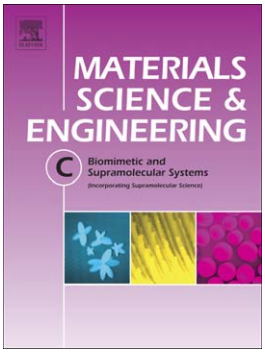

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Two dimension (2-D) graphene-based nanomaterials as signal amplification elements in electrochemical microfluidic immune-devices: Recent advances

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ABSTRACT

Graphene is a 2-D carbon nanomaterial with many distinctive properties that are electrochemically beneficial, such as large surface-to-volume ratio, lowered power usage, high conductivity and electron mobility. Graphene-based electrochemical immune-devices have recently gained much importance for detecting antigens and biomarkers responsible for cancer diagnosis. This review describes fabrication and chemical modification of the surfaces of graphene for immunesensing applications. We also present a comprehensive overview of current developments and key issues in the determination of some biological molecules with particular emphasis on evaluating the models. This review focuses mostly on new developments in the last 5 years in development of chip architecture and integration, different sensing modes that can be used in conjunction with microfluidics, and new applications that have emerged or have been demonstrated; it also aims to point out where future research can be directed to in these areas.

Keywords: graphene; microfluidics; global health; immune-device; bioelectronics

1. Introduction

Advancements in nanotechnology have revolutionized nano-medical research in clinical science, resulting in innovative nano-devices and nano-systems that depend on the design and the precise integration of functional nanomaterials[1]. Among different carbon allotropes, graphene is a novel and potentially useful nanomaterial, the application of which in biomedical sciences and biotechnology is beginning to be realized. Graphene materials have been explored for various biomedical applications, such as biosensors, tissue engineering, and drug delivery[2, 3]. Although graphene was isolated for the first time in the 1960s [4], its synthesis based on the mechanical exfoliation of graphite was reported in 2004 by Geim and co-workers[5]. This achievement boosted the interest on this carbon family member and turned graphene into one of the most investigated materials in the recent years due to its unique thermal, mechanical and electrical properties [6, 7]. Graphene is composed of a single layer of carbon atoms in sp^2 hybridization arranged in a honeycomb shape with six-membered rings, forming two dimension (2-D) crystals[8] . This structure can be used as building block to obtain other carbon-based shapes, as carbon nanotubes, fullerene or graphite [9].

Among the several applications of graphene, one of the most promising and explored examples involves its use as electrochemical sensor [10]. Due to the fact that in a graphene layer all carbon atoms are located on the surface, the molecular interactions and electron transference are highly favored [11]. These properties make graphene a material with high conductivity and excellent catalytic activity for electrochemical reactions [12]. Many procedures concerning the fabrication of electrodes containing graphene for use as electrochemical sensor are reported in literature. Among these methods, the most widely

used is the modification of the electrode surface, where graphene is usually dispersed in solvents or polymer solutions which are used for electrode modification [13-18].

The advantages offered by the electrical properties of graphene turn it into a very attractive material to be used as working electrode for sensing applications in microchip bioelectronics devices. When compared to conventional systems, microchip bioelectronics devices offer several advantages including shorter analysis times, lower sample consumption, higher throughput capability, portability, and possibility of integrating multiple analytical steps in the same platform. Currently, microchip bioelectronics devices have been largely applied in medical, pharmaceutical and forensic analysis, environmental monitoring and food quality control, coupled with a variety of detection methods, and electrochemical modes as amperometry.

The field of biosensors is vast and complex to begin with. If you add to it the possibility of combining a biosensing element with microfluidic sample handling capabilities, the number of realizations described in the literature and being the subject of current research efforts becomes almost, if not quite, astronomical. One reason for the complexity of the field of biosensors is that the term itself is often rather loosely defined, or, at least, means different things to different people. In fact, it is often considered that a biosensor is a device that senses the presence of a biomolecule or perhaps a cell. In other words, there has to be a biological response (which can be triggered by any type of analyte), which then is converted to an electrical signal by means of an optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical transducer. In this review, we have attempted to adhere to this definition in order to bring down the inherent complexity. Other authors have also commented on the ambiguity of the term

"biosensor" and in particular the confusion of the terms "sensor" and "probe"[19]. It is beneficial for a number of reasons to combine biosensor designs with microfluidic circuits to improve the overall performance of the sensing system. The main motivation here is certainly to improve transport of analyte from the sample volume to the biorecognition element, in particular for surface-bound sensing elements. The reduced dimensions and volumes in microfluidic channels allow first of all to work with much less sample than what otherwise might be used, making analysis on drops of blood or even the contents of single cells possible. But, more importantly, the reduced distances for analyte molecules to diffuse to the biorecognition elements immediately yield a great gain in response time, significantly improving conditions for diffusion-limited processes. Moreover, specially designed channels can further improve convective transport to the sensor surfaces by, e.g., including flow focusing or helical flows [20]. Expanding from pure microfluidics to more fully developed lab-on-a-chip solutions, entire sample preparation procedures such as separation, enrichment, and labeling, to name but a few, can be incorporated prior to the sensing, . All of these pre-sensing steps can improve the actual detection step by removing interfering species or by increasing the concentration of analyte in the detection volume. When searching the literature, one finds indeed a wealth of papers that utilize microfluidics in order to transport sample containing the analyte(s) of interest, but it is somewhat surprising that most of the realizations are very basic, often only comprising a single channel with an inlet and an outlet and thus allowing not much more than to flush sample across the sensor surface, or to bring two or more reagents together. Only rarely do we find more advanced solutions or systems where several (other) functionalities are integrated to come closer to the goals of the lab-

on-a-chip philosophy. The reasons for sticking with a simple microfluidic cell can be manifold and differ from case to case, ranging from putting an emphasis on the biosensor design to a deliberate reduction of complexity and thus sacrificing sophistication for the sake of reliable operation. However, it is dangerous to view the microfluidic channel (and its design) as a separate part, while it is in fact an integral part of what makes the entire biosensing system work as intended. Some of the most important design considerations for combining (planar) sensors with microfluidic systems are laid out and explained in an article by Squires and coworkers [21]. Here, the interplay between channel dimensions, sensor dimensions, flow velocities, diffusional transport, advective transport and reaction rate are explored and the consequences of a bad design on the overall performance of the sensor system are investigated. Undoubtedly, there are a number of examples in the literature where microfluidic (bio) sensors underperform on account of a flawed design or because they are operated under sub-optimum conditions. Serendipitous cases of sensor designs performing better than expected are further indications that a better theoretical understanding and improved models are still lacking.

For this review article, we have collected examples from the literature describing immunosensors utilizing a biorecognition element according to the definition given above. We have attempted to achieve a rough structure by sorting the described systems according to the used transduction principle, as these are just slightly less numerous and less diverse than the number of biorecognition elements exploited and investigated. The latter include, among others, enzymes, antibodies, DNA strands, aptamers, peptides, receptors, bacteria and other cells, and either direct or indirect reactions and interactions of these with the analytes of interest. The complexity of the matter combined with the

ingenuity of the researchers in finding novel approaches makes it almost impossible to avoid inclusion of "borderline" cases on the one hand, and missing some papers entirely, on the other hand. There are, in particular, many cases where an enzyme is used to turn over a substrate and where the product of this enzymatic reaction is then detected. Such instances are clearly in a "greyzone" when held against the definition mentioned earlier, especially when the enzyme is added like a reagent and not really part of the sensor as such. We hope that the readers will find this review nonetheless a great resource and a starting point for further explorations.

Microfluidics is a technology that facilitates the manipulation of small volumes of fluids in the range of mL to aL (10^{-6} to 10^{-18} liters). Microfluidics is most often implemented in planar substrates bearing enclosed channels with lengths, widths, and depths on the B10 mm, B100 mm, and B10 mm scales, respectively. The technology was popularized in the early 1990s [22] for applications related to chemical separations, but in the intervening years, it has been applied to an incredible array of applications, ranging from genomics [23]and synthesis [24]to music [25]and mazes [26]. A particularly attractive vision for the microfluidics community has been the development of integrated ''lab on a chip'' systems that reproduce laboratory-scale processes with reduced cost, less time, and with substantially smaller footprints than their conventional counterparts [27].

Microfluidic platforms can be classified into a number of different categories, including (**i**) channel-based microfluidics, (**ii**) paper-based microfluidics and (**iii**) digital microfluidics. There are many alternative classifications (*e.g.*, one might include a Slip Chip 48 category, or split a ''droplet microfluidics'' category out from ''channel-based microfluidics''), but these categories suffice for the purposes of this review.

Based on our previous review articles [27-36] and with the selected latest research articles from 2012 to May 2016, we summarized various graphene-based electrochemical immune-devices comprehensively in this review. More importantly, we discussed several outstanding properties of the immune-devices and their research opportunities as well as the development potential and prospects. Therefore, this review describes fabrication and chemical modification of the surfaces of 2-D graphene for immunesensing applications. We also present a comprehensive overview of current developments and key issues in the determination of some biological/pathogenic molecules with particular emphasis on evaluating the methods. This review shows how 2-D graphene has made significant contributions in the development of electrochemical immune-devices. In addition, different aspects of the electrochemical immune-devices such as type of nanomaterials, detection techniques, analytes and the corresponding sensitivity and sample matrix, as well as several noticeably prominent characteristics have been discussed in detail. Accordingly, research opportunities and future development trends in these areas are discussed.

2. Types of application

Electrochemical immuno-devices

There is continuing demand for fast, simple analytical methods for the determination of many clinical, biochemical and environmental analytes. In this respect, immunoassays and immunosensors that rely on antibody-antigen interactions provide a promising means of analysis due to their specificity and sensitivity. Electrochemical immunosensors and immunoassays have recently attracted considerable interest because of their high sensitivity, low cost, and inherent small size [14, 28]. For immunosensors, the

immunologic materials are immobilized on an electrochemical transducer. The emergence of nanotechnology is opening up new horizons for the use of nanomaterial labels for signal amplification in electrochemical immunosensors [34]. Electrochemical-affinity sensors based on antibodies offer great selectivity and sensitivity for early cancer diagnosis. A range of electrochemical transducers includes amperometric, potentiometric and impedimetric or conductivity devices. Amperometric and potentiometric transducers have been used most commonly, but much attention in recent years was devoted to impedance-based transducers, since they are classified as label free detection sensors [31, 32]. Recently, there has been growing interest in the use of electrochemical immune-devices for clinical diagnosis [37]. Electrochemical methods have the advantage of being highly sensitive, rapid, simple to handle and inexpensive compared to the other methods. For immune-devices based on electrochemical methods, it is also possible to miniaturize the electrode for field applications [38]. Electrochemical immunosensors utilize various types of electrode [*e.g.*, as screen-printed carbon electrodes (SPCEs), carbon-paste electrodes (CPEs) and glassy-carbon electrodes (GCEs)]. The antibodies were immobilized by physical adsorption or chemical immobilization. These types of electrodes can be used only once.

With the selected latest research articles from 2012 to May 2016, we summarized various grapheme-based electrochemical immune-devices for detection of different biomarkers in this review. More importantly, we discussed in detail different aspects such as type of nanomaterials, injection and detection techniques, labels, analytes and the corresponding sample matrix, and sensitivity. Consequently, we discussed several outstanding properties of the nano-immunosensors and their research opportunities as well as the development

potential and prospects. Also, we summarized below examples of nanostructured material applications in electrochemical immunesensing biomarkers in whole blood reported so far in the literature, with their advantages and limitations, and their potential for future development in this field was stressed.

The development of microfluidic or paper-based electrochemical immune-devices offers great opportunities for rapid detection, separation of interfering agents and effective measurement. Recently, graphene-based microfluidic immunosensors for the detection of biomarkers has become a promising area for point-of-care monitoring and diagnostics. The integration of signal amplification strategy with a microfluidic immunosensor was developed for the multiplexed measurement of cancer biomarkers in serum samples from patients [39]. The device was divided into eight individual lines modified by graphene nanomaterials, and each was extremely sensitive to the specific analyte. Silica NPs were used as probes to label the signal antibodies. A positive identification was indicated by the electrochemical current signal and was visually compared with the on-chip reference. Various cancer biomarkers in human samples can be detected due to reaction variability and product stability. Because ambient conditions have tremendous influences on accurate analysis, many attempts have been made to design calibration standards and new amplification protocols without optimal conditions. Wu and coworkers [40]improved their graphene-based microfluidic immune-device by triggering a controlled amplification-by-polymerization on the immune-device surface and achieved a self-calibrating system. This novel signal amplification strategy was claimed to enhance electrochemical signal output significantly to minimize ambient condition impacts and achieve ultrasensitive detection. Another notable study reported that vertically aligned

ZnO nanowire arrays were fabricated on a graphene foam-modified electrode in one microfluidic system to selectively detect the biomarkers of Parkinson's disease [41]. According to the results of this research, an extremely low detection limit for each analyte was achieved by the modified electrode because of its high surface area and high selectivity. Also, the good stability was explained by the high protective ability for the occupied biomolecules of the graphene foam-modified electrode. These studies demonstrated that graphene materials would greatly accelerate research on developing immunoassays for sensitive detection of biomarkers.

In addition, Lu and co-workers [42]prepared an NP-based paper microfluidic biosensor in detail, as shown in scheme. 1A. As describe by this chinese researcher, a paper sheet was first patterned with a wax printing, in which every rectangle space was penetrated with wax as a hydrophobic barrier, except the central circular portion (marked A and B) that was reserved as the sensing interface (Sheet A). Then, carbon ink was printed onto area A as working electrode (WE) while carbon ink and Ag/AgCl ink were printed onto area B as counter electrode and reference electrode, respectively (Sheet B). The treated Sheet B was cut in the shape of Sheet C, which could be folded along the middle line to form three-electrode systems. Finally, the three-electrode system was inserted into a transparent device holder that comprised two circuit boards (Board A and Board B) with conductive pads on them to form a 3-D paper-based EC microcell, in which the wire on the device holder was used to connect the EC workstation. In this paper, an amino-functionalized graphene nanosheet (NH_2-GN) and AuNPs were deposited in order on the surface of the WE, followed by the addition of the DNA capture probe (S1) to form the S1/AuNP/GN/WE sensing interface. This nanocomposite-modified paper biosensor was

used to detect target DNA (S2) using a traditional sandwich DNA hybridization reaction, where detection DNA (S3) and signal reporter thionine (TH) were co-immobilized on nanoporous gold (NPG) to form S3-TH/NPG complexes. In the presence of S2, the DNA hybridization reaction (S3-TH/NPG-S2-S1/AuNPs/GN/WE) was completed. Most importantly, with the contribution of NPs, the EC signal could be greatly amplified and this DNA paper biosensor exhibited excellent sensitivity and good linear relationship in the range 8×10^{-16} to 5×10^{-10} mmol/L with an LOD of 8×10^{-16} mmol/L.

Also, Wu and coworkers [39]prepared a paper microfluidic biosensor, in which multiplex WEs were established on the pre-printed area of paper and all of them employed the same counter electrode and reference electrode. In this multiplex electrode system, four different cancer biomarkers were immune-detected at the same time with the incubation steps similar to those in traditional immunoreaction. As displayed in scheme. 1B, the mixed solution of graphene oxide (GO) and chitosan was first dropped onto the WEs, followed by EC reduction to form a more conductive reduced GO (RGO) interface. Then, the modified electrodes were incubated with glutaraldehyde to bind different capture antibodies onto the specific WE. In this report, Wu and coworkers applied SiO_2 NPs as carriers to co-immobilize detection antibodies and HRP molecules, which greatly amplified the EC signal.

Also, Pt-Ag alloy nanoparticles were used as signal amplifier for the highly sensitive determination of carcinoembryonic antigen in human serum samples. In this work, Bossi and coworkers [43]introduced an electroluminescence immune-device into a folding cellulose fiber paper based on gold/graphene modified screen-printed electrode (SPE) to develop an electroluminescence microfluidic origami immunodevice. Graphene was used

to modify the working electrode for its fast electron transportation, excellent mechanical stiffness and good biocompatibility. A dense gold layer was formed for the first time through the growth of gold nanoparticles on the graphene layer, to further enhance the sensitivity, stability and effective surface area of the SPE. On this as-prepared SPE, phenyleneethynylene derivative modified nanotubular mesoporous Pt-Ag alloy nanoparticles were used as signal amplifier for the highly sensitive determination of carcinoembryonic antigen in human serum sample with a detection limit of 0.3 pg/mL [43] .

Interestingly, Wu and coworkers [39]reported the combination of a signal amplification strategy with a microfluidic paper-based analytical device for the quantitative analysis of four kinds of cancer biomarkers as model analytes, namely, alpha-fetoprotein, carcinoembryonic antigen, cancer antigen 125, and carbohydrate antigen 153. Signal amplification was achieved through graphene modification of the immunodevice surface to accelerate the electron transfer and also the use of silica NPs as a tracing tag to label the signal antibodies. Using the horseradish peroxidase-O-phenylenediamine-H_2O_2 electrochemical detection system, the potential clinical applicability of this biosensor was demonstrated in the detection of four candidate cancer biomarkers in serum samples from cancer patients.

Detection of biomolecules with the aid of electronic devices such as FET, where the high conductivity (also large surface area) of graphene can be utilized, can supplement the electrochemical sensing of biomolecules. In this method, high sensitivity can be achieved down to picomolar concentrations (pM). Further, the other recent detection strategies such as optical, plasmonic, nanomechanical and microfluidic technologies [7, 44, 45] are

employed on graphene-based detection systems. Recently, gold nanoparticles have been integrated with various graphene-based FET sensors [45-47]. Scheme 2 is the schematic of a typical sensitive and selective FET biosensor using vertically oriented graphene labeled with gold nanoparticles-antibody conjugates. The vertical morphology of graphene device facilitates the deposition of gold nanoparticle-antibody conjugates on the sensor while still ensuring that the entire surface area is accessible to the analyte molecules. This new morphology of graphene allows the development of graphene based protein sensors with a high sensitivity of 2 ng/mL or 13 pM and selectivity to specific proteins (analyte protein complex, immunoglobulin (IgG)).

Some researches on the preparation and application of a glucose enzyme biosensor based on novel graphene materials have been reported. For example, Rattanarat and coworkers [48] described a novel centrifuge based microfluidic device coupled with an electrochemical detector using graphene-polyaniline nanocomposite modified electrode as working electrode for the determination of glucose in control human serum. Especially, microfluidics is highlighted here, it is a multidisciplinary field intersecting engineering, physics, chemistry, biochemistry, nanotechnology, and biotechnology, with practical applications to the design of systems in which low volumes of fluids are processed to achieve multiplexing, automation, and high-throughput screening [49]. Typically, micro means one of the following features including small volumes (μL, nL, pL, fL), small size, low energy consumption and effects of the micro domain. Therefore, combing with the good performances of graphene electrode, microfluidic devices will show excellent properties including low-cost, little sample, small size, fast response and

high sensitivity, and will have bright application prospect in electrochemical sensing [50].

More recently, hybrid polymer/graphene-based electrodes were end-channel coupled to the microfluidic system to improve the analytical performance [51]. D-Met and D-Leu were successfully detected becoming this proof-of-the-concept a promising principle for the development of point of- care (POC) devices for in situ screening of *V. cholerae* related diseases.

Also, Lucca and coworkers [52] describes the development and application of a novel graphene modified electrode to be used as amperometric sensor in microchip electrophoresis devices. The performance of the modified electrode for the amperometric detection on microchip electro devices has been demonstrated by the separation and detection of an anionic mixture containing iodide and ascorbate. The graphene-modified electrode provided significantly higher sensitivity (896.7 *vs.* 210.9 pA μmol/L for iodide and 217.8 vs. 127.8 pA μmol/L for ascorbate), better separation efficiencies (3,400 *vs.* 700 plates m^{-1} for iodide and 10,000 *vs.* 2,400 plates m^{-1} for ascorbate), enhanced peak resolutions (1.6 *vs.* 1.0) and lower limits of detection (1.5 *vs.* 5.3 μM for iodide and 3.1 *vs.* 7.3 μM for ascorbate) in comparison with the unmodified Pt electrode.

Also, Indian researchers transferred chemical vapor deposition (CVD) graphene onto paper without any intermediate layers to yield G-paper [53]. Resistive gas sensors have been fabricated using strips of G-paper. These sensors achieved a remarkable lower limit of detection of ~300 parts per trillion (ppt) for NO_2 which is comparable to or better than those from other paper based sensors. Ultraviolet exposure was found to dramatically

reduce the recovery time and improve response times. G-paper sensors are also found to be robust against minor strain, which was also found to increase sensitivity.

Recently, a novel flow-through microfluidic device based on magneto-controlled graphene sensing platform has been designed for homogeneous electronic monitoring of pyrophosphatase (PPase) activity, coupling with enzymatic hydrolysate-induced release of inorganic copper ion (Cu^{2+}) from Cu^{2+}-coordinated pyrophosphate ions (Cu^{2+}-PPi) complex[54]. Magnetic graphene nanosheets (MGNS) functionalized with negatively charged Nafion were synthesized by using the wet-chemistry method. The Cu^{2+}-PPi complexes were prepared on the basis of the coordination reaction between copper ion and inorganic pyrophosphate ions. Upon target PPase introduction into the detection system, the analyte initially hydrolyzed pyrophosphate ions into phosphate ions, and released the electroactive copper ions from Cu^{2+}-PPi complexes. The released copper ions could be readily captured through the negatively charged Nafion on the magnetic graphene nanosheets, which could be quantitatively monitored by using the stripping voltammetry on the flow-through detection cell with an external magnet. Under optimal conditions, the obtained electrochemical signal exhibited a high dependence on PPase activity within a dynamic range from 0.1 to 20 mU mL^{-1}, and allowed the detection at a concentration as low as 0.05 mU mL^{-1}. Coefficients of variation for reproducibility of the intra-assay and inter-assay were below 7.6 and 9.8%, respectively. The inhibition efficiency of sodium fluoride (NaF) also received good results in pyrophosphatase inhibitor screening research. In addition, the methodology afforded good specificity and selectivity, simplification, and low cost without the need of sample separations and

multiple washing steps, thus representing a user-friendly protocol for practical utilization in quantitative PPase activity.

Interestingly, a three-dimensional (3D) graphene incorporated electrochemical sensor was constructed for sensitive enzyme based phenol detection [55]. In this work, polydimethylsiloxane (PDMS) micropillars were fabricated in the microchannel by using a conventional photolithography and the surface was modified with 3-aminopropyltriethoxysilane. Then, the negatively charged graphene oxide sheets were electrostatically adsorbed on the PDMS micropillar surface, and reduced in the hydrazine vapor. The resultant 3D graphene film provides a conductive working electrode as well as an enzyme-mediated sensor with a large surface area [Scheme 3]. After bonded with an electrode patterned glass wafer, the graphene based electrochemical sensor was produced [Scheme 4]. To utilize the graphene as an enzyme sensor, tyrosinase enzymes were immobilized on the surface of the graphene micropillar, and the target phenol was injected in the microchannel. The enzyme catalytic reaction process was monitored by amperometric responses and the limit of detection for phenol was obtained as 50 nM, thereby suggesting that the graphene micropillar structure enhances the enzyme biosensing capability not only by increasing the surface area for enzyme immobilization, but also by the superlative graphene conductivity property.

In addition, a new type of high-throughput and parallel optical sensing platform with a single-color probe based on microfluidic chip electrophoresis combined with aptamer-carboxyfluorescein/graphene oxide energy transfer was reported by Lin and coworkers [56]. According to this report, FRET process involved two basic steps in this proposed system. First, fluorescein-based dye (FAM)-labelled ssDNA of aptamers assembled non-

covalently onto GO. Due to the strong interaction between the aptamers and GO, the fluorescence of the labelled FAM was quenched by FRET between GO and FAM. Second, upon the introduction of proteins, the specific binding between the aptamer and its corresponding protein target resulted in a change of the interaction between the aptamer and GO, and then the release of the ssDNA probe from the surface of GO. As a result, the fluorescence of the FAM-labelled aptamer was restored. Otherwise, there was no recovery of the fluorescence without the target protein. The schematic diagram of chip-CE assisted biosensor for the simultaneous detection of protein is given in Scheme 5. Label-free protein multi-targets were detected, even in challenging complex samples without any pre-treatment.

From this report it's found that, there are three striking characteristics: (**i**) there was no immobilization involved, unlike in the strategy of sensor array biochips; (**ii**) there was no sample pre-treatment or pre-labeling involved; (**iii**) no multicolor DNA probes are required. This methodology should in theory have wide applicability because, with sufficient specific sensing elements, the number of targets would be considerable as long as the separation resolution allows. Secondly, a high detection sensitivity with low background noise was ensured by the super-quenching ability of GO. Furthermore, ratiometric imaging is also expected to increase the signal-to-background ratio, and thus afford a greater sensitivity in fluorescent digital imaging. Meanwhile, the irreplaceable advantage of integration makes the micro-fluidic chip a good candidate to realize high-resolution separation with further improved sensitivity detection. The lowest detection limit of our method and how to increase the number of targets detected are now under way with further experiment optimization. Third, one emphasis in bioassays nowadays is

the move to methods that are quick, simple, use a small sample size and portable equipment. Micro- and nano-fluidic devices are becoming the focus of investigations. The unique characteristics of speed, high separation performance, low-volume sample consumption and simple operation bestowed by the chip, combined with the design flexibility of recognition biosensors, promise great potential capacity for any high-throughput recognition in real complex-sample assays.

At the previous year, Tuteja and coworkers [57]report lithium ion intercalation mediated efficient exfoliation of graphite to form monolithic graphene sheets which have subsequently been investigated for the development of highly sensitive label-free electrochemical detection platform for cardiac biomarker, Troponin I (cTnI). The spectroscopic and morphological analysis demonstrated the formation of defect free graphene sheets which were successfully employed to fabricate an inter-digited micro-device in a drain-source configuration on a silicon biochip. In this report, the functionalized carboxylated graphene, as synthesized by Li^+ intercalation process was dropcasted across the interaction area of the microfabricated gold electrodes (Scheme 6). The pure graphene showed ohmic behavior as expected. The graphene gated biochip functionalized with anti-cTnI antibodies used in label free detection of cTnI which exhibited an excellent sensitivity in the picogram range ($\sim$1 pg mL^{-1}) for cTnI without the use of any enzymatic amplification that promises its potential applicability for bio-molecular detection in clinical diagnosis.

From this report it's found that, this novel synthesis route of functionalized graphene with minimal surface defects has successfully been demonstrated first time in the resistive/DCT transduction mode for enzyme-free “point-of-care” immunosensing of

highly charged cTnI protein with high sensitivity in the picogram range. The ability to detect such low concentrations using the reported sensor would find potential applications in routine monitoring of cTnI in blood samples.

In addition, a novel electrochemiluminescence (ECL) DNA sensor based on graphene-modified porous Au-paper working electrode (GR/Au-PWE) and calcium carbonate/carboxymethyl chitosan ($CaCO_3$/CMC) hybrid microspheres@luminescent silver nanoparticles (AgNPs) composites was developed by Li and coworkers [58]. In this work, Complementary ssDNA sequence was covalently bound to AgNPs on the surface of $CaCO_3$/CMC hybrid microspheres. Enhanced sensitivity could be obtained by the increase of AgNPs loading per DNA sensor. The $CaCO_3$/CMC@AgNPs labels were brought to the surface of the PDDA-GR/Au-PWE through subsequent sandwich DNA hybridization. The $CaCO_3$/CMC@AgNPs composites exhibited 3.6 times higher ECL intensity than the pure AgNPs-labeled reporter DNA. Taking advantage of dual-amplification effects, the paper-based DNA sensor could detect the target DNA quantitatively, in the range of 4.0×10^{-17} to 5.0×10^{-11} M, with a limit of detection as low as 8.5×10^{-18} M, and perform excellent selectivity. The simple, low-cost, sensitive device could be easily applied for point-of-care testing, public health and environmental monitoring in remote regions, developing or developed countries.

From this report, it's found that the μ-PAD DNA sensor exhibited excellent analytical performance and had acceptable application potential in human serum assay. The proposed method provided a promising platform for accurate gene diagnostics at home and in the field.

Also, a microfluidic device was fabricated incorporating the SPE sensor for real-time glucose detection in human urine samples [59]. The excellent sensing performance, operational characteristics, ease of fabrication, and low cost bode well for this electrochemical microfluidic device to be developed as a point-of-care healthcare monitoring unit. In this report, the mold for PDMS chamber and the device holder were prepared using acrylonitrile butadiene styrene (ABS) plastic. A 10:1 mixture of Sylgard 184 (Dow Corning Co.) was poured on top of the mold and peeled off after curing for three days at room temperature. Two holes were drilled in the PDMS chamber to accept inlet and outlet tubes (760 μm OD, 250 μm ID, Cole-Parmer Co.). The detailed device setup is shown in Scheme 7. The PDMS chamber was placed on top of the sensor and fixed by the ABS holder. The microfluidic chamber embedded in PDMS chamber was carefully aligned with the active area on the modified SPE sensor. One of the two holders has square or circular shape through the hole so that the inlet and outlet tubes can be connected to the PDMS chamber. The whole device stack was firmly held by Quick Grip® Micro Bar Clamp and Spreader (American Tool Inc.) to prevent any possible leaking from the chamber. The inlet tube was connected to a 10-mL microsyringe for sample injection; the inlet tube can also be connected to a pump, micropipettor or dropper, as needed. Samples could be injected manually at a slow and steady rate or by an adjustable syringe pump. Based on the dimensions of the PDMS chamber (7 mm diameter, 0.2 mm height), the calculated sample volume required for detection using this device is 7.7 μL. After measurements, the used SPE can be easily removed and replaced with a fresh one.

From this work, it was found that, a simple, rapid, and convenient one-step approach was used to synthesize chitosan-reduced graphene oxide-nickel nanoparticles from the self-assembled nanocomposite precursor solution on SPEs, initiated simultaneously by pH-responsive electrodeposition of CS, electrochemical reduction (and deposition) of soluble GO into insoluble RGO as well as reduction of metal precursors into metal NPs. Deposited GO was electrochemically reduced *in situ* without any pre- or post-reduction steps. Uniformly distributed and interconnected NiNPs with relatively homogenous particle sizes could be observed in the CS and wrinkled ERGO scaffold. The resulting nanocomposites exhibited fast electron transfer kinetics and high electrocatalytic activity against glucose, with good sensitivity, selectivity and stability. A pocket-size, point-of-care electrochemical microfluidic sensing device was fabricated using PDMS molding and ABS plastic milling to incorporate the as-prepared disposable SPEs for real-time glucose monitoring in small sample volumes. Results of glucose measurements in human urine samples using the device were correlated well with those obtained by HPLC-electrochemical instrument. The simple material synthesis, enzyme-free nature, portability, affordable cost and strong sensing performance of the reported device offers out-of-lab and beaker-free glucose sensing in healthcare.

In the current year, some of reports was published which is necessary for discussion. Recently, a nano-interfaced microfluidic exosome (nano-IMEX) platform based on a unique coating of graphene oxide (GO) and polydopamine (PDA) for ultrasensitive exosome detection (Scheme 8) was reported by Zhang and coworkers [60]. These researchers adapted the method of the mussel-inspired self-polymerization of dopamine because of the following reasons: it provides a very simple surface coating method

applicable to virtually any material; its amine and catechol functional groups ease surface modification and bioconjugation; its highly hydrophilic PDA coating possesses excellent biocompatibility and resistance to biofouling; and lastly, the kinetics of PDA coating can be well controlled by tuning the reaction conditions such as pH, temperature, choice of oxidants and incubation time. However, most existing PDA coating methods are slow and require tens of hours to produce relatively thick surface coatings. Compared to these methods, this microfluidic coating approach markedly expedites the PDA deposition kinetics, which could promote the greater application of this promising coating material.

From this report it's found that, that the nano-interface greatly enhances the immuno-isolation efficiency, while at the same time effectively suppressing the effects of non-specific exosome adsorption. This novel interface enables the development of an ultrasensitive and specific ELISA assay for molecular analysis of exosomes. According to finding of researchers of this report, the applications of this nano-IMEX platform in molecular profiling and in the quantitative detection of exosomes purified from a colon cancer cell line or directly in plasma samples from ovarian cancer patients were demonstrated. The chip is scalable for the multiplexed analysis of exosomes and for the high-throughput screening of clinical samples. Therefore, this platform should provide a useful tool to facilitate exosome research and the clinical utilization of exosomes for disease detection and treatment.

More recently, a highly sensitive label-free paper-based electrochemical immunosensor employing screen-printed working electrode (SPWE) for detection of carcinoembryonic antigen (CEA) was fabricated [61]. In order to raise the detection sensitivity and immobilize anti-CEA, amino functional graphene (NH_2-G)/thionine (Thi)/gold

nanoparticles (AuNPs) nanocomposites were synthesized and coated on SPWE. The principle of the immunosensor determination was based on the fact that the decreased response currents of Thi were proportional to the concentrations of corresponding antigens due to the formation of antibody–antigen immunocomplex. Experimental results revealed that the immunoassay enabled the determination of standard CEA solutions with linear working ranges of 50 pg/mL to 500 ng/mL, the limit of detections for CEA is 10 pg/mL and its corresponding correlation coefficients were 0.996. Furthermore, the proposed immunosensor could be used for the determination of clinical serum samples. A large number of clinical serum samples were detected and the relative errors between measured values and reference concentrations were calculated. Results showed that this novel paper-based electrochemical immunosensor could provide a new platform for low cost, sensitive, specific, and point-of-care diagnosis in cancer detection.

3. Concluding Remarks and Outlooks

This review presents an overview on recent advances in the development and the application of 2-D graphene-based nanomaterials toward construction of microfluidic bioelectronics immune-devices. Such devices are extremely useful for delivering clinically relevant information in a simple, fast- and low-cost fashion, and are thus uniquely qualified for meeting the demands of POC testing. One of the challenges is to avoid non-specific adsorption which causes false response errors and consequent decreased sensitivity. Another issue to be addressed is the integration and automation of the technology as well as development of appropriate sample preparation methods. Moreover, successful development of POC systems will require continued improvement and validation of biomarkers and development of bio-receptors for those biomarkers. In

short, while there is still a long way to go for POC testing, microfluidic biosensors will eventually become one of the strongest candidates for a real-world tool.

Although much progress has been made towards the application of 2-D graphene-based nanomaterials in the bioelectronics immune-devices since the conception of microfluidics, the current state-of-the-art technologies in this field are not yet capable of being employed for field or point-of-use applications; in contrast, microfluidics developed in academic labs over the years made tangible contributions to basic sciences. As the health care costs continue to escalate, inexpensive, reliable, and easy-to-use devices are likely the next-generation tools that biomedical diagnostic industries and field users anticipate. We believe academic research in microfluidics should continue to push the frontiers of the development into new materials, processes, and functionalities; furthermore, research should be directed towards standardization, automation, and high throughput.

Significant progress has been made during past years; however, the research on 2-D graphene-based bioelectronics microfluidics devices is still at an early stage. More efforts will be needed in this field to help it become a more matured platform technology in diagnostic and point-of-care applications. Despite many potential future directions in this research, here, we hope to convey to the reader just a few of the perspective directions that we think are relevant and attractive in this field.

As it is presented in this survey, the usage of 2-D graphene-based microfluidic devices for diagnostic purposes is in the center of interest of many researchers in the world. It is obvious that 2-D graphene-based microfluidic devices cannot replace sophisticated laboratory equipment, including spectrometers or chromatographs, which are used in

laboratories around the world. However, in specific situations, especially in developing countries, these devices may be a very good alternative, when access to hi-tech equipment is very limited. This becomes even more real, when such methodology will be included in the scope of telemedicine services. Under such conditions, 2-D graphene-based microfluidic bio-devices can be instantly transferred thousands miles away from analysis place to specialized laboratory. Due to their enormous advantages, microfluidic-based electro-analytical devices can play vital roles in improving diagnostics and treatment of various immuno-diseases in resource-limited areas of the world, which are financially incapable of having advanced technologies. A low-cost electrochemical microfluidic biochip can fulfill the need of people in developing and poor regions of the world, which lack facility of proper infrastructure and trained healthcare professionals to the site of patients.

Finally, it is important to point out; the use of 2-D graphene-based nanomaterials for construction of electrochemical microfluidic immuno-devices is still in its infancy. Due to the minority of research being on the development of new microfluidic for immunesensing, more electrochemical techniques should be involved in this area. With 2-D graphene-based nanomaterials, it will be possible for electrochemical immune devices to be applied to pre-warning and real-time detection of diseases.

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Figure Legends

Scheme 1. (A) Fabrication of paper biosensor and (B) paper-based biosensing device for detection of analytes [39].

Scheme 2. (a) Differential Pulse Voltamogram of (i) 1.0 mM AA, (ii) 1.0 mM ST, (iii) 1.0 mM DA, and (iv) 1.0 mM AA+1.0 mM ST+0.1 mM DA (simultaneous detection) at graphene electrodes. (**b**) (top) Schematic of the vertically aligned graphene based FET sensor by direct growth of graphene between the drain and the source electrodes. Probe antibody is labeled to the vertically aligned graphene (VG) surface through Au NPs. (Bottom) Transistor measurement results of the VG sensor modified with Au NP-anti-IgG conjugates, blocking buffer (BB), and IgG (2 ng/mL) [46].

Scheme 3. (a) Detailed design and dimension of the microchannel in the PDMS layer and the patterned electrodes in the glass substrate (unit: μm). (b) Illustration of a 3D graphene micropillar incorporated electrochemical sensor device. The microfluidic channel was patterned in the PDMS layer for sample loading, and the graphene micropillar was fabricated in the middle of the microchannel. The working (W), counter (C) and reference (R) electrodes were deposited on a glass substrate. C 1s XPS spectra of (c) the GO on the PDMS micropillars and (d) the reduced GO on the PDMS micropillars. [55]

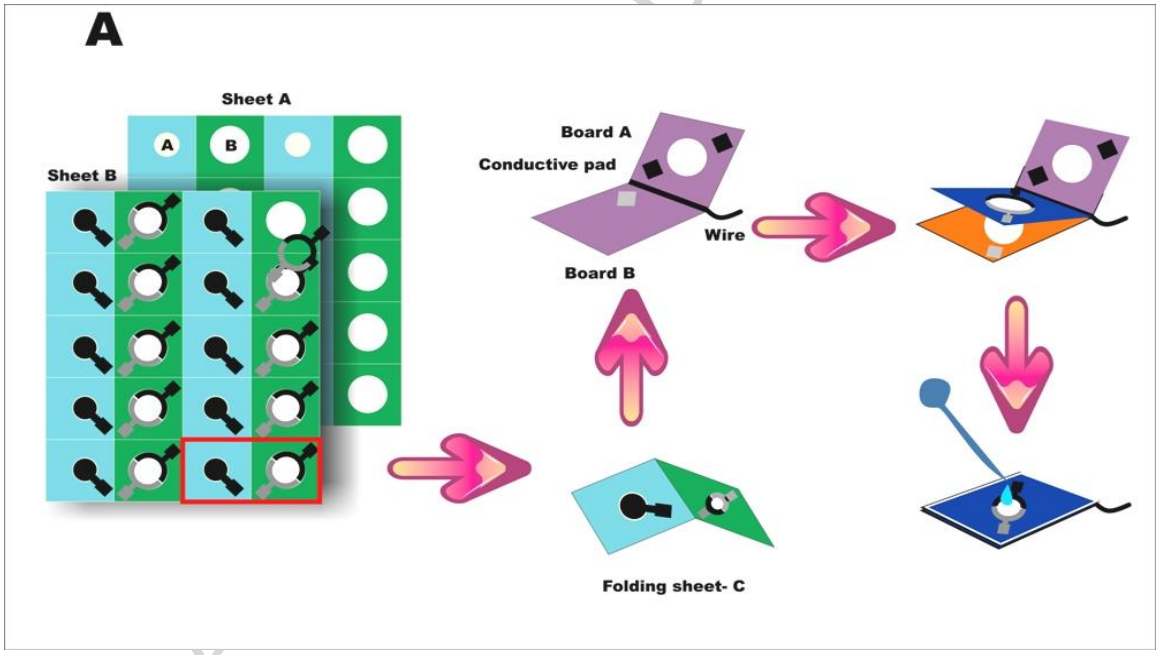
Scheme 4. (a, b) Low- and (c) high-magnification SEM images of the graphene micropillars. (d) The PDMS micropillars uncovered with graphene are shown bright due to the charging effect. (e) Digital image of the graphene micropillar integrated electrochemical sensor device for phenol detection. [55]

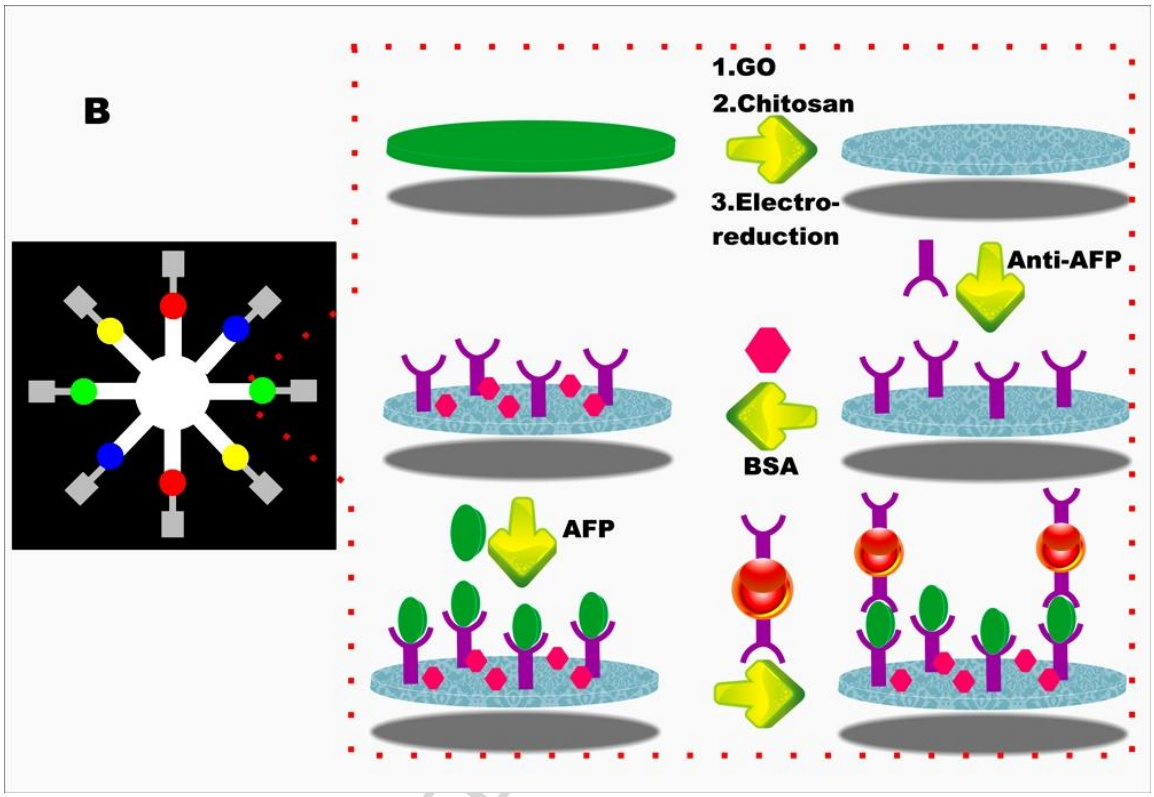
Scheme 5. The schematic diagram of chip-CE-assisted biosensor for the simultaneous detection of protein [56]).

Scheme 6. Micro-fabricated graphene gated biochip. Electrodes were fabricated by standard lithography procedure on a silicon chip electrode. Inset shows the SEM image of fabricated electrodes[57].

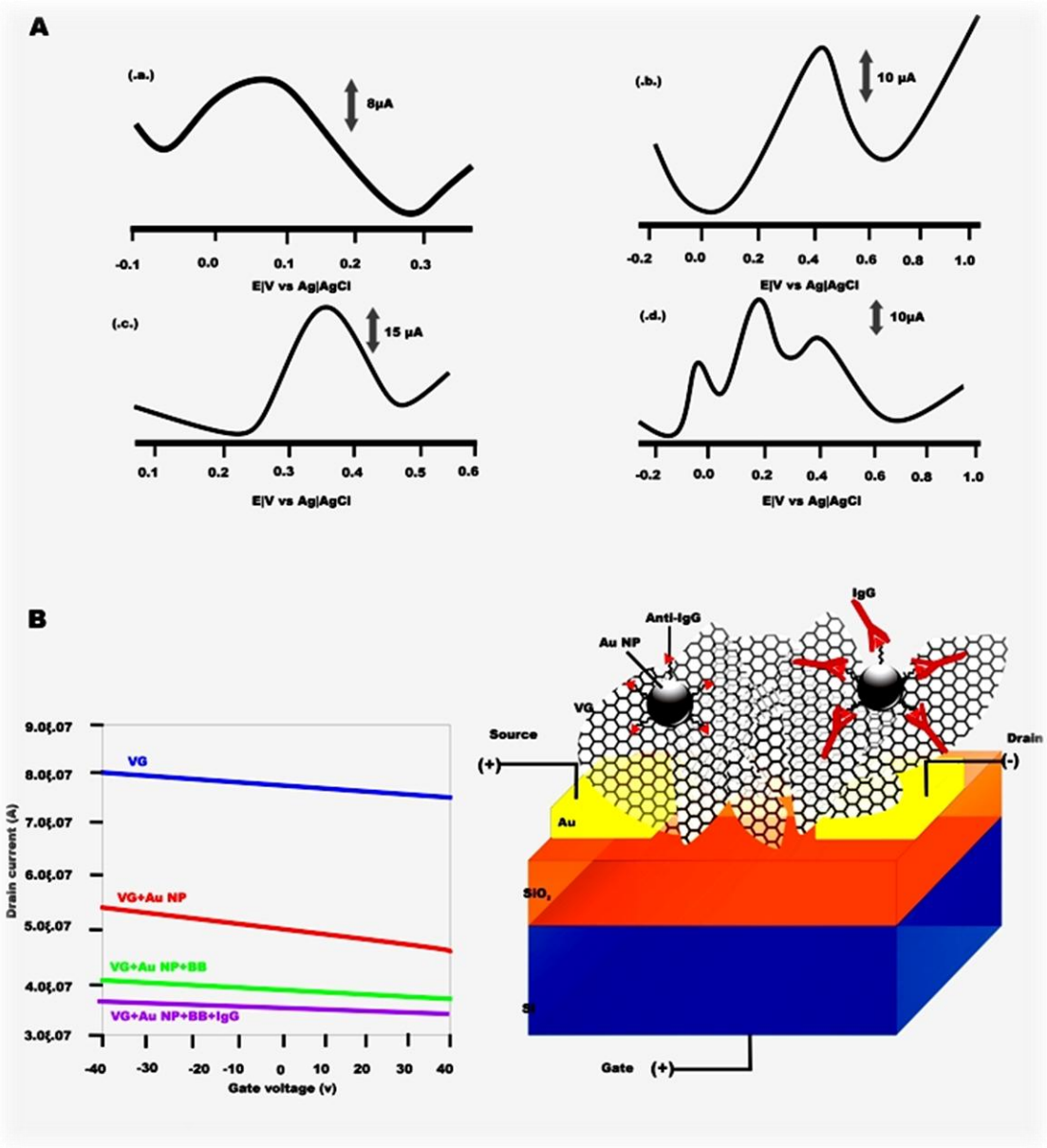
Scheme 7. (a) Schematic of the device stack setup. **(b)** Photograph of the as-fabricated microfluidic electrochemical device for sensing Glc. (1) PDMS chamber (inset in (b) shows an enlarged view) (2) CS-RGO-NiNPs modified SPE (3) top piece of plastic device holder (4) bottom piece of plastic device holder (5) outlet tubing (6) inlet tubing connected to a sample injector (syringe or pump) (7) Micro bar clamp/spreader (8) holes for connecting inlet and outlet tubes (9) circular chamber fit to the sensing area[59].

Scheme 8: The nano-interfaced microfluidic exosome platform (nano- IMEX). (A) Schematic of a single-channel PDMS/glass device, with the exploded-view highlighting the coated PDMS chip containing an array of Y-shaped microposts. (B) Surface of the channel and microposts coated with graphene oxide (GO) and polydopamine (PDA) as a nanostructured interface for the sandwich ELISA of exosomes with enzymatic fluorescence signal amplification. (C) The procedure for surface functionalization of the microfluidic chips [60].

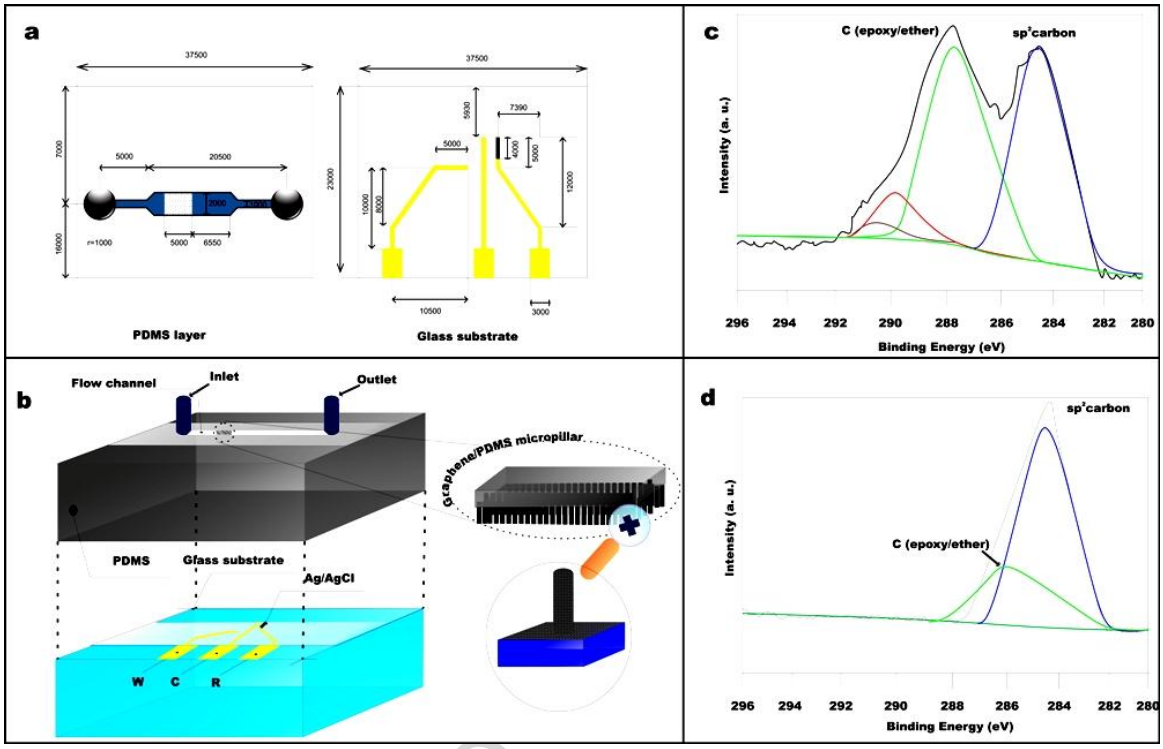




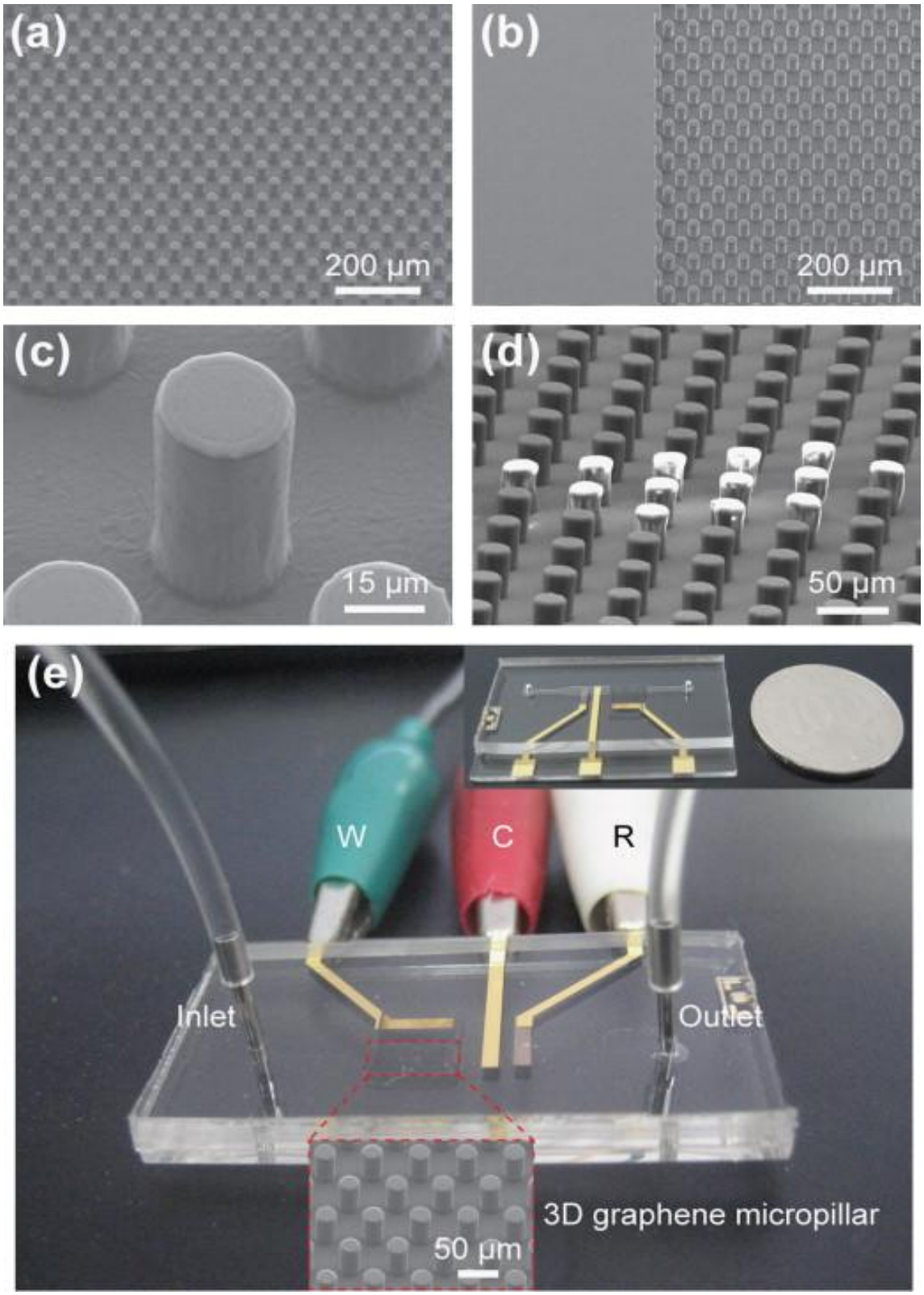
Scheme 1



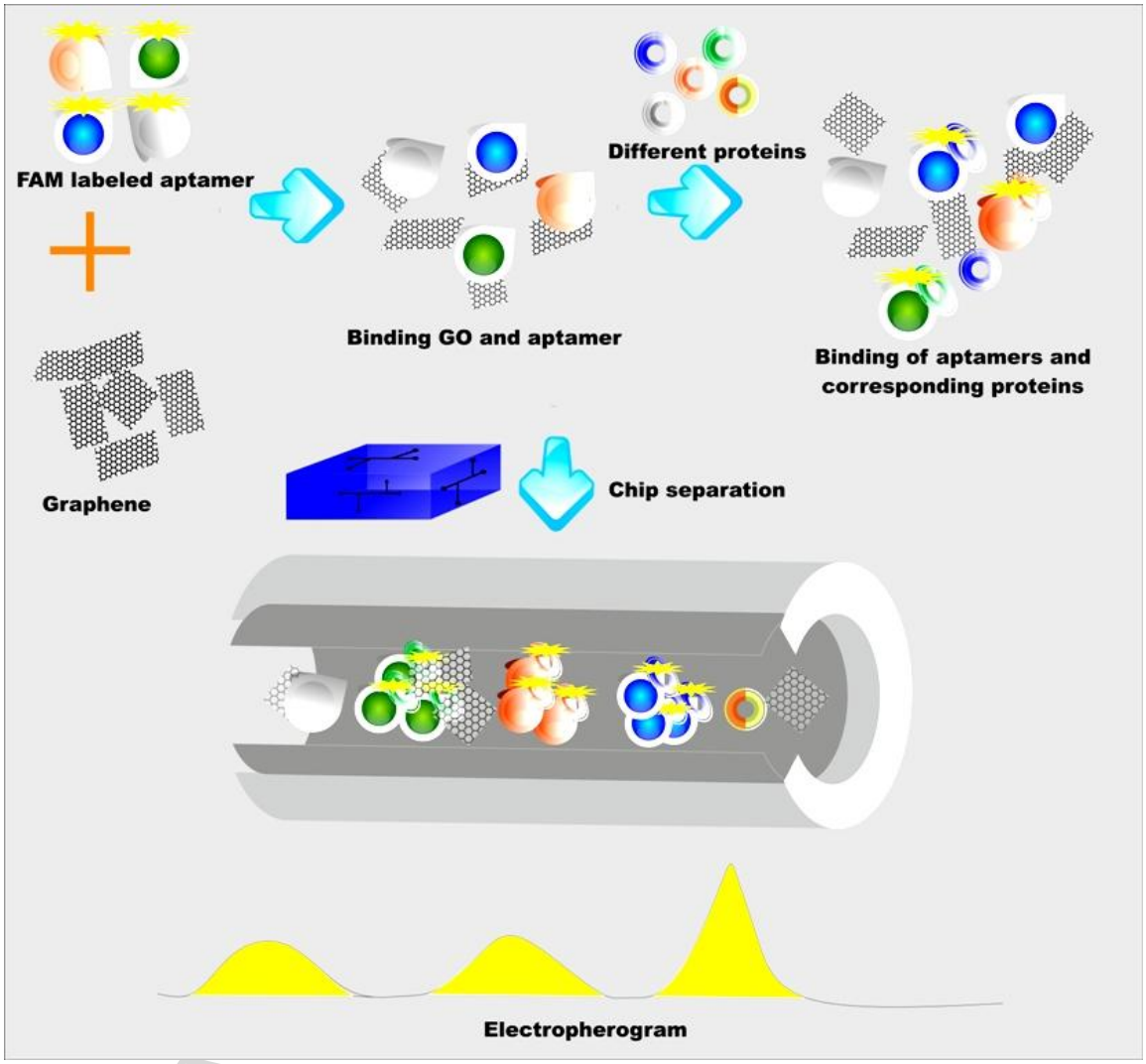
Scheme 2



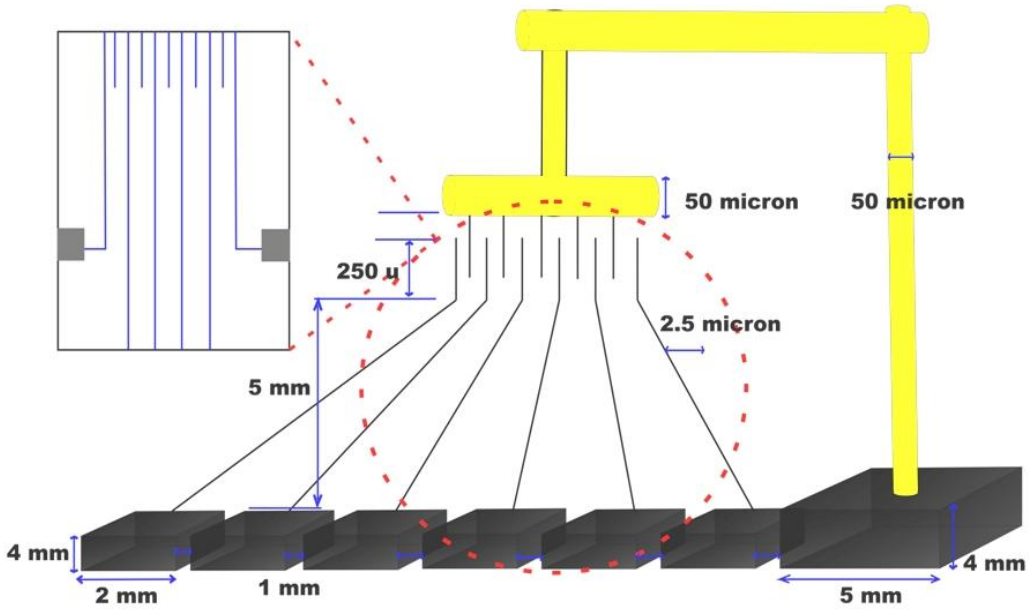
Scheme 3


(a)
200 μm
(b)
200 μm
(c)
15 μm
(d)
50 μm
(e)
W
C
R
Inlet
Outlet
3D graphene micropillar
50 μm

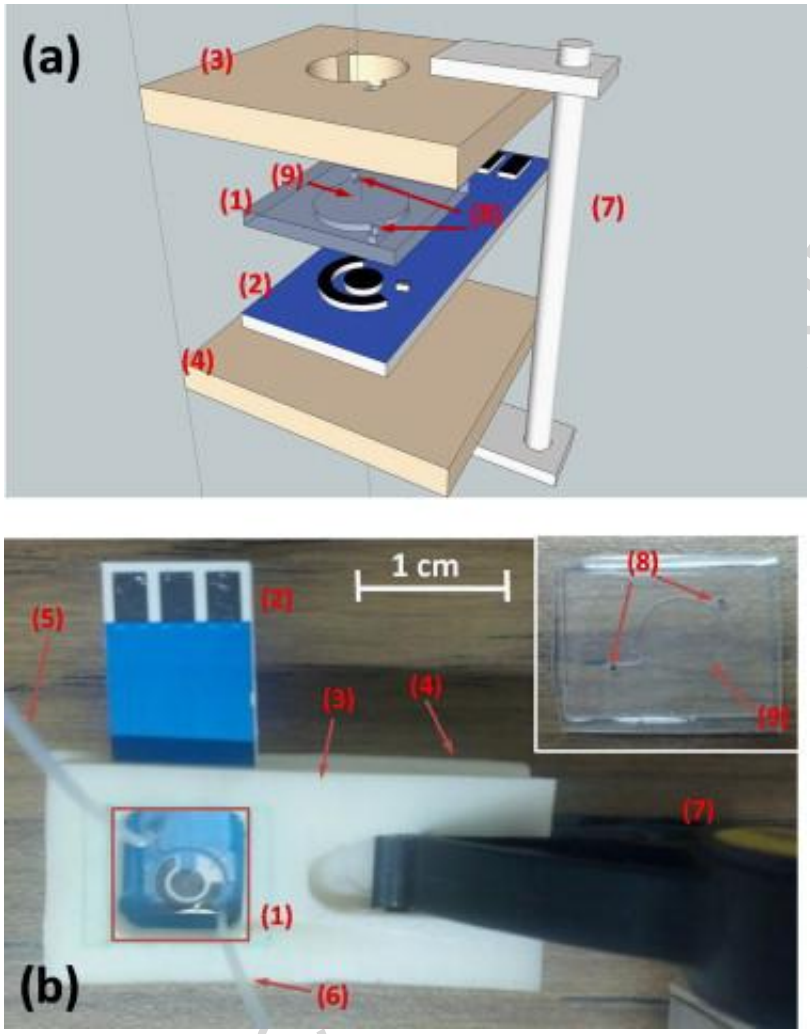
Scheme 4



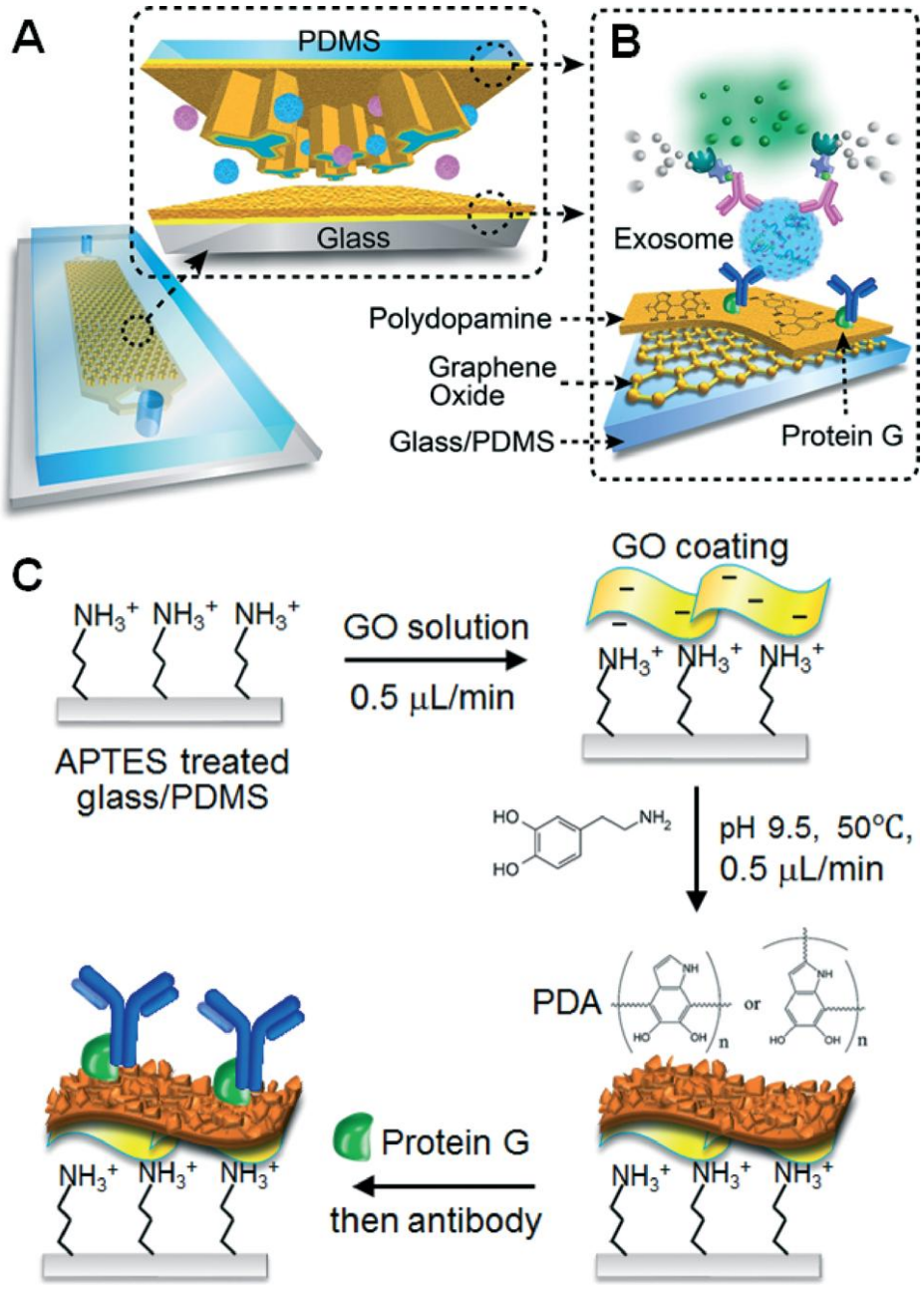
Scheme 5



Scheme 6



Scheme 7



Scheme 8

Highlights

Graphene-based immune-devices have been used for biomedical testing.
Two dimension (2-D) graphene-based immune-devices were discussed.
Current state-of-the-art in graphene-based immune-devices was reflected.