion and sonication may be required to ensure the reproducibility of electrodes. In addition, combining interface materials (between graphene and electrode) and chemical activation of electrodes also play an effective role in improving the reproducibility and stability of the electrodes due to graphene's inert nature and lack of interactions with electrode materials. Interface materials, such as chitosan, nafion or metallic nanoparticles, could form stable composites and anchor graphene nanosheets tightly onto the activated electrode surface [88, 89]. With respect to the assembly of antibodies and blocking agent layers, it is similar to graphene electrical immunosensors, where the reproducibility can be improved by using the orientated antibodies with small physical dimension and blocking agents with higher blocking capacities. Other parameters, such as the quality of electrode base [90], supporting electrolyte [91] and storage conditions [92] all have their contributions to producing reproducible results.

## 4. Graphene Optical Immunosensor

### 4.1 Optical Sensing Mechanism

Optical techniques such as surface plasmon resonance (SPR), surface-enhanced Raman spectroscopy (SERS) and fluorescence spectroscopy have been widely explored for the detection of bio-analytes, as shown in Fig. 5. The performance of these optical immunosensors strongly depends on the physio-chemical properties of the sensor materials, which may lead to higher sensitivity, selectivity and specificity toward the detection of protein biomarkers in specific settings.

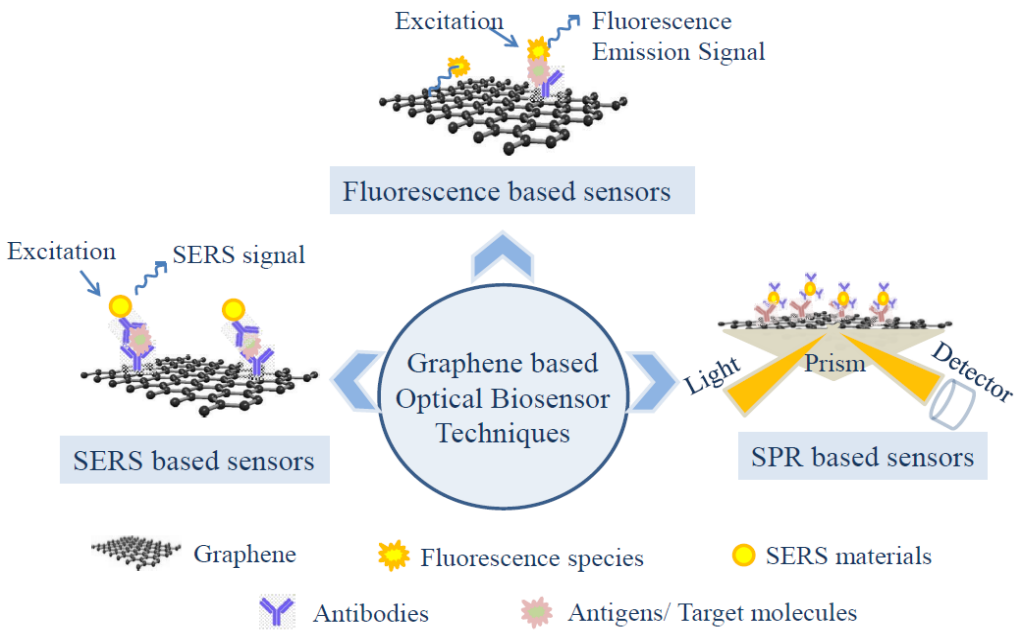


Figure 5. Schematics of graphene-based optical immunosensor.

**Surface Plasmon Resonance (SPR):** SPR is a phenomenon based on the interaction of a monochromatic light source (laser) and the oscillations of electrons at the surface of metals. In most cases in SPR, the classic Kretschmann configuration is used where the metallic thin film, typically 40 nm thick, is sputtered onto one face of a 45° or 60° prism. The prism should have a high refractive index if possible, such as 1.737 for an N-SF11 prism, compared to standard borosilicate prisms at around 1.5. In this case an evanescent (vanishing) electromagnetic wave propagates along the interface, with an intensity that decreases exponentially away from the surface. This evanescent wave interacts with the oscillating electron cloud (plasmon) to produce a surface plasmon. As the angle of incidence is varied it is seen that the total internally reflected beam reduces in intensity – i.e. becomes attenuated. At a specific angle, the reflected intensity is minimised and can become zero. This is the resonant SPR angle, and this angle is very sensitive to the optical properties of the layers on the surface of the metallic layer. The characteristics of this angle with respect to changes in the layer's properties are the basis of

SPR sensing. If Au or Ag nanoparticles are used, as in SERS, then the SPR becomes localised SPR or local surface plasmon resonances (LSPR). SPR systems rely on the design of the sensor to determine how this attenuated return beam responds.

An SPR immunosensor includes the prism with a metallic layer on one face, as previously discussed, upon which the surface is subsequently functionalised with biorecognition elements such as antibodies. The configuration and surface functionalisation are similar to those in GFETs or electrochemical immunosensors if mono or few layers of graphene are placed onto the Ag or Au plasmonic layer. The optical detection system is highly responsive to changes in refractive index at the proximity within 30-40 nm of the metallic plasmonic layer, in this case, attributed by the binding of corresponding antigen molecules [93].

Fig. 6 below shows the SPR configuration used. The prism is inverted in this instance but can also be on the horizontal plane. Using microfluidics, a number of sensor channels can be configured and functionalised with different antibodies, with the probing beam split accordingly, to simultaneously detect a number of different immunosensitive interactions.

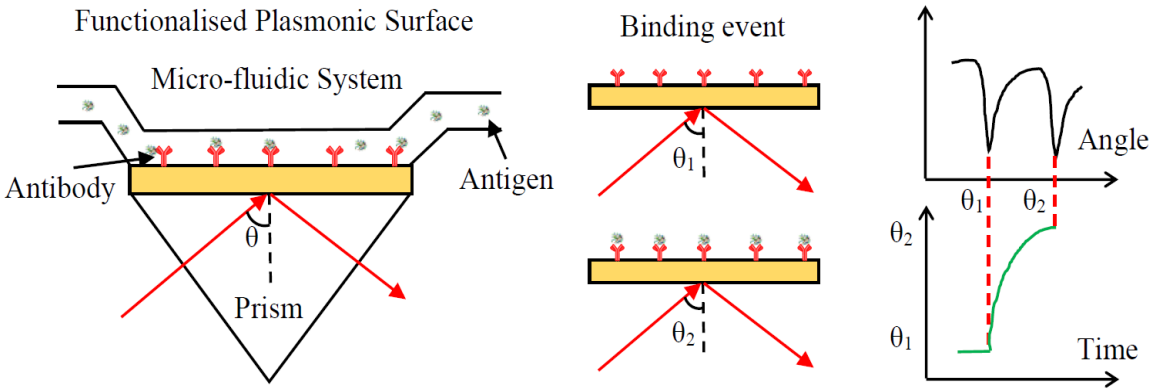


**Figure 6.** Schematic view of SPR immunoassay technique.

The sensor may be reused by flushing the system with solution pH of 9-11 or 3-1 depending on whether the system has overall negative or positive charge. In SPR immunosensors, the metallic layer is normally gold, however silver has a sharper SPR response, but subsequent oxidation of silver significantly reduces this sensitivity. Placing monolayer graphene onto the metallic layer prevents silver from oxidation. In addition, the sensitivity and responsivity increase by enhancing the probing electric field and the affinity to biomolecules to bond on both silver and gold films [94, 95]. This is comprehensively covered in a review paper by one of the authors [96].

In graphene-based SPR immunosensors, the sensitivity and detection accuracy can be

optimised by carefully tuning the number of graphene layers relative to the thickness of the metallic layer [94], since a greater number of layers will disturb the SPs due to the large imaginary dielectric constant [97]. In addition, a different GO-based SPR configuration was presented by Chiu [98] as a sensing platform. In this work, GO was chosen as the medium owing to its high covalent binding affinity for protein molecules, which lead to a LOD of 100 pg/mL for the BSA detection. The sensitivity and selectivity can also be improved by functionalising the GO surface with negatively charged carboxyl groups. In this case the carboxyl-GO based SPR sensor provided high affinity and stronger binding of peptides, and this is very significant for a non-immunological label-free mechanism. By doing so, Chiu et al achieved a LOD of 1.15 pg/mL for the detection of human chorionic gonadotropin in clinical serum samples [99]. Although graphene SPR sensors have yet to be converted as immunosensors for the detection of neurodegenerative biomarkers, the excellent LOD in detecting proteins with similar molecular weight indicates that this is a promising direction to explore [100].

**Surface-enhanced Raman spectroscopy (SERS):** The core science of SERS is the Raman effect, which is inelastic scattering of incident photons by molecules upon illumination with electromagnetic radiation. The fraction of incident photons, which are scattered with a different frequency from that of the excitation source, and thus determine the intensity of the Raman signal, is only one in about $10^{10}$ [101]. However, the efficiency of the Raman scattering can be enhanced by a factor up to $10^{14}$ via the dramatically increased electromagnetic field localised in the nanoscale features of plasmonic materials such as Au, Ag or Cu [102], which allows single molecule detection in immunosensors.

A graphene-based SERS immunosensor utilises a sandwich format for the detection of protein biomarkers, as shown in Fig. 5. It consists of a Raman signal measuring unit, complex SERS nanostructure (Ag or Au coated rods, or chiral structure) modified detection substrate, and SERS nanotags, where the latter two are functionalised with corresponding antibodies for the detection of specific biomarkers. When being used in such an immunosensor configuration, one of the primary roles of monolayer graphene has been protection of the metallic layer beneath from oxidation. As described previously, sensitivity is better with Ag, and the chip surface can be coated with transferred monolayer graphene to maintain detection responsivity. In addition, graphene derivatives can be also functional in the same way as in other immunosensors, allowing a strong local electric field, enrichment and uniform adsorption of target molecules, while preventing aggregation of nanotags and background

fluorescence signals. Even with gold as the metallic layer, where oxidation is not an issue, the monolayer of graphene increases the affinity of the surface [103].

As a successful example, Demeritte et al discussed the importance of the SERS effect as a rapid and reliable tool for the diagnosis of AD biomarkers from clinical samples [104]. The SERS effect was investigated on iron oxide magnetic core-gold shell nanoparticles. As in LSPR, gold nanoparticles (AuNPs) can enhance the electric field. Hot spots are highly localised regions of intense local field enhancement attributed to LSPR, in the interstitial crevices between closely spaced nanoparticles. Such hot spots have been claimed to provide extraordinary enhancements of up to 15 orders of magnitude to the surface-enhanced Raman scattering (SERS) signal. GO is able to enhance the SERS signal by a chemical mechanism (charge transfer), yet it has not been rigorously explored. Kim et al showed that that there is a preferential route for charge transfer responsible for chemical enhancement, which is an enhancement process in non-plasmonic SERS only [105]. This enhancement in the Raman signal is explained through a chemical enhancement effect, the $sp^2$-structure favourable for aromatic molecular interaction via π-π interaction and the highly electronegative oxygen on the surface of GO increase the local electric field of the molecules absorbed on the surface. It shows a 25 times increase in Raman intensity for GO than core-shell nanoparticles. As a result, the LODs of SERS immunosensor have been determined to be 100 fg/mL for both amyloid-β and tau, which is higher than the ELISA 0.312 ng/ mL and 0.15 ng/ mL in the work.

**Fluorescence spectroscopy** is another interesting optical method for the detection of protein biomarkers. In fluorescence immunosensors, graphene and its derivatives are mainly used as general quenchers for a range of fluorescence species, such as fluorophores, quantum dots and nanoclusters [106]. The mechanism is that when the fluorescence species approach a graphene surface, the excited electrons interact with the planar π electrons to go through a photo-induced electron transfer process, rather than releasing photon. While in the presence of the target molecules, the fluorescence species detach from the surface of graphene due to a stronger interaction between probes and targets, producing sharply increased fluorescence to be used as detection signals. The efficiency of this long-range fluorescence resonance energy transfer (FRET) from dyes to graphene heavily depends on the distance between them. In the case of using graphene as a quencher, the effective quenching distance can reach 30 nm, which is much higher than conventional materials and make it a unique candidate for biosensor development [107]. The shorter the distance between graphene and the

fluorescence dyes, the better the sensitivity will be, which is similar to the situation in GFET and electrochemical immunosensors [108]. In addition, the quenching efficiency (sensitivity) also depends on the chemical moieties on the GO surface, where a higher C/O ratio leads to enhanced efficiency, as oxygen-containing moieties occupy the free $sp^2$ transport domains [109]. Inversely, GO has a broad fluorescence emission peak, making it also an ideal candidate to be used as a fluorescence species. In a FRET process, GO can act as an energy donor, in which its fluorescence can be quenched by other energy acceptors, such as metallic nanoparticles.

Using this sensing mechanism, Huang et al developed an ultrasensitive fluorescence immunosensor for tau protein detection. The sensing platform, GO, is functionalised with anti-tau probes. When the fluorescein isothiocyanate-labelled tau (tau-FITC) is captured by the probe, the optical signal is effectively quenched by the GO nanosheets. Whilst both normal tau and fluorescence-labelled tau are present, they compete for the binding sites and leave part of the tau-FITC free standing in solution for fluorescence generation. The resulting change of fluorescence signal can be used for the quantitative detection of tau proteins with a LOD of 0.14 nM in a buffer solution [110]. He et al also employed GO as a quencher for the detection of AD biomarkers [111]. Differently in their work, GO was firstly cultured with resveratrol, which acted as both fluorescence dye and probe molecules for amyloid β binding. This complex presents no fluorescence signal due to the π-π interactions and FRET effect in the absence of target amyloid β molecules, whilst the signal can recover when photoexcited dyes become encapsulated by the target molecules. The configuration is not a representative antibody-antigen immunosensor but paves a new path for the design of fluorescence immunosensors for neurodegenerative biomarker detection. Although this sensing mechanism has been widely explored for the detection of various targets, such as DNA [112], RNA, dopamine, thrombin, Mucin 1 and even viruses [113], limited efforts have been focused on the development of neurodegenerative immunosensors, giving researchers expansive space to explore the potential of using graphene fluorescence immunosensors to address this social challenge.

### 4.2 Barriers to Graphene Optical Immunosensors in Clinical Application

Optical techniques such as SPR, SERS and fluorescence labelling have been widely explored with graphene nanomaterials for the development of immunosensor platforms, which have demonstrated a series of promising outcomes. However, many challenges need to be addressed for the adoption of graphene-based optical immunosensors in clinical diagnostics

[114]. For SPR immunosensors, the optical focusing step used in measuring wavelength or angle change has been determined to limit the sensitivity, LOD as well as dynamic range in real-time measurement. It is also difficult to balance the response speed and the sensitivity of an SPR immunosensor due to their conflicting nature. For example, the SPR sensor measuring intensity presents at a faster response speed but low resolution, whilst the sensor measuring angle or change shows higher sensitivity at the cost of lower response speed [115]. In addition, the high cost, mass production difficulties and issues with mechanical and electromagnetic interference make SPR immunosensors struggle to be applied in frontline clinical settings.

For graphene-based SERS immunosensors, the response heavily depends on moieties on the graphene surface. However, similar to the graphene materials used in electrochemical sensors, electrochemical or chemical processes used in graphene production generate epoxy, carboxyl and hydroxyl groups randomly distributed on the graphene surface. Yin et al. found that the Raman intensities of rGO decreased with the increase in the duration of reduction. For reduction times from 2 to 60 min, the strongest Raman intensity of R6G molecules was observed for a 10 min-rGO monolayer substrate and that on the GO monolayer substrate was the lowest. The results indicate that single-layered rGO sheets with a lower degree of reduction could be better substrates than GO [12].

For fluorescence immunosensors, the main challenge is the over-estimation of the target fluorescence signal due to the high background signal from the complex clinical samples, particularly in serum. In a report presented by Bahamonde *et al* [116], various targets have been detected using graphene in combination with other materials as immunosensors, where the electrochemical route is highly preferred in lower background signals, compared to fluorescence-based sensing. This means, to get good sensitivity and selectivity using fluorescence detection, some sample preparation (e.g. separation and preconcentration) is necessary before the final analysis of target biomarkers. GO also presents broad fluorescence peaks in the presence of different oxygen-containing moieties, when being used as fluorescence dyes. When a narrow fluorescence peak is required in sensing, it requires extra work for GO in terms of selective functionalisation and purification. In addition, the nature of fluorescence detection determines that the time-consuming labelling process of either probe or target is inevitable. These make the graphene-based fluorescence immunosensor challenging to be used in the analysis of clinical blood samples.

## 5. Strategies Toward Clinical Applications

### 5.1 Unified Standards for Graphene Reproducibility

Although many methods have been developed for the production of different graphene nanomaterials [117], so far the practical applications are limited to those in which consistent quality and properties are not strictly required, such as additives in composites [118] and batteries [119]. To address the inconsistent qualities and properties of raw graphene materials, large-scale production requires precise controls of procedure parameters, standard manufacturing techniques, and the quality of final products [120]. For example, the production capacity of CVD graphene is enlarged by stacking the metal foils efficiently in the growth chamber [121]. However, when stacked together, the microscopic growth environment, such as heat and mass transport along the gas flow direction between different layers would be significantly different within and between foils. The resulting graphene would suffer from non-uniformity in thickness, number of layers, defect density and domain size. Therefore, controlling growth parameters on different foil areas, and between different batches and providers, remains a challenge. This currently presents the main barrier to transfer laboratory progress into practical applications, including biosensing technology. In addition, like the Si industry, the graphene providers need to set unified application-specified standards with clear grading systems and datasheets to demonstrate the reliability and the reproducibility of their products. For example, transparent optical films and FET-based applications require clear structural and performance information, including number of layers, detect density, electrical mobilities, uniformity and average grain size in a continuous film. Whilst electrochemical probe-based application requires information like elemental composition, functional moiety distribution, sheet size distribution, layer thickness and concentration in different solvents.

On the other hand, unified guidance for the fabrication and characterisation of certain types of clinical immunosensor will be helpful for the large-scale adoption of the technology. This is unlike research that pursues extreme performance using bespoke recipes in laboratories. In order to reproduce and compare the results obtained by different individuals or primary care practitioners under various environmental conditions, a standard operatng procedure is needed to ensure that the graphene devices are subjected to identical influence factors and exposed to the clinical samples from the same baselines.

## 5.2 Signal Amplification for Sensitivity Improvement

Although a few neurodegenerative biomarkers have been successfully detected using graphene immunosensors, many other biomarkers have not yet been investigated or cannot be detected at low clinical concentrations due to inadequate sensitivities of the sensors, as shown in Table 2. Therefore, signal amplification has been considered an essential strategy in achieving lower detection limits and higher sensitivity. To date, many nanomaterials and enzymes tags have been used for signal amplification [122], shown in Fig. 7.

Table 2. Comparison of LODs of different immunosensors for neurodegenerative blood biomarker detection. M indicates the median concentration in health controls. Q indicates data from Quanterix Company. Units are pg/mL except where stated otherwise.

| **Biomarker** | **Clinical Range** | **ELISA** LOD | **SIMOA** LOD | **Graphene** LOD |
|---|---|---|---|---|
| IL-1β | 1.22-6.61 [123] | 0.14 [124] | 0.0051 [124] | 5 [125] |
| IL-2 | 0.52(M)-53.5 [124, 126] | 0.25 [124] | 0.089 [124] | --- |
| IL-4 | 0.18 (M)-18.8 [124, 126] | 0.22 [124] | 0.0093 [124] | 80 [127] |
| IL-6 | 0.82–7.28 [128] | 0.11 [124] | 0.0043 [124] | 1 [60] |
| IL-10 | 1.28 (M)-206.7 [121, 126] | 0.17 [124] | 0.0048 [124] | --- |
| TNFα | 9.28-26.02 [123] | 0.191 [124] | 0.013 [124] | 5 [125] |
| MMP-9 | 51-813 ng/mL [129] | 1 ng/mL [129] | 0.581 (Q) | 5 fg/mL [62] |
| IFNγ | 0.1-40.3 [130] | 0.69 [124] | 0.017 [124] | 83 pM [131] |
| Aβ1-40 | 2.2-645.7 [132] | 1.91 [132] | 0.044 [133] | 1 [134] |
| Aβ1-42 | 3.7-728.5 [132] | 2.04 [132] | 0.522 [133] | 0.41-5 [59, 61] |
| Total tau | 0.43-18.9 [135] | 60 [136] | 0.02 [135] | 0.15 nM (tau441) [63]<br>75 fM (unk isoform) [57] |
| GFAP | 0.8–129.6 [123] | 62.5 [137] | 0.8 [123] | 1 [138] |
| NFL | 78.0–252 [139] | 78.0 [139] | 0.62 [139] | --- |
| ApoE | 2.19-4.22 μg/mL [140] | 3.1 ng/mL [141] | --- | --- |
| α-Synuclein | 4.16-36.23 ng/mL [142] | 0.1 ng/mL [143] | 0.44 (Q) | 1.2 pM [56] |
| MBP | 40-2010 [144] | 30 [144] | --- | 0.3 nM [63] |
| Fibrinogen | 0.51-3.68 g/L [145] | --- | 12.5 (Q) | 5 μg/mL [146] |
| S-100B | 0.1-1.79ng/mL [147] | 20 [147] | --- | 0.15 [148] |

Due to the small physical dimensions (from 1-100 nm), metallic nanoparticles have been used to enhance the electron transfer efficiency and the surface-to-volume ratio on working electrodes [149]. Among them, AuNPs have been integrated into electrodes since their excellent electrochemical activity allows electrons to transport freely from valence to conduction band. Bernard et al reported a glutathione-protected AuNP electrochemical immunosensor for the detection of interleukin-6 in serum. The sensor employs a sandwich

configuration and shows three times higher sensitivity and two times wider linear detection range than carbon nanotube modified electrodes [150]. Silver nanoparticles (AgNPs) have also been used as electrode material due to the good electrochemical activity and lower cost. A report demonstrated 1.7 times higher sensitivity and more than 10 times lower LOD for the detection of microcystin-LR, compared to the electrode without AgNP modification [151]. More recently, nanoparticles have been used collaboratively with other nanomaterials for the fabrication of hybrid electrodes. For example, AuNPs have been chemically integrated with thiolated rGO film and deposited onto the surface of screen-printed electrodes for cancer prognosis antibody detection. This synergistic improvement leads to a low LOD of 0.088 pg/mL [152]. Notably, metallic nanoparticles are electrically unstable, meaning they may form aggregations in the presence of salts. Therefore, optimal functionalisation of the sensor is needed before they are used in clinical samples with high salt concentration. In addition, signal amplification achieved by introducing metallic nanoparticles is inconsistent among studies. Therefore, strict quality control over nanoparticle fabrication and electrode processing will be required for higher reproducibility and reliability [153].

Signal amplification has also been achieved by labelling the detection antibodies with enzymic tags in combination with electron mediators in an electrochemical immunosensor. The enzyme has excellent catalytic activity towards the specific substances. The most commonly used method is to immobilise a number of enzyme molecules onto a detection antibody to form a dendrimer. Xiong's group developed a signal tag which is composed of a dextran amine skeleton with more than 100 HRPs and 15 secondary antibodies for the detection of alfa-fetoprotein [154]. The nanocomplex on the electrode surface allows a large amount of HRP to amplify the electrocatalytic current in the presence of hydrogen peroxide, leading to a 2 pg/mL LOD and a 0.005–0.2 ng/mL linear range in undiluted serum. Yoon et al constructed a multi-layered enzymic film on Au electrode surface [155]. The film was fabricated by depositing poly(amidoamine) dendrimers and periodate-oxidized glucose oxidase (GOx) layer-by-layer alternatively, resulting in an enhanced electrocatalytic response correlated to the number of deposited bilayers. Signal amplification via enzyme labelling has also been achieved by adding a redox mediator into the electrolyte. Lai et al reported an immunosensor that uses chitosan-ferrocene-antibody, antibody-GO-HRP and 4-chloro-1-naphthol as capture, detection and redox mediator components respectively [156]. After the sandwich complex forms, both the dielectric detection component and the multi-enzymatic precipitate contributed to the signal decrease, resulting in 5 orders amplification of LOD at

0.54 pg/mL.

Enzyme-labelled immunosensors have obvious advantages in producing highly specific, sensitive and reliable signals as described. This approach, however, suffers from some drawbacks for electrochemical immunosensors. These include a relatively narrow dynamic range, and a time-consuming incubation process for detectable signals. More importantly, a stronger interference signal is expected when performing detection in complex clinical samples, which contains analytes that may react with the enzyme labels. Therefore, a reduced diffusion limitation of the substrate, an optimised enzymic reaction environment as well as the removal of potential reactive substances with enzymes prior to the detection will be crucial for a reliable and sensitive diagnosis.

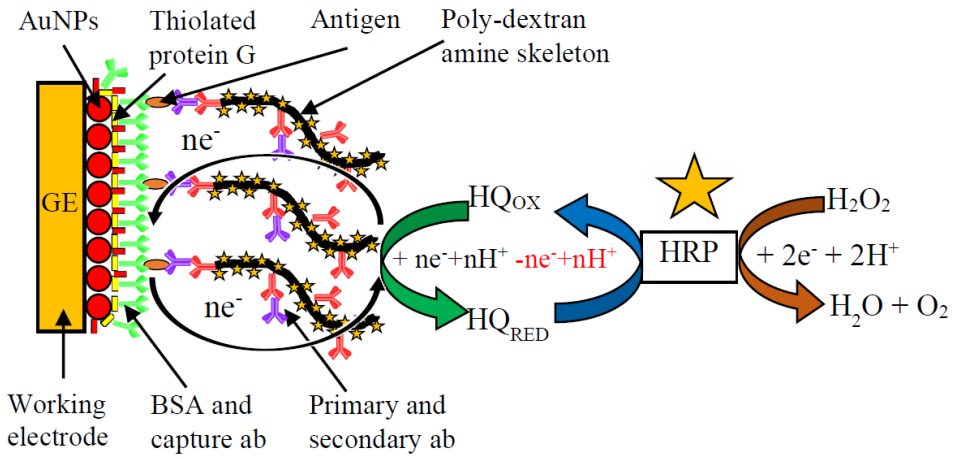


Figure 7. Signal amplification using nanoparticles on substrate, nanostructures and enzyme labels as signal enhancer. Adapted from Ref. [154]. Copyright © 2012 by P. Xiong, N. Gan, Y. Cao, F. Hu, T. Li and L. Zheng. Licensee MDPI, Basel, Switzerland.

### 5.3 Challenges in Sample Preparation

In addition to the globally well-established sampling infrastructures, the advantages of using blood samples for neurodegenerative biomarker detection includes the largely constant concentrations in cellular and extracellular constituents and the detectable change of biomarkers before and during the onset symptoms [157]. However, the disadvantages of blood samples are also clear, such as high viscosity, multiple blood components, complex matrix, high background as well as a wide dynamic range of the biomarkers. A reproducible immunosensor platform surely can contribute to a more reliable detection, but appropriate sample preparation, such as separating the disruptors and enriching the biomarker concentration, has increasingly been treated as an essential step to translate the research into clinical applications [158].

Separating the disruptors has been commonly used in clinical assays as an initial step, by dividing the blood sample into plasma and blood-cell rich components. The conventional laboratory-based high throughput separation is centrifugation using dedicated equipment, which make it difficult for adoption in primary care settings. Alternatively, the Zweifach–Fung bifurcation effect has been employed in microfluidics for the separation of plasma from finger prick blood samples [159, 160]. The working mechanism is that when blood cells encounter a branch point in a microfluidic channel, they selectively go into the higher flow rate channel, leaving plasma concentrating in the lower flow rate channel, as shown in Fig. 8 (a). In parallel, another group developed a hydrophobic wax filter-based paper substrate for lab-on-chip applications, which filter out aggregated blood cells and allows the plasma to flow through onto the sensing area at high efficiency, as shown in Fig. 8 (b) [161]. However, the matrix effect could be heavily affected by many other interfering compounds in serum, such as non-target proteins, immunoglobulins, debris and salt ions, which could either increase the background signal or reduce the target signal, resulting in low detection sensitivities in clinical samples [162]. Diluting the blood samples may reduce the matrix effect, but more dedicated equipment is normally required for the removal of these smaller molecules, limiting its use in primary clinical settings. Therefore, high efficiency and easy-to-use separation techniques need to be further developed to integrate with graphene-based immunosensors for the detection of neurodegenerative biomarkers.

On the other hand, increasing the concentration of target biomarkers within clinical samples could be another effective strategy for neurodegenerative diagnosis. Microbeads have been demonstrated for high efficiency concentration of biomarkers in microfluidics [124, 163]. The outstanding surface-to-volume ratio and fast diffusion rate in liquids offers the capture agent functionalized-microbeads with enhanced probabilities to interact with the target molecules. Furthermore, combined with magnetic manipulation, the beads can be easily recollected, and the target molecules can be eluted into a new solvent to increase the target signal and reduce the matrix effect (noise). Notably, the integration of any forms of enriching procedure requires corresponding equipment, expertise as well as some processing time, it remains a challenge to find balances between a well-prepared sample and the feasibility of doing so in primary clinical settings.

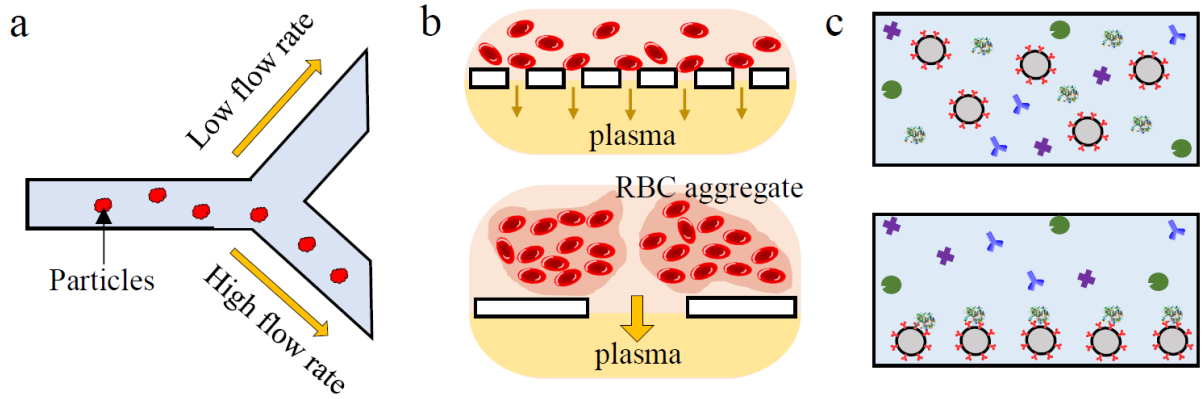


Figure 8. Commonly used separation and concentration techniques in biosensing. (a) Zweifach–Fung bifurcation effect-based separation - particles go to the higher flow rate channel. (b) Large blood cell aggregates can be filtered out through micropores with optimal size to allow higher filtering efficiency. Reproduced from Ref. [161] with permission from The Royal Society of Chemistry. (c) Magnetic microbead-based separation. The beads quickly diffuse into the mixture and interact with target molecules. The target concentration can be increased by tuning the magnetic field and recollecting target-bound beads.

### 5.4 Multiplexed Detection for Reliability Improvement

Diagnostic reliability and accuracy can be improved by using multiplexed immunosensors. Although many of the above techniques are suitable for multiplexed detection [164-166], the most commonly used configurations are the electrochemical immunosensors [167]. The construction of multiplexed immunosensors can be achieved using two methods. The first is multiplexed electrode arrays, where individual electrodes are functionalised with different antibodies for capturing the corresponding antigens [168, 169]. This configuration requires several sensing, reference and counter electrodes to perform independent measurement with a n-channel analyser. The advantages are that detection can be label-free (or single label), and crosstalk between the adjacent electrodes can be effectively eliminated. As an example, Cui et al developed a multiplexed immunosensor employing graphene-modified electrodes and mesoporous platinum nanoparticle for the simultaneous detection of three tumour biomarkers (CA125, CA153 and CEA) [170]. This sensor array consists of three separate working electrodes, where each electrode is individually modified with a different capture antibody to allow simultaneous detection without crosstalk between electrodes. Platinum nanoparticles modified with corresponding antibodies have been used to form the sandwiched complex for generating signals through the electro-reduction of $H_2O_2$, leading to LODs of 0.002 U/mL, 0.001 U/mL and 7.0 pg/mL for detection of above biomarkers, respectively. The second

method is barcode configuration, where different capture antibodies are immobilised on the same electrode and use multiple labels to generate distinct signals at specific potentials. In this configuration, multiplexed detection can be performed in a single electrochemical scan. However, signals from different labels may not be sufficiently separated, resulting in crosstalk for the detection of multiple antigens. A multiplexed graphene immunosensor developed by Li's group has been deployed for the simultaneous detection of two interleukin biomarkers [171]. The surface of the graphene electrode was functionalised by the mixture of anti-IL-6 and anti-IL-17 for capturing two different antigens. Upon their binding with IL-6 and IL-17, anti-IL-6 functionalised polystyrene-cadmium ion and anti-IL-17 functionalised polystyrene-ferrocene are introduced to the electrode surface to identify the formation of each corresponding sandwiched complex in SWV scans. By analysing the intensities of these redox peaks at different potentials, clinically meaningful LODs of 0.5 and 1 pg/mL have been achieved for the detection of IL-6 and IL-17 respectively.

In electrochemical immunosensors, signal multiplexing also reduces the physical dimensions of the area required for containing sensors that share a common electronic interface for simultaneous analysis of either multiple biomarkers (labels), or increased spatial resolution for the same label [172]. In many applications the sensing area needs to be kept as small as possible due to the limitations on the sample quantity available for analysis (e.g. blood, sweat and saliva) [173]. Detection of multiple biomarkers or other proteins can then be achieved in a single run [174], allowing faster screening times, lower equipment cost and enabling point-of-care diagnosis [175]. To achieve multiplexed functionality, electrical signal traces (conductors) can be patterned or deposited over the sensing area [176], with the measurement interface adjacent (2D sensor array) or within a different geometry level (3D array). Detection of a single biomarker typically involves fabrication of a specific type of sensor, with a single access-port to the interface electronics for signal amplification, conditioning, or stimulation. When layering a large number of the same sensors, one may also require additional interface electronics, which may be impractical in terms of physical space occupied, or availability of interface materials and components [177]. A more efficient solution is to use multiplexed and addressable electronics. This is standard semiconductor technology which is realised by top-down fabrication techniques like screen-printing [178], photolithography, microcontact printing and electron beam lithography [179]. The multiplexing operation also introduces additional electric impedance in series with the sensor's outputs that must be accounted for during the design of the interface electronics. In

multiplexing, for smaller impedance values, amplitude attenuation is more tolerable, since the voltage drop developed across the multiplexer is minimised, and therefore the signal output is determined by the transfer function of the amplifier. In terms of temporal control, fast switching times for the multiplexer and amplification electronics are essential to interrogate all sensors in the sensing area within a reasonable time frame for measurements [180]. For slowly varying concentrations of biomarkers within body fluids, the temporal constraints for multiplexing can be lifted. Recent advances in electronics allow multiplexing to be performed in the order of nanoseconds, while signal acquisition is sub-nanoseconds, without compromising signal amplification and resolution. For a 1024 sensor array (32 x 32), an acquisition rate of 10 million samples per second requires around 10 ms to complete one data frame acquisition with roughly 50 samples per sensor. This is without taking into consideration the time for sample transference, storage or for visualization inside the acquisition system. For cases where more than one type of sensor is required to detect multiple biomarkers, strategies exist that divide the sensing area into different sensing units specific to each biomarker, and are connected only to the respective interface electronics. For more details about multiplexed detection based on graphene immunosensors, the reader is directed to ref. [167] where several relevant clinical biomarkers are discussed.

The multiplexed strategy is particularly promising for the detection of blood neurodegenerative biomarkers. This is because the most frequently reported biomarkers are not only discriminative for neurodegeneration, but also overlapping with patients undergoing normal ageing or with underlying conditions, such as cardiovascular, respiratory, renal and rheumatic disease. These comorbidities all affect protein profiles in blood, therefore no single definitive biomarker exists [181], making the diagnosis of neurodegenerative disease using blood biomarkers inherently complicated. In this case, the multiplexed immunosensor allows a simultaneous description of the full spectrum of multiple independent biomarkers, which can lead to a combinational conclusion, thus improving the diagnosis accuracy.

Tremendous progress has been made with multiplexed immunosensors to improve their reliability for biomarker detection. However, some issues need to be carefully addressed before the technology can be used in primary clinical settings. One of them is crosstalk and interaction between individual electrodes, which requires effective spatial separation of electrodes with addressable signal readout [172]. In addition, the threshold levels of target biomarkers could be different in clinical samples, and attention should be paid to analysing strategies for determining biomarkers at very different concentrations. Despite several

challenges to address, multiplexed electrochemical immunosensors show extreme promise for the development of the reliable and feasible diagnostic technology in clinical settings.

### 5.5 Disposable Immunosensors

Although graphene nanomaterials exhibit great promise in immunosensors, the dissociation of antibody-antigen complexes is not a spontaneous process, hence the graphene-based immunosensors cannot be reused in primary clinical settings and are intended to be single-use instruments. After a single use, the sensing element would need to be replaced. This is, however, a limitation in most biosensors, and is not unique to graphene-based systems. Furthermore, once the sensor is contaminated with a biological sample from a subject, cleaning and reuse are generally considered to be unsafe. To address these issues, graphene-based disposable immunosensors have been developed to provide an affordable and easy-to-use platform for single-shot measurements. The current strategy for disposable biosensors is to split the sensing system into a disposable sensing unit, such as a chip, cartridge or strip, which can be provided by the supplier in large quantity and low price; and a relatively inexpensive readout device, which is portable and reusable for a long period [182]. Electrochemical glucose tests are a well-known example for this testing strategy, in which diabetics can check their blood glucose using an inexpensive handheld reader and disposable strips which they use for sampling and measurement. In this section, we will focus on the disposable sensing unit, more specifically the materials for the substrate and technologies for the fabrication of graphene-based layers for signal transduction.

There are no one-fits-all substrate materials for the development of different types of immunosensors. However, there are two popular choices of substrate for the construction of graphene-base biosensors. i) Standard MEMS materials, used for large-scale semiconductor production, have also been adopted for the fabrication of disposable graphene-based sensing systems [183]. The main MEMS substrate materials used are inorganics and include silicon, glass and ceramics. Because MEMS technology is high precision and low-tolerance, the materials used are also more expensive; however, they have excellent electrical, optical and mechanical properties, with high processability and the potential to yield a large number of functional devices in a single run (larger yields result in lower cost/device). Because most substrates used in MEMS are rigid, they are generally not suitable for the fabrication of graphene-based flexible wearable devices. ii) Synthetic and natural polymers have been explored as potential alternatives to MEMS substrates for the construction of graphene-based biosensors. The most notable synthetic polymer in this application is polydimethylsiloxane

(PDMS) [184]. PDMS, a crosslinking silicone polymer, has been used in the construction of microfluidic graphene-based immunosensors due to its transparency, chemical stability and ease-of-fabrication. PDMS, however, continues to be used mainly in small-scale laboratory prototypes since fabrication with PDMS is slower than thermoplastics, which can be patterned through injection moulding or hot embossing. PDMS is also highly permeable to water vapour (the sample may evaporate; an issue for longer, higher temperature reactions), and presents stronger non-specific adsorption of biomolecules in clinical samples, leading to the higher background signal. PDMS-based microfluidics also requires a pump to drive the liquid around, adding more complexity to the system. Cellulose paper, a natural polymer, has emerged as a viable alternative to the materials mentioned above for the construction of graphene-based immunosensors. Unlike PDMS-based microfluidics, in paper, "active" pumping is replaced by "passive" capillary action, enabling fabrication of low-cost disposable devices. Paper is flexible, porous, biodegradable and devices can be fabricated at large scale using a vast array of printing technologies [185]. For example, Wang et al. developed label-free graphene-based paper analytical devices (μPADs) using screen-printed working electrode for the detection of carcinoembryonic antigen [186]. By immobilising amino-functionalised graphene/thionine/AuNPs nanocomposites together with antibody layers onto the electrode surface, a LOD of 10 pg/mL was achieved. In addition, a number of other novel graphene-based materials or material complexes are being investigated for the development of disposable biosensors [187], which has become a hotspot in the field.

As for the fabrication of graphene-based electrodes for disposable sensors, the most promising, scalable and low-cost approach is probably screen-printing (Fig. 9(a)). Screen-printed electrodes with graphene-based nanomaterials have been employed in the detection of a wide range of biomarkers [188, 189]. Screen-printing involves depositing graphene-containing inks onto a solid substrate through a pre-patterned screen, which defines the geometry of the sensing component(s). In addition to graphene inks, screen-printing allows printing of a wide range of other materials for the fabrication of electrodes and conductive traces e.g., Ag, Ag/AgCl, Au [190-192]. As an alternative to screen-printing, which requires a mask for patterning, inkjet printing technology offers maskless patterning for the fabrication of graphene-based disposable biosensors (Fig. 9 (b)). Similar to screen-printing, there are a large number of ink formulations available for the printing of conductive traces and electrodes; however, unlike screen printing, inkjet printing is a digital technology rendering it more versatile than screen printing although the instrumentation may be costlier [193]. For

instance, a single inkjet system can deposit different patterns of inks without major modifications; screen printing requires separate, precisely aligned screens for each layer. These features make inkjet printing an attractive option both for mass production and prototyping. Furthermore, inkjet printing is more efficient, in terms of the amount of material required, as the ink is deposited only where it is needed [194]. Just like any technology, inkjet printing also has some shortcomings; printheads used in inkjet printing are susceptible to clogging and require frequent maintenance. Ink formulations also require more optimization (e.g., viscosity) to improve printability. Regardless of its disadvantages, adoption of inkjet printing will likely continue in the coming years for the fabrication of disposable graphene-based biosensors.

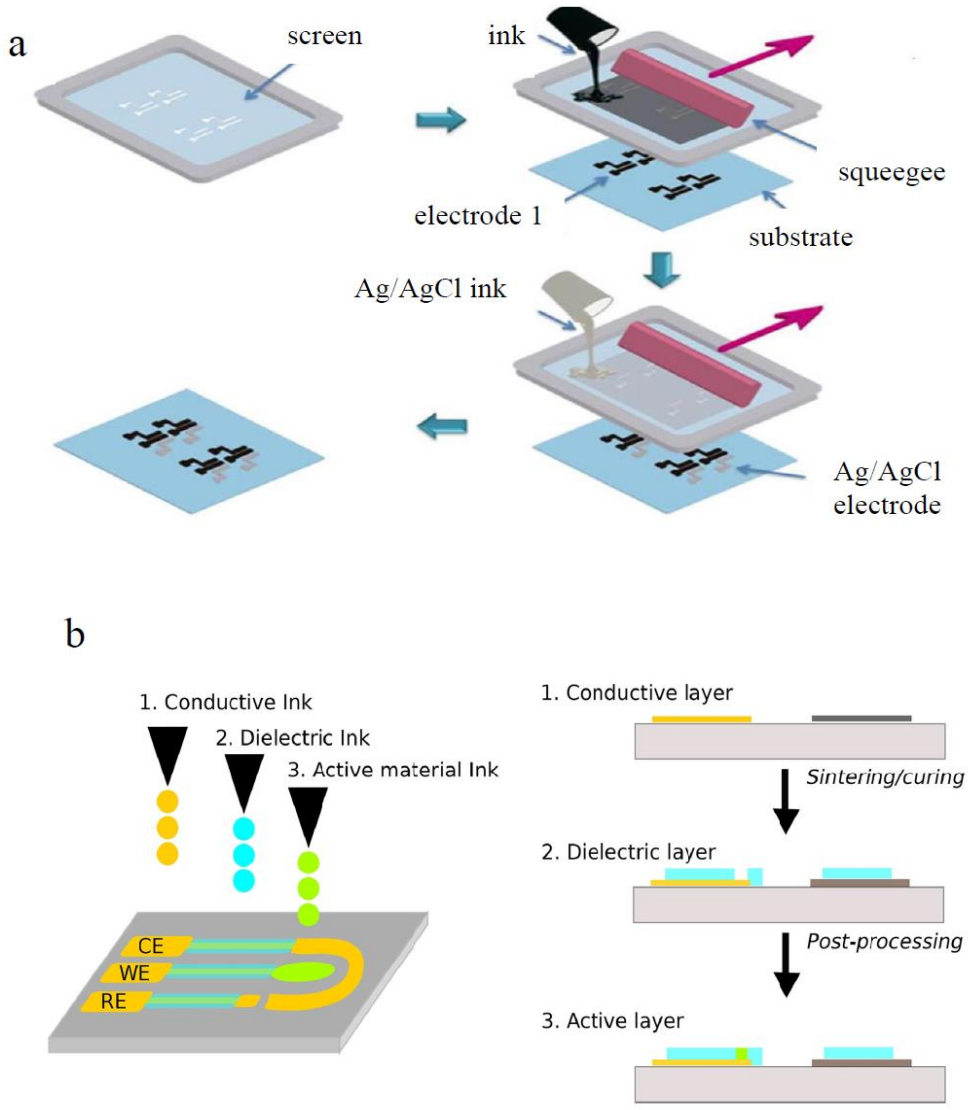


Figure 9. Schematic for different printed biosensors. (a) screen-printed biosensors [195] Reprinted by permission from Springer Nature: [Springer] [Lab-on-a-Chip Devices and Micro-Total Analysis Systems, J. Castillo-Leon and W. E. Svendsen], © 2015 Springer

Nature. (b) Maskless inkjet-printed electrochemical biosensors [193]. Reprinted from *Current Opinion in Electrochemistry*, vol. 3(1), A. Moya, G. Gabriel, R. Villa, F. J. del Campo, Inkjet-printed electrochemical sensors, pp. 29 - 39, Copyright (2017), with permission from Elsevier.

## 6. Conclusions and Prospects

The clinical adoption of biosensing technology needs to stand on the basis of large-scale production of sensors with the emphasis on cost, accessibility, measurement selectivity as well as accuracy and reproducibility. This review summarises the most recent achievements in developing graphene-based immunosensors for the detection of blood-based neurodegenerative biomarkers. In particular, the advantages and disadvantages have been outlined for graphene-based devices as electrical, electrochemical and optical immunosensors against the standards of being used in primary clinical settings. To promote their use in the diagnosis of neurodegenerative disease, further development strategies have been proposed in parallel.

Blood neurodegenerative biomarkers have great early diagnostic value in clinical practice and their corresponding detection will lead to significant social and societal impacts. Since the concept arose, many graphene immunosensor prototypes have been developed using different sensing mechanisms with their own advantages and drawbacks. Primary results have been obtained from both artificial and clinical samples. However, none of these technologies have yet to be used as practical diagnostic tools in clinical settings. Considering it has only been a few years since the transition of lab-based research into practical application, clinical graphene biosensing technology will be a long journey with no shortcuts to success, although its low-cost, extreme sensitivity and high selectivity show the potential to allow the early real-time detection of low concentration biomarkers. It is still difficult to predict exactly when the graphene immunosensor will be adopted as a clinical diagnostic tool. However, with continuous improvements in production and fabrication techniques, device measurement strategies and data analysis algorithms, graphene immunosensors for neurodegenerative detection can look forward to a brighter future.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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