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Graphene–protein field effect biosensors: glucose sensing☆

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Chronic diseases are becoming more prevalent, and the complexities of managing patients continue to escalate, since their care must be balanced between the home and clinical settings. Diabetes is the most advanced example, where self-monitoring has been shown to be necessary. Glucometers are point-of-care (POC) devices that have become standard platforms at home and clinical settings. Similarly, many other POC biosensors have also been developed. Enzymes are often used in these sensors because of their specificity and the reaction products can be electrochemically transduced for the measurement. When enzymes are immobilized to an electronically active substrate, enzymatic reactions can be transduced by direct electron transport. This paper describes an approach for the development of graphene-based POC devices. This includes modifying enzymes for improved performance, developing methods to bind them to the graphene surface, incorporation of the functionalized graphene on a field-effect transistor (FET), and integration into a microfluidic device suitable for home use. This paper describes an approach for the development of a graphene-based POC biosensor platform using glucose as an example of target molecule.

Introduction

Serum glucose, cholesterol, triglyceride and glycated hemoglobin (HbA1C) monitoring are valuable tools in the management of diabetes and cardiovascular diseases. Point-of-care (POC) devices, continuous biomedical monitoring systems and non-invasive monitoring systems have significantly improved over the last twenty years. An obvious example is glucose sensors, which have been developed and refined significantly during that time period. However, even with substantial effort, there continues to be several challenges related to the achievement of accurate and reliable glucose monitoring [1,2]. Currently, glucometers are required under an international standard to produce results within a 20 percent margin of error, and the Food and Drug Administration (FDA) is contemplating more stringent standards. For this reason

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coupled with the knowledge gained from the previous research done on glucose measurement, the paper uses glucose as a representative target biomarker. An approach functionalized graphene-based FET for the development of a POC platform based on functionalized graphene-based Field Effect Transistor (FET) sensors is presented in this chapter.

Graphene-protein sensors combine the exceptional electrical properties of graphene, and the selectivity of proteins with the processing power of nanoelectronics and fabrication to offer new powerful diagnostic tools with much greater precision in medical science. Early blood glucose meters have already used an enzyme, glucose oxidase, because it specifically reacts with glucose and produces gluconic acid and hydrogen peroxide, which can be amperometrically or colormetrically transduced. While measuring the hydrogen peroxide is relatively easy, it can have problems with interferences from other electroactive molecules such as acetaminophen and ascorbic acid [3]. Later generations of glucose meters have increased accuracy, but further technical improvements in glucose biosensors, standardization of analytical goals for their performance, and continuous assessment and training of lay users are required [4]. With this motivation the development of integrated, highly efficient, lab-on-a-chip devices based on graphene protein FET sensors that will measure clinically relevant analytes from a single drop of blood would have broad applications for bedside monitoring of patients. The nano-dimensions of graphene and its electronic properties make it an ideal substrate candidate for anchoring the proteins for biochemical sensing. Yang et al. have discussed the advantage of graphene over carbon nanotubes (CNTs) in the design of biosensors [5].

Over recent years, research and development for clinical diagnostic systems based on lab-on-a-chip technologies has proliferated significantly. Miniaturization can save reagents, enable rapid and inexpensive assays, and reduce the need for skilled personnel. These characteristics are important for monitoring patients at home. Home-based diagnostics simply should not require large volumes of blood samples or processing for plasma, as they are going to be distributed widely, and venipuncture and centrifugation require trained personnel and equipment. Home-based systems must be able to work with samples that are easy to acquire, such as a drop of blood or saliva. Thus, POC systems must also be able to either to use the raw sample or have the ability to perform sample preparation autonomously.

Lab-on-a-chip microfluidic devices [6] designed by integrating graphene with proteins exploit the full potential of proteins in acting as nanosensors, nanofilters, and excellent electrical properties of graphene auger well for the next generation of handheld, ultra-portable devices for personal health care monitoring by the patient. One could envision with the revolution in wireless based mobile multi-media [7], these hand-held devices might be able to transmit the information to the physician or hospital for timely intervention. For instance, early detection of infections for patients at a home setting by monitoring can minimize chronic cases and allow potential infections to be addressed early at an acute phase [8–13]. This type of monitoring minimizes costs and hospitalizations, resulting in overall savings for the economy. Another potential application is screening tests for early detection of cancer biomarkers; such detection could enable treatment prior to metastasis, increasing the odds of survival [14].

In general, proteins and biological macromolecules offer extreme stereo-specificity and sensitivity and can be incorporated into cutting edge protein-based biomedical devices [15]. The larger outcome in using these platforms is the possibility of using them for any pathological/metabolic disorder that has enzyme detection or assay titration as the basis for diagnosing, monitoring and treating diseases. This could include comprehensive metabolic panels such as liver enzymes, miRNA, aspartate aminotransferase–alanine aminotransferase (AST/ALT), kidney tests such as BUN/creatinine, cardiac biomarkers, and thyroid function tests – the prospects are endless. Saliva, as well as other bodily fluids, could be explored for health and disease surveillance and the approaches described here may be modified for salivary diagnostics, which would be especially useful in pediatrics [16]. This paper explores the use of combined graphene – glucose oxidase sensors for glucose measurements as an example of a graphene – protein field effect biosensor platform.

Graphene platform

There are two critical aspects of carbon-based sensor systems: first, the surface to volume ratio of the substrate, and second, the electronic transfer characteristics of the material. For sensors of a similar overall size, the surface to volume ratio controls the number of proteins that are in contact with the sample and can react with the target molecules. The efficiency of the electronic interactions between reacting proteins and the substrate directly affect the measurement. Large surface area coupled with high mobility and conductivity are potential advantages of graphene for electronic and electrochemical biosensors.

Electrochemical biosensors are one of the most important classes of biochemical sensors, and there are various forms available in the market. For these types of sensors, a carbon based material is considered as an ideal electrode material due to its wide anodic potential range, low residual current, chemical inertness, easy availability, and reduced cost [17]. Moreover, carbon-based electrodes exhibit fast response time and can easily be fabricated in different configurations and sizes. These electrodes possess attractive electrochemical reactivity with high mechanical stability, and various forms of carbon are also available in nature. Recent advances in graphene research can overcome the issues related with CNTs based sensors such as signal interference due to the presence of metal, by providing graphitic metal free edges with high surface area [18]. These advantages motivated researchers to make graphene based glucose and bio-analyte sensors.

Apart from *in vivo* and *in vitro* imaging and drug delivery applications, graphene and related materials-based biosensors have been reported in the literature [19–25]. Large surfaces area of graphitic surfaces will be highly sensitive to electronic/electrochemical changes happening in its surroundings. This enables graphene based sensors with high sensitivity, selectivity and with short responsive time. For example, Graphene Oxide (GO) can interact with nucleotides through π–π stacking, similar to the intercalation of DNA with CNTs, but on a much faster time scale *vis-à-vis* CNTs. Graphene has high sensitivity to electrical perturbations due to its ultra-thinness and high carrier mobility at room temperature. Combining its unique transport properties and structure, graphene is attractive for a variety of sensing applications, when it is covalently attached to a protein, which confers the stereo-specificity for a specific analyte. A number of studies have

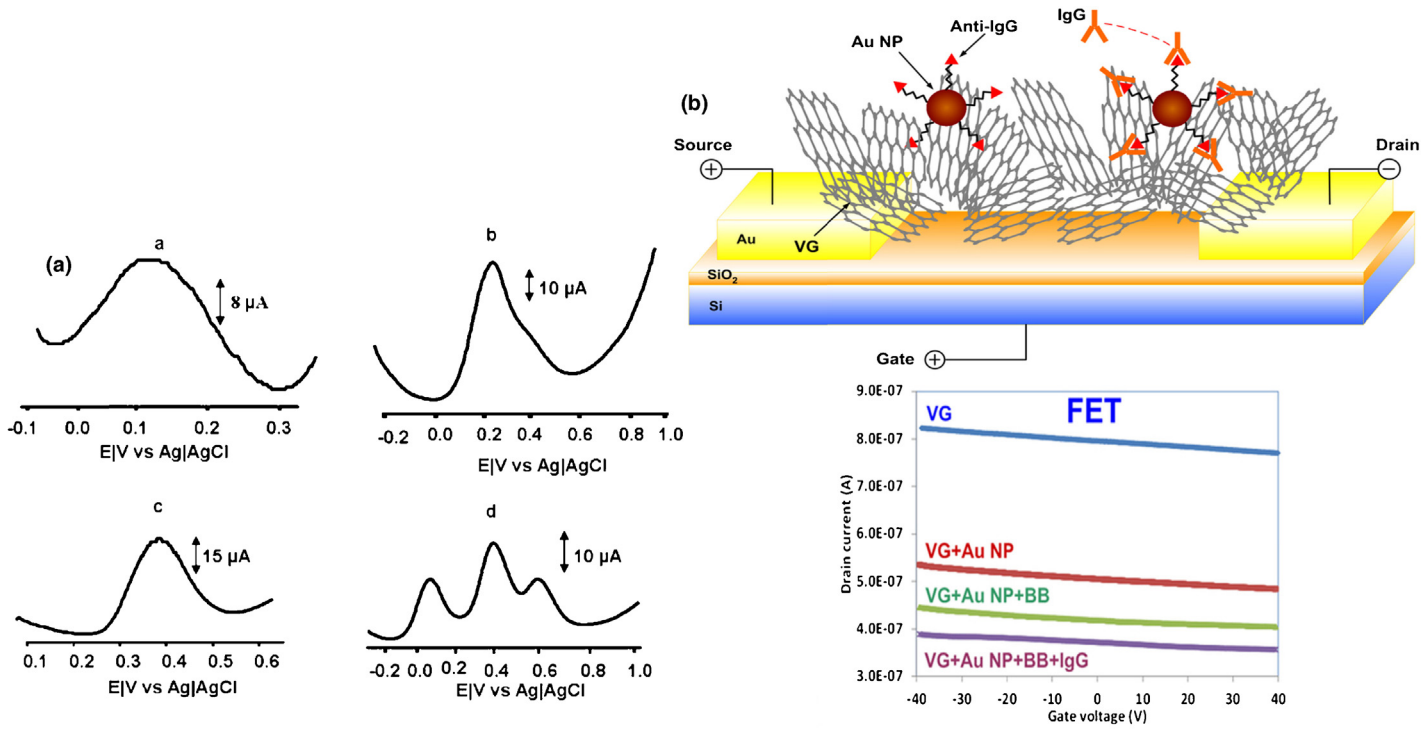

FIGURE 1

(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 Li et al. Reproduced with permission [17]. Copyright American Chemical Society (2009). **(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). Reproduced with permission [18]. Copyright (2013), Rights Managed by Nature Publishing Group.

been reported on graphene biosensors, and electrical detection of biomolecules using ultrathin 2D graphene sheets can potentially achieve high sensitivity. This is because graphene is extremely sensitive to electronic perturbations and its surrounding environment, and also because graphene has a large specific surface area, which enables surface functionalization with probe proteins [21,26,27]. For example, Alwarappan et al. [17] demonstrated the suitability of graphene surface for the electrochemical detection of dopamine and serotonin. They compared the sensitivity and reliability of graphene based electrodes in comparison to single-wall CNTs and they found that graphene exhibit better signal-to-noise ratio and stability than single-wall CNTs for electrochemical detection. It is also reported that graphene based electrodes are suitable for simultaneous multi-analyte detection say, dopamine (DA), ascorbic (AA) acid and serotonin (ST) making them ideal for '*in vivo*' applications [17].

Although graphene-based biosensors present excellent performance in biomolecular detection, the sensor performance should be further improved to meet demanding requirements in terms of sensitivity, selectivity, and stability. Graphene sensor fabrication methods also should be suitable for large-scale manufacturing, which is critical for commercialization. Graphene surfaces provide a biocompatible and highly conductive platform for anchoring proteins, although functionalization of graphene for covalent attachment of proteins or by electrostatic attachment poses daunting challenges.

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 [28–31] are employed on graphene-based detection systems. Recently, gold nanoparticles have been integrated with various graphene-based FET sensors [18,22]. Figure 1B 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)).

Engineering graphene: doping

Recent attempts on doping graphene with other elements lead to the development of various multifunctional graphene forms. Doping has immense implications in tuning the physico-chemical properties of graphene thereby getting new-layered material having desired properties with similar morphology and structure [32]. Some of those functional derivatives are shown in Fig. 2. Those include semiconducting nitrogen (N), boron (B), and sulfur (S) doped graphenes' and bandgap tunable BCN structures, fluorine (F) doped and hydrogen (H) doped graphenes.

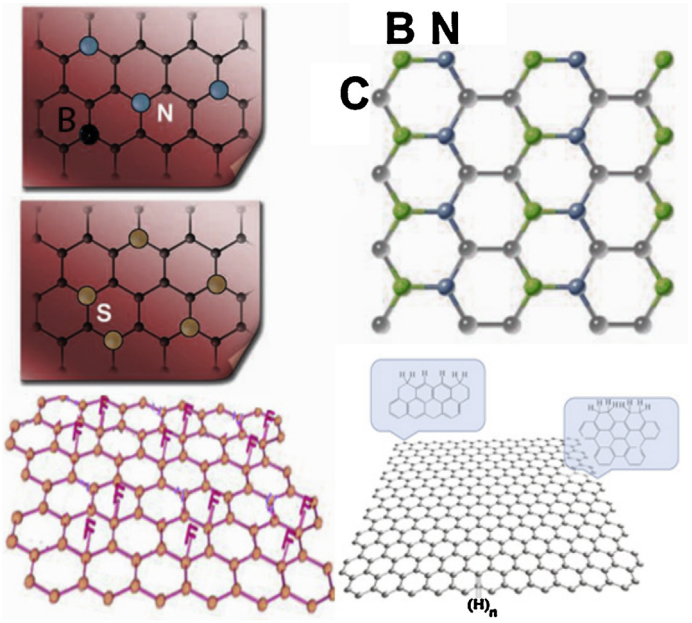

FIGURE 2

Schematics of various functional derivatives of graphene. These include N/B doped graphene, BCN systems, S-doped, F-doped and H-doped graphenes'.

One such functional derivative containing fluorine, where F is covalently attached to carbon lattice forming sp^3 hydridization, is known as fluorographene and is projected to have huge advantages in biomedical sensors and diagnosis over others [33]. For example, recently, oxidized fluorographene (fluorinated graphene oxide) has been demonstrated for applications in multimodality imaging along with drug carrier ability [33]. Presence of F- makes GO paramagnetic, otherwise it is a diamagnetic material, enabling its use for Magnetic Resonance Imaging (MRI). Moreover, use of fluorinated materials for MRI also helps ^{19}F based imaging techniques, which increases localized high contrast compared to proton based MRI. Hence the development of fluorinated graphene oxide led to discovery of single material based multitasking agent, that is, a theranostic agent with drug carrying capacity, it was previously realized using hybrid materials. Though various graphene and graphene derivatives were studied for biomedical applications, recent reports suggest that various negatively charged functional groups present on graphitic surfaces can induce thromboembolism (aggregation of platelets in blood) [34]. It is reported that surface charges on graphene can play a key role in the interaction between graphene and platelets. It has also been shown that positive surface charges on graphitic surfaces can avoid thrombotic and hemolytic predisposition. Fluorine like elements can modify the electronic properties of graphene by reducing the charge in the conducting π orbitals. Hence, low level inhomogeneous doping of highly electronegative elements like F can induce partial positive surface charge in graphene lattice, thereby providing a positive surface without further functional groups, thereby making safer usage of graphene *in vivo* applications.

Synthesis of single layer graphene and transfer

Large area continuous single layer (almost) graphene films can be synthesized on 25 μm thick copper (Cu) foils by the chemical vapor deposition technique using hexane as a carbon precursor [35]. Cu foil is loaded in a quartz tube and pumped down to 10^{-2} Torr, before flowing in Ar/H_2 mixture gas at a pressure of ~8–9 Torr (with a flow rate of ~400 sccm). Cu foil is then heated to 950 °C inside the quartz tube in Ar/H_2 atmosphere. When the temperature becomes 950 °C, Ar/H_2 flow is stopped and hexane vapor is passed in the quartz tube to maintain the tube pressure of 500 mTorr for 4 min. The flow rate of hexane is maintained at ~4 mL/h (~2–3 sccm). After 4 minute passage of hexane, the furnace is allowed to cool to room temperature suddenly. The graphene Cu foils are taken out and further processed for transferring the film.

The film is first spin-coated with a thin layer of poly(methyl methacrylate) (PMMA) as a scaffold. This film is separated from the Cu foil by dissolving the foil in dilute nitric acid. After dissolution, the PMMA-supported graphene, which remains floating on the solution, is carefully washed by placing it in a Petri dish of DI water, and is then transferred onto the desired substrate (SiO_2/Si or indium tin oxide or glass substrates). After transferring, the PMMA film can easily be dissolved using acetone, leaving behind pure graphene on the desired substrate [35].

Functionalization of graphene

Functionalization of graphene for applications in bionano-devices depends on the exfoliation of graphite, chemical or thermal reduction of GO, intercalative expansion of graphite, chemical vapor deposition, and epitaxial growth.

Glucose oxidase (GOx) used in the current study was initially obtained from EMD Biosciences Inc. or derived from *Aspergillus niger* by fermentation under standard procedures (NBS Fermenter, 500 rpm, pH 6.0, 30 °C, 96 hours) [36]. Commercial GOx was used extensively purified by HPLC while expressed GOx using *Pichia pastoris* system [37,38] was purified as follows: after 96 hours expression, the culture was centrifuged (4 °C, 15 min, 7500 × *g*), and two volumes of 95% ethanol were added to the supernatant to precipitate GOx. Further centrifugation (4 °C, 15 min, 10,000 × *g*) yielded a precipitate that was dissolved in PBS buffer and then subjected to super filtration (160 kDa MWCO) and anion exchange chromatography on DEAE Sepharose (GE Healthcare). GOx purity was monitored by 12.5% SDS-PAGE and the protein was lyophilized for storage prior to covalent attachment to single layer of graphene.

Glucose oxidase is immobilized onto graphene film *via* a linker molecule (1-pyrenebutanoic acid succinimidyl ester) which on one end firmly attaches to the graphene surface through π–π interaction with a pyrene group and on the other end covalently reacts with the amino group on the enzyme by an amide bond, GOx is incubated with a 5 mM linker molecule (1-pyrenebutanoic acid succinimidyl ester, i-DNA Biotechnology) in dimethylformamide (DMF) for 2 hrs at room temperature, and washed with pure DMF and deionized (DI) water.

Molecular biology of glucose oxidase

Glucose oxidase (EC 1.1.3.4), which catalyzes the oxidation of glucose to D-glucono-δ-lactone and hydrogen peroxide. It contains one very tightly, non-covalently bound, flavine adenine dinucleotide (FAD) cofactor per monomer, and a homodimer with molecular mass of 130–320 kDa, depending on the extent of

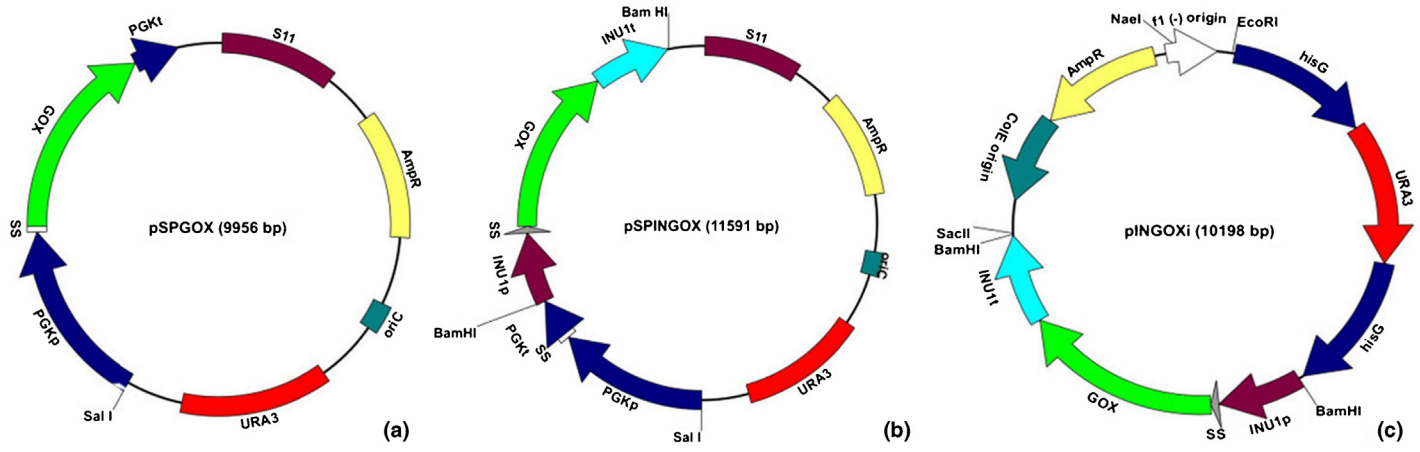

FIGURE 3

Expression systems (31). **(a)** pSPGOX provides episomal expression of GOx under control of the ScPGK promoter; **(b)** pSPINGOX is an episomal vector and GOx expression is performed under control of the KmINU1 promoter; **(c)** pINGOXi is an integrative plasmid that confers the cells capacity of GOx expression under control of the KmINU1 promoter. (a and b) derive from pSPGK1 and (c) corresponds to pNADFL11 plus the expression cassette [39]. p, promoter; t, terminator; SS, secretion signal sequence. Reproduced with copyright permission [39].

glycosylation. The native enzyme is glycosylated, with a carbohydrate mass percentage of 16–25% [39]. The enzyme expressed *Saccharomyces cerevisiae* leads to a highly glycosylated form, with a carbohydrate mass percentage approximating 60% [39]. Rocha et al. [39] have evaluated the potential of *Kluyveromyces marxianus* as a host organism for production of GOx, not only in terms of protein expression levels, especially in terms of glycosylation and thermokinetic properties of the heterologous protein, by cloning the gene for glucose oxidase from *A. niger* BT18 into *K. marxianus* CBS 6556 and into *Kluyveromyces lactis* CBS 2359, using different episomal and integrative constructs. Three different genetic constructs were used by Rocha et al. [39] to direct heterologous glucose oxidase expression, which was also followed in the reference yeast *K. lactis* system, allowing for a direct comparison between the two organisms (Fig. 3).

Protein engineering GOx to enhance thermal stability, catalytic activity, and interaction with graphene

Redox proteins are neither evolved nor optimized for applications as sensors. Therefore, they require fine-tuning to operate efficiently in ambient temperatures by rational protein engineering [40]. Design of thermally robust proteins is critical in the design of protein-based sensors. Principles of design of thermally robust proteins require an understanding of inter-molecular forces contributing to the free energy of proteins and thermodynamics of protein stability. Precise correlation of thermal stability and protein sequence is not well understood There are at least three major issues governing thermal stability: (i) the upper limit on the temperature at which proteins remain physiologically active and are useful for technological applications, (ii) the use of hyperthermophilic enzymes as molecular templates to design highly stable enzymes that have high activity at physiological temperatures, and (iii) the correlation between protein sequence and thermostability and activity. Recent research reports show the importance of the enhancement of protein thermal stability [15,38,41].

GOx has an apparent transition temperature of $55.8 \pm 1.2\ ^{\circ}C$ and an activation energy of ~280 kJ/mol [42] derived from circular dichroism spectroscopy (CD), differential scanning calorimetry (DSC), and fluorescence quenching studies. Protein thermodynamic stability, ΔG, is determined by a multitude of intra- and inter-molecular interactions, which in turn depend on the primary amino acid sequence of the protein. The primary sequence determines all secondary and tertiary structural features. Hence, it is the sequence determining protein thermodynamic stability. At a structural level, this will consist of essential stability determined by the packing of the secondary structural elements, which will be complemented by the interactions of and between side chains. Berezovsky and Shakhnovich [43] recently concluded that most strategies for rational design of thermally stable proteins are of two types: (i) non-specific, structure-based strategies, with contributions from different stabilizing interactions, and (ii) specific, sequence-based strategies, using only dominating factors for adaptation to extreme temperatures. The rapidly increasing number of fully sequenced genomes of thermophilic proteins will be an invaluable help in deciphering which sequence variations among homologous proteins are related to stability and which ones are simply a result of evolution.

We have at least three important goals in protein engineering studies of GOx: (i) enhanced thermal stability, (ii) enhanced catalytic activity, and (iii) optimizing the covalent or pi-pi stacking of GOx on graphene surface.

Holland et al. [44] have reported site-directed mutagenesis and directed evolution studies to enhance the catalytic activity of recombinant *A. niger* GOx. Four mutants with improved catalytic properties were identified out of a library of approximately 4800 mutant (T132S, T132S/T56V, T132S/V42Y, and V42Y) with a 3–4-fold improvement in specificity constant over the parental strain. V42Y showed a modest improvement in enhancement of thermal stability and catalytic activity compared to wild type GOx. Holland et al. [44] have compared the thermal stability of six mutants and all of them showed a modest improvement in thermal stability over the parental strain. One of these mutants, V42Y, had the largest enhancement in thermal stability; Table 1 reproduced from Holland et al. [44], with copyright permission.

TABLE 1

Thermal stability of parental and mutant GOx strains incubated at 50 °C.

GOx strain	$T_{70\%}$ (h)[a]	$T_{1/2}$ (h)[a]	Corr. coeff. (R^2)[b]
Biozyme	10.4 ± 0.5	20.1 ± 0.9	0.990
Parent	14.8 ± 1.1	28.8 ± 1.9	0.992
T132S	19.8 ± 1.3	38.5 ± 2.4	0.986
T132S/T56V	20.4 ± 1.0	39.6 ± 2.4	0.981
T132S/V42Y	17.4 ± 0.7	33.8 ± 2.5	0.974
V42Y	20.7 ± 1.1	40.3 ± 2.3	0.991

[a] Error corresponds to the standard deviation of GOx kinetic rate measurements performed in triplicate.
[b] Correlation coefficients obtained from an exponential least squares regression fit of GOx kinetic rates measured in triplicate vs. time.

X-ray crystallographic study offers some insight into why T132S, may have yielded increased performance. The structure for the flavin–oxygen adduct intermediate was solved for choline oxidase, a closely related GMC oxidoreductase, using a combination of visible spectroscopy and X-ray spectroscopy under cryogenic conditions. In the published structure [44] (Fig. 4), the isoalloxazine ring undergoes a large conformational change upon addition of the oxygen adduct.

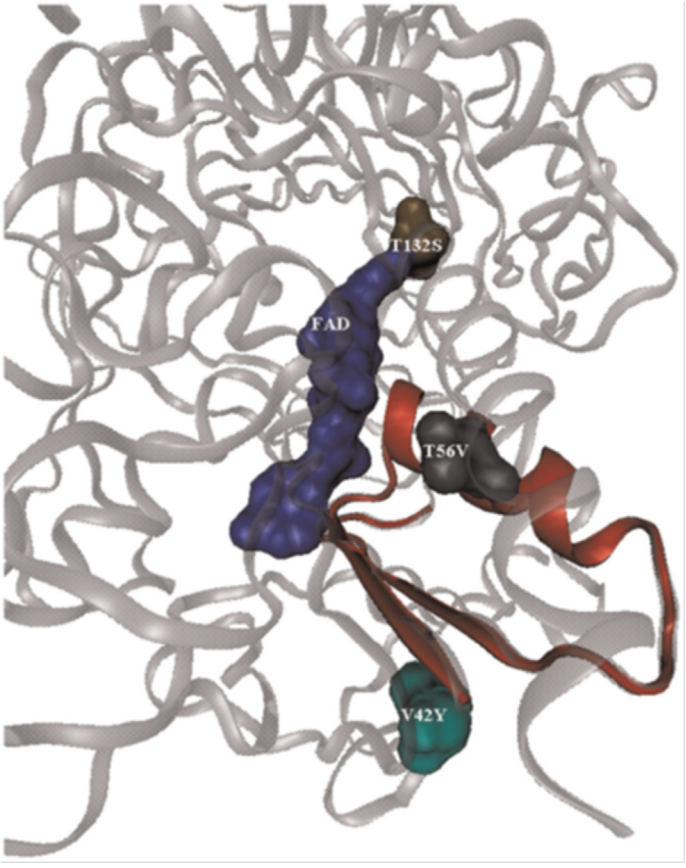

FIGURE 4

View of amino acid mutations that enhanced GOx kinetic activity in relation to the Flavin Adenine Dinucleotide (FAD) cofactor: T132S, T56V, and V42T. The monomer protein is shown as ribbons. The FAD group and mutated amino acid residues are shown as space-filling models. The FAD binding peptide region, containing amino acids T56V and V42T, is colored red [44]. Reproduced with copyright permission.

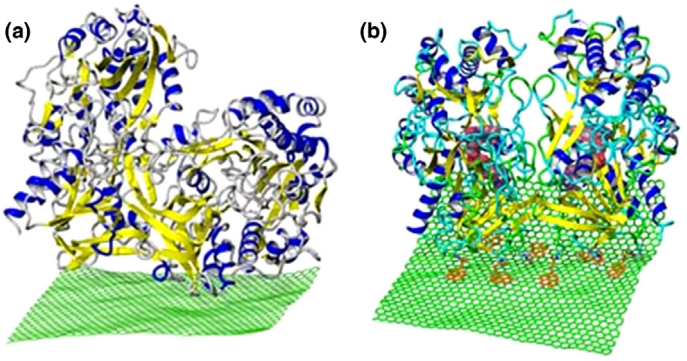

FIGURE 5

(a) A snap shot of 100 ns molecular dynamics (MD) simulation of GOx grazing on the surface of graphene, **(b)** the GOx dimer bound to a single layer of graphene *via* linker 1-pyrenebutanoic acid succinimidyl ester (colored in orange) covalently bound to lysine residues. A cofactor FAD is colored in purple. The structure of the complex was obtained after 50 ns of molecular dynamics simulation in water environment. All MD simulations were performed using Gromacs v 4.5.5 [46–48].

By comparing the crystal structure of GOx (PDB ID: 1CF3) with the choline oxidase structure (PDB ID: 2JBV) [45], it was observed that the threonine 132 residue in GOx (an isoleucine in the choline oxidase structure) is directly adjacent to this warped region of the isoalloxazine ring, as shown in Fig. 5. Replacement of threonine by the less bulky serine could reduce the steric hindrance of this rearrangement. Molecular dynamics studies of encapsulation of GOx by graphene at 100 ns resolution and 50 ns have yielded a wealth of data on the residues in GOx proximal to graphene surface. A snap shot of GOx grazing the surface of graphene is shown in Fig. 5.

Covalent binding of GOx to functionalized graphene

The linker-modified graphene is then incubated with 10 mL. GOx in Na_2CO_3–$NaHCO_3$ buffer solution (pH 9.0) overnight at 4 °C followed by rinsing with DI water and phosphate buffered saline solution (PBS), pH 7.2. To deactivate and block the excess reactive groups remaining on the surface, the device was incubated with 0.1 M ethanolamine solution (pH 9.0) for 30 min and then rinsed by DI water. Infrared spectroscopy and Raman spectroscopy are used to determine shifting of the carboxyl stretching.

The activity of the glucose oxidase immobilized on the surface of the graphene in presence of glucose was confirmed by measuring the amount of H_2O_2 produced by the breakdown of glucose by glucose oxidase. Briefly, the sensors were incubated with buffer solutions containing glucose. Discrete samples were taken from the solution over an experimental period of 1 hour and the concentration of H_2O_2 was measured (using a modified catalase activity assay fluorimetric assay kit (Sigma Aldrich MAK165)) (Fig. 6).

Device fabrication, microfluidic chip

As stated previously, once active enzymes, or biomolecules, are correctly attached to the graphene, any charges resulting from a reaction should be transmitted to the graphene. Because of its two-dimensional characteristics and sensitivity, the resulting signal should reflect the number of reactions with a high degree of precision and relatively high signal to noise. One method of transducing the signal is using functionalized graphene in a FET

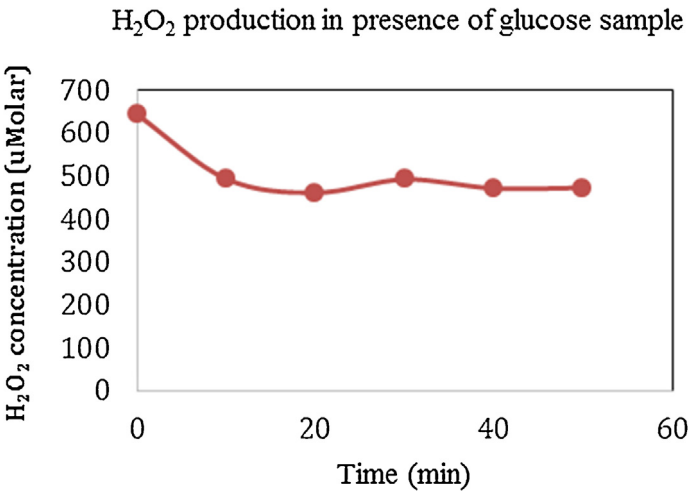

FIGURE 6

Initial performance of the functionalized graphene chips demonstrating the activity of the graphene-bound GOx through the production of H_2O_2 in the presence of glucose. The H_2O_2 concentration, measured using a modified catalase activity assay, was demonstrated to be constant over almost an hour.

mode. Reactions of the attached biomolecules will change the charge characteristics of graphene, and this will change the conductance of the FET as they do with carbon nano-wire FETs [49]. In their review, Nicu and Leichle point out that the sensitivity of nanoscale sensors can be similar to their macro-scale counterparts. However, reducing the size significantly reduces the number of attached biomolecules, making it potentially less sensitive, and makes these devices hard to work with. As a result, the graphene is on the order of 1 cm on a side that is generally supported on a SiO_2/silicon substrate, which includes space for bond pads for electrical contacts and mounting the FET in a biosensor. Clearly, an ideal approach would be to determine a standard size and layout so that different biosensors could be used in a 'standard' microfluidic system.

Any new biosensor system that will be useful for home will have to be useable by the patient and cannot be dependent on any form of laboratory infrastructure. This scenario is ideal for a completely self-sufficient lab-on-a-chip approach. It is more likely in the near future that the system would not only involve a small lab-on-chip, but have some form of 'box' that would provide an external source of power and, possibly, reagents. It could also measure the sensor output, potentially in the form of an electronic or fluorescent signal, convert it to a clinically relevant quantity, and transmit the information to the patient and/or doctor [50–52].

The use of two components for a biosensor is similar to a point-of-care glucometer, where the sample acquisition and preparation as well as the test chemistry are combined into a strip while the raw result is 'read' by the handheld device. Similarly, the two components of the system described in this chapter should not change the operational simplicity that is a requirement for a home-health device. Specifically, the final operational complexity should be roughly equivalent to current meters.

Thus, an obvious approach would be to have a simple plastic card that could contain a well for the biological sample, fluid transport channels, additional reagents, and the graphene-based biosensor chips [53,54]. The chip, with a blood sample, would be inserted into a larger, non-disposable processing system that contains the power supply, pumping system, and data acquisition/processing. The potential to have multiple biosensor chips in the card provides a significant improvement over the glucometers that were used as an example above. Multiple measurements of a single analyte would improve measurement accuracy. Possibly even more useful would be using different sensors so that data fusion could be used to determine better clinical information. Figure 7 shows a CAD design of a microfluidic device, in which four silicon-based biosensors are integrated. Electrical connections are allowed through the top plastic layer providing the necessary sealing of the sensor.

One of the most difficult and critical processes that must be transparent to the user is sample preparation. In the case presented here, the sample is serum or plasma that is separated/pre-processed from a small blood sample. For normal laboratory measurements plasma is used because the red blood cells generally interfere with sensor function: for these measurements, blood is obtained by venipuncture, and the plasma is separated by centrifugation. Not only is the idea of home centrifuges outside of the realm of practicality, but the acquisition of several milliliters of blood is an impossible request. Samples must be easily and reliably acquired by the patient. Sample preparation must be done by the POC device. Similar in current glucose meters, the simplest approach is to acquire a small drop of blood that is then put into the biosensor. Several methods of separating plasma from the red blood cells have been reported in the literature and include methods such as size-exclusion techniques that include weir-type structures [55], membranes [56], and packed columns for filtering out blood cells; active energy sources such as ultrasonic waves or electrical currents to retain or direct the flow of erythrocytes in microfluidic channels [57]; and utilizing hemodynamics for separation, taking advantage of flow behaviors such as the Zweifach–Fung effect [58], plasma skimming, centrifugal microfluidics [59], or a 'filter trench' that exploits complex flow field effects created by a sudden contraction [60]. Figure 8 shows a microfluidic device fabricated by means of hot embossing with an imbedded graphene biosensor chip, which is 1 cm on a side.

Major challenges in the high quality fabrication of microfluidic platforms are high costs and long fabrication times. The application of electrodeposition and hot embossing technologies to the fabrication of microfluidic devices is promising. This method enables inexpensive and rapid fabrication microfluidic devices of a wide variety of thermoplastic materials. This fact allows researchers to address some of the requirements needed for POC diagnostic devices such as transparency, chemical resistance, biocompatibility, and hydrophobicity. Even more importantly, this fabrication process is scalable from the lab bench to the industrial level.

An exemplary fabrication process is depicted step-by-step in Fig. 9. Specifically for the mentioned designs, molds were fabricated by electroplating nickel on steel wafers (90 mm diameter 304 stainless steel #8 0.03 in. thick, finished mirrored 22G, Stainless Supply, NC). A photoresin was used to pattern the microfluidic network by means of photolithographic process. Different types of photoresins can be used to create the electrical insulator layer, that is, positive or negative, liquid or dry, or even adhesive precut films. The photoresin selection depends on the desired feature size, namely the channel width and depth. Furthermore, a multilayer

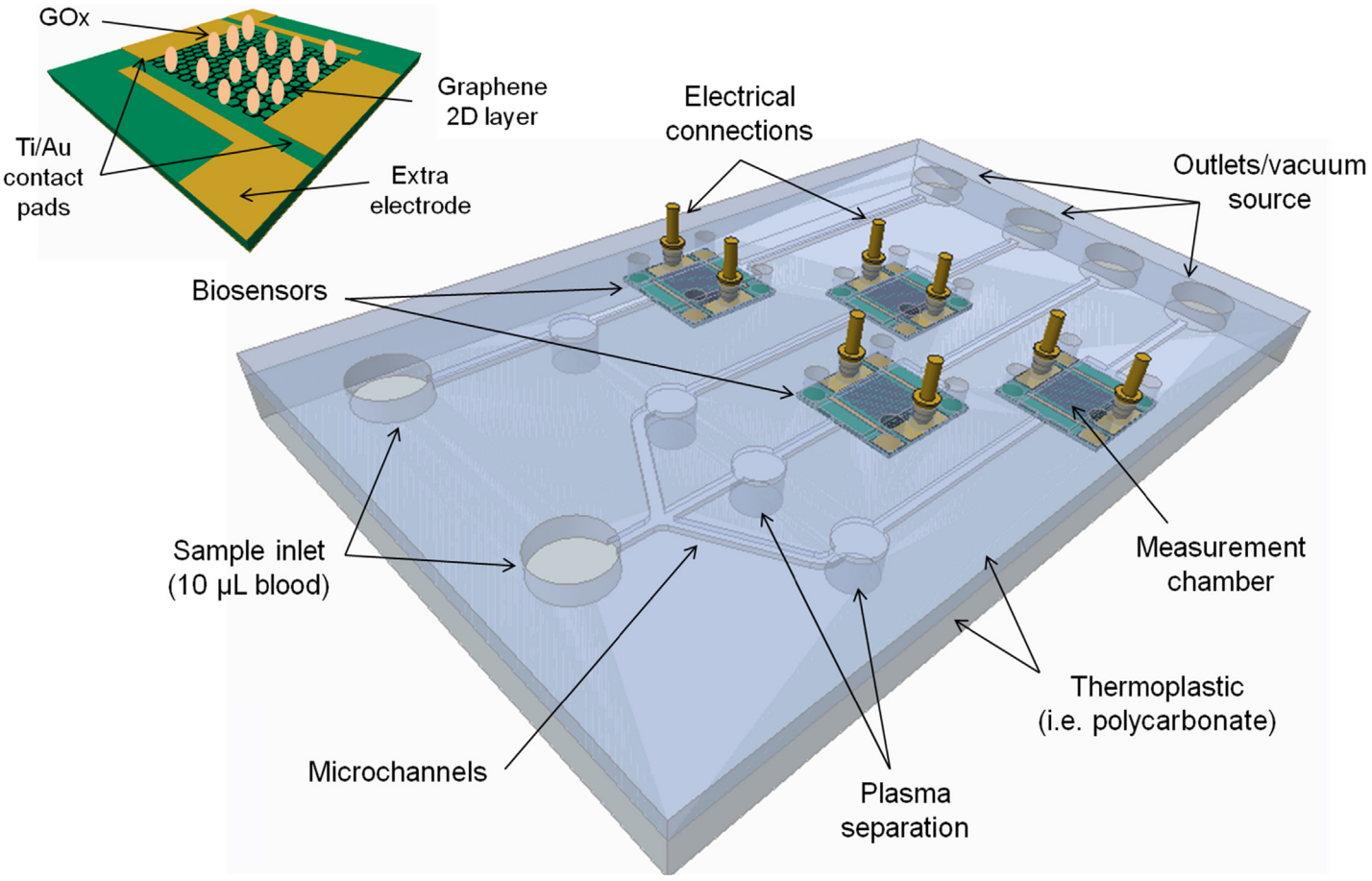

FIGURE 7

Example of a plastic-based design for a microfluidic platform for multiple, standardized biosensor chips (i.e. graphene FET sensor).

approach can be used to achieve different structure heights on the mold, which will be transferred to the plastic device. In this case, a 75 μm thick dry photoresin was used to pattern the microfluidic network (Riston® GoldMaster Series, Dupont). Specifics about the electroplating process can be found in the recent paper by Novak et al. [61], which describes rapid prototyping methods for fabricating plastic microfluidic devices using hot embossing.

The electroplated structures comprise the negative mold for the microfluidic device, with the final plastic devices produced *via* hot

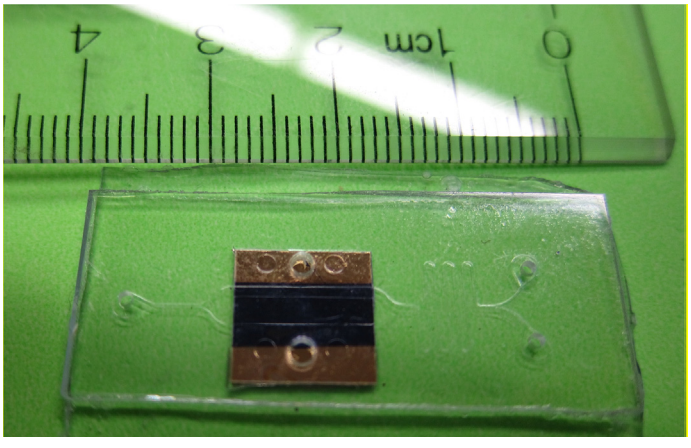

FIGURE 8

Microfluidic test system made from polycarbonate using hot embossing. The fluid channels are 100 μm wide and 75 μm deep. In the middle of the device is a graphene FET sensor with gold contacts that are accessed through the holes in the plastic cover.

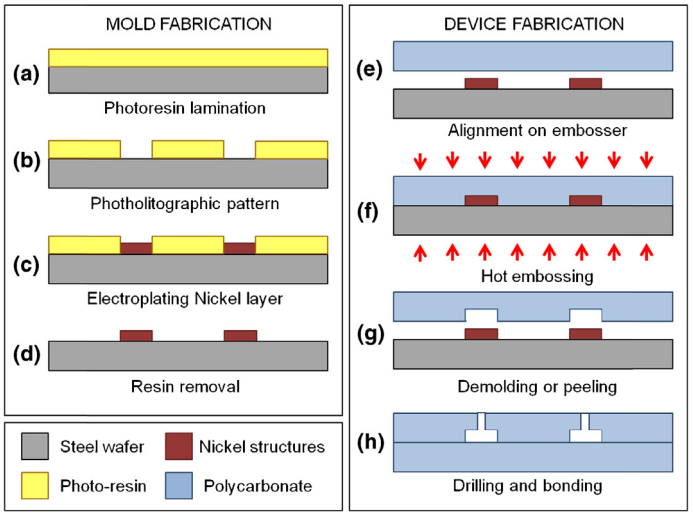

FIGURE 9

Workflow of the fabrication process. The mold fabrication **(a–d)** is based on a photolithographic processes and electrodeposition of Nickel on the steel substrate. The microfluidic device is made by hot embossing **(e–g)** a thermoplastic sheet with the mold. A final assembly of the plastic device **(h)** will finish the fabrication.

embossing. This method takes advantage of the viscosity properties of thermoplastics above the glass transition temperature (Tg). The thermoplastic material will accommodate to the shape of the mold under the conditions of pressure (commonly between 5 and 25 kN) and temperature (at least 10 °C below Tg). 1.5 mm thickness polycarbonate sheets (McMaster Inc.) were employed to emboss the microfluidic devices with approximately 20 kN and 160 °C. A final assembly of the device is required to seal the open channels after drilling the inlets and outlets. Among other bonding processes, plasma or oxygen surface activation treatment, thermal bonding or solvent-assisted bonding and gluing are some of the more popular processes.

The integration of silicon-based sensors within the microfluidic network must be carried out before assembling the device. This can be performed by either directly embedding the chip during the hot embossing process or placing and gluing the chip after the embossing step. Contraction of the plastic during cooling and sealing performance after bonding must be carefully considered for the design of the fabrication process. Precise control of the dimensions of the measurement chamber could be used to maximize the performance of the sensor.

For the initial tests of a graphene glucose sensor, a gasket with dimensions similar to that of the functionalized graphene chip was created in a polycarbonate (PC) substrate using hot embossing as shown in Fig. 9. The functionalized graphene chip was then gently placed inside the gasket and the edges were sealed by applying very small amount of glue. In order to create the electrical connection to the graphene, a mask was prepared and placed on top of the graphene sensor embedded in PC substrate. The gold pads were then created *via* gold sputtering. A microfluidic channel with desired geometry was printed on a double-sided tape and placed on top of the sensor. Finally, the sensor was sealed using another PC substrate, which was placed on top of the device leaving two openings for electric connection and two openings for inlet and outlet of the microfluidic channel. The functionalized graphene surface that was exposed to the flow was 2 mm wide by 13 mm long. The chamber depth was 100 μm.

The glucose sensor was tested under flow conditions. Varying concentrations of glucose were continuously infused into the device inlet utilizing a peristaltic pump, which was set to 50 μl/min. This process removed the hydrogen peroxide generated by the glucose–GOx reaction and maintained constant conditions in the test chamber. The resistance of the sensor was measured continuously using an Agilent 34401A digital multimeter. The resistance across the graphene decreased with increasing glucose concentrations as shown in Fig. 10. Three runs with the same device were performed with subsequent experiments showing similar response characteristics but with reduced signal response corresponding to an increase in resistance at the highest glucose concentration from 2.37 to 3.30 kΩ. It is likely that the reduction in signal results from damage to or loss of the surface-bound GOx enzymes during washing between experiments, but this needs to be verified.

Even without using the graphene chip as a FET, the device showed high sensitivity especially at low glucose concentrations where the resistance changed by 1 kΩ when the glucose concentration increased from 0.1 to 1.0 mM. Although reduced at higher concentrations, the sensor exhibited easily measurable resistance

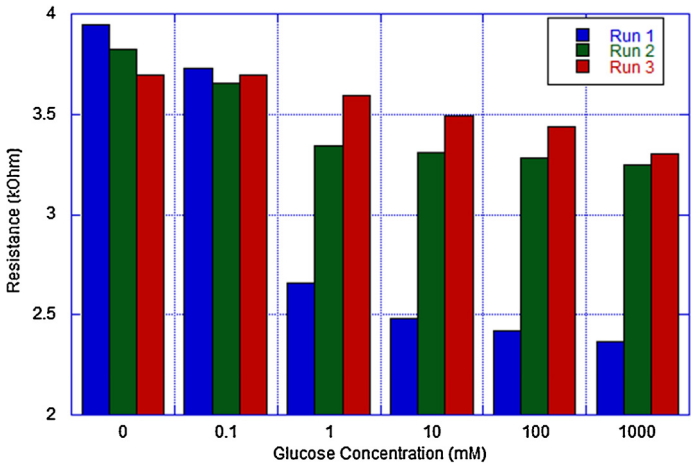

FIGURE 10

Graphene-based sensor response to glucose concentration. Resistance (kΩ) as a function of glucose concentration (mM) for a single sensor are shown for three sequential experimental runs. Between each experiment the sensor was flushed with distilled water.

changes (180 Ω and 62 Ω) for glucose concentrations of 100 mM and 1 M, which are well beyond normal physiological range of around 3–10 mM. While this is not clinically useful for glucose, it indicates that as a platform the proposed graphene-based biosensor could be used for a variety of biomedical applications. Using the sensor chip as a FET by applying a bias voltage to the sensor chip should increase its sensitivity due to the better control over gate voltage than bias voltage in the case of resistance measurement. While this is not necessary for the current experiments, it would make it possible to reduce the size of the sensor while maintaining system sensitivity.

Conclusion

The enhanced sensitivity, improved response time and smaller size of nanomaterials make them more suitable than traditional materials for various applications. Noteworthy among these applications, is their inherent value as sensors, and carbon nanomaterials are particularly interesting for this application. In particular, biosensing is of paramount importance in improving the quality of human life. Biosensors are able to detect a wide range of molecular species with enhanced sensitivity and selectivity with applications in security, health care for point-of-care analyses of diseases, and environmental safety.

Graphene, a single monolayer of graphite, is a particularly interesting 2D nanomaterial, which finds increasing applications including nanoelectronics, photovoltaics, and chem/biosensing. Its high surface area and capability to be fabricated in many shapes and sizes, especially by chemical vapor deposition, makes graphene more useful for specific applications compared to other carbon nanomaterials such as nanotubes and nanowires. Furthermore, the robust nature of carbon–carbon sp^2 bonds forming a honeycomb lattice makes graphene chemically stable and biocompatible. Graphene is extremely strong, flexible, transparent and conductive. Other nanomaterials such as boron nitride have also emerged as candidates for enhanced biosensor applications. The development of graphene or other 2D material based technologies for biomedical applications, either in the form of a POC device or

as a drug carrying/therapeutic material, should thoroughly follow existing regulatory and approval framework implemented by national and international agencies [62]. Hence, well designed studies are needed to be conducted for this new class of materials with cells, tissues and organisms, and such a study will help guide the best choices for the use of this exciting family of materials [62].

Most of the existing nanoelectronic sensors rely on charge detection mechanism where the molecular binding changes the charge density of the active material (nanomaterial) of the sensor and which leads to a sensing signal [63]. A charge transfer will happen between adsorbed molecules and the nanomaterial, and this in turn changes the charge density of the latter. This causes the changes in the Fermi energy of the material and hence conductance of the sensor. Charge carrier density can be varied by doping, and graphene, being a 2D material, is extremely sensitive to the chemical and physical variations occurring in its surroundings. The adsorption of organic molecules (bio-analytes) can form additional scattering centers in graphene and they can perturb the local molecular electric fields [64]. Hence the presence of external molecules can vary its conductivity and this variation can either be monitored using a simple chemiresistor or by a transistor based sensor [65]. The sensitivity of both methods can be tuned by the biasing voltage or gate voltage.

Microfluidics has opened new vistas in the design of practical devices, but to date, very few lab-on-a-chip based technologies have been introduced into the market. POC diagnostics will strongly benefit from miniaturization based on microfluidics, emerging 2D nanomaterials, nano fabrication, and combining the stereo specificity of biological macromolecules, to accomplish the goal of ultra-sensitive micro sized diagnostics, within the reach of patient, physician, emergency room, hospital use in static and dynamic continuous monitoring mode.

Conflict of interest

Dr. Utkan Demirci is a founder of, and has an equity interest in: (i) *DxNow*, a company that is developing microfluidic and imaging technologies for point-of-care diagnostic solutions, and (ii) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions. Dr. Utkan Demirci's interests were viewed and managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

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