



Polyaniline-graphene based α -amylase biosensor with a linear dynamic range in excess of 6 orders of magnitude

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ABSTRACT

α -amylase is an established marker for diagnosis of pancreatic and salivary disease, and recent research has seen a substantial expansion of its use in therapeutic and diagnostic applications for infection, cancer and wound healing. The lack of bedside monitoring devices for α -amylase detection has hitherto restricted the clinical progress of such applications.

We have developed a highly sensitive α -amylase immunosensor platform, produced *via in situ* electropolymerization of aniline onto a screen-printed graphene support (SPE). Covalently binding an α -amylase specific antibody to a polyaniline (PANI) layer and controlling device assembly using electrochemical impedance spectroscopy (EIS), we have achieved a highly linear response against α -amylase concentration. Each stage of the assembly was characterized using a suite of high-resolution topographical, chemical and mechanical techniques. Quantitative, highly sensitive detection was demonstrated using an artificially spiked human blood plasma samples. The device has a remarkably wide limit of quantification (0.025–1000 IU/L) compared to α -amylase assays in current clinical use. With potential for simple scale up to volume manufacturing through standard semiconductor production techniques and subsequently clinical application, this biosensor will enable clinical benefit through early disease detection, and better informed administration of correct therapeutic dose of drugs used to treat α -amylase related diseases.

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1. Introduction

α -amylase was the very first enzyme to be discovered and characterized (Whitcomb and Lowe, 2007) and has been used for the past two centuries as a disease biomarker. α -amylase is a well-established marker for the diagnosis, monitoring of progression and outcome of pancreatic disease, a common cause of hospital admissions with high morbidity and mortality (Seetharaman and Bertoft, 2012). Despite this historic utility, it is only very recently that a substantial expansion of its role into therapeutic and diagnostic applications in other areas of high clinical demand including inflammation, infection, cancer and wound healing has occurred (Azzopardi et al., 2013, 2014, in press; Duncan, 2014). In particular, amylase has been extensively investigated as a trigger for

controlled, bioresponsive release of advanced therapeutics such as dextran conjugates of growth factors, antibiotics, and anticancer agents (Duncan, 2008; Hardwicke et al., 2008). It is also exploited as a replacement enzyme, whilst some of its inhibitors are clinically licenced for the treatment of diabetes and weight management.

Current α -amylase assays in clinical use are laboratory based, utilize analytical equipment with a large footprint, have an appreciable turnaround time (TAT), measure activity rather than concentration, and are susceptible to hemolysis and inactivation. These substantial limitations restrict further expansion of α -amylase based diagnostics and theranostics into viable clinical practice. Moreover, the host of controlled release medications that have been developed on the premise of α -amylase activated release would benefit from rapid, accurate, point-of-care quantification (Hardwicke et al., 2008; Gaspar and Duncan, 2011; Azzopardi et al., 2014, in press). Furthermore, availability of bedside diagnostics would push the scope of these applications from

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secondary into primary care, leading to improved clinical and economic outcomes (Price 2001).

Due to their ease of manufacture, high-sensitivity and potential for miniaturization, electrochemical immunosensors have emerged as a major technology platform for point of care /at the bedside (POC/B) biomarker detection (Tothill, 2009). Electrochemical impedance spectroscopy (EIS) enables detection and signal output due to its ability to measure subtle changes in the electrochemical properties of materials at their interface with conducting electrodes (Ensafi and Karimi-Maleh, 2010; Beitollah et al., 2012; Shahmiri et al., 2013; Moradi et al., 2013; Karimi-Maleh et al., 2013, 2014). Gold, zinc oxide, iron oxide, and carbon are the main substrates that are being developed for use in such sensors (Pena-Pereira et al., 2012). Carbon nanostructures, and more recently graphene, are now being widely adopted as alternatives to gold as electrode substrates (Pumera et al., 2010; Price, 2001; Song and Xuefeng 2012; Wenjing et al., 2013; Toktam et al., 2014). Graphene is of particular interest as a biosensor platform due to intrinsic properties such as its large surface area, high electrical conductivity and biocompatibility (Li et al., 2008; Li et al., 2009; Qin et al., 2011). Graphene based devices have been developed that can measure minute changes in analyte concentration levels, and have been used to detect for cancer tumor markers, human chorionic gonadotrophin (hCG) and markers of cardiac diseases (Yasufumi et al., 2010; Hyun Jung et al., 2010; Nguyen Xuan et al., 2013; Zhang et al., 2015).

Composed of sp^2 carbon, graphene is chemically unsaturated. Intrinsically, it can undergo covalent addition to change the carbons from sp^2 to sp^3 following hybridization, however, carbon atoms in the graphene basal plane are protected by their π -conjugation system, the motion of which is constrained by surrounding carbon atoms. Therefore, basal plane covalent addition usually encounters large energy barriers, and reactive chemical groups, such as atomic hydrogen, fluorine, and pre-cursors of other chemical radicals, are usually needed as reactants. The controlled functional association of biomolecules with graphene is therefore key to developing any high throughput biosensor platform. The chemical modification of graphene is difficult to control, with reactions occurring predominantly at the surface edge thereby limiting surface reactivity and limits of detection (Martin, 2011). Additionally, defects can be introduced to the graphene through functionalization procedures, which are required to immobilize biomolecules to the graphene surface.

Polyaniline (PANI) is a conductive polymer and has been widely used as an additive transducer layer in sensors to avoid the introduction of graphene surface defects (Ates, 2013). In addition the use of PANI improves antibody attachment to sensor electrodes while preserving optimal electrical characteristics (Wang et al., 2011; Ates, 2013). In addition, PANI has excellent acid/base sensitivity, a huge range of tunable conductivity, (Srinives et al., 2015) redox sensitivity, environmental stability (Ruecha et al., 2015; Zhang et al., 2015), short reversible response times (Ruecha et al., 2015), and is easily synthesized (Ruecha et al., 2015) and functionalized. A detailed description of state of the art sensors under development or fully developed is provided in the [Supplementary material](#).

The present work describes an approach toward the development of an α -amylase specific immunosensor, using a combination of electro-polymerization of PANI on a graphene support and subsequent antibody binding to the polymer film. The biosensor platform enables fully quantitative analysis of analyte concentrations in a simulated biological sample and in human plasma. The device displays a linear response to increasing α -amylase concentration between 1 and 1000 International Units/L (IU/L), and a LOD of 0.025 U/L.

2. Experimental section

2.1. Chemicals and materials

All chemicals used were of analytical grade and water was ultrapure grade. Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), *N*-Hydroxysuccinimide (NHS), bovine serum albumin (BSA), Phosphate Buffered Saline (PBS) and aniline were obtained from Sigma Aldrich-UK; α -amylase and anti- α -amylase antibody was purchased from Abcam (UK); A 0.1 M aniline solution was prepared in 2.5 M H_2SO_4 . EDAC, NHS and antibody solutions were prepared in PBS buffer. A redox probe solution was prepared in PBS buffer to a concentration of 5.0 mmol/L of $[Fe(CN)_6]^{3-}$ and 5.0 mmol/L of $[Fe(CN)_6]^{4-}$.

2.2. Apparatus

Electrochemical measurements were conducted with a potentiostat/galvanostat, Metrohm Autolab, PGSTAT302N, controlled by Nova software and equipped with a Frequency Response Analysis module. Graphene-SPEs were purchased from DropSens (DRP-110GPH) and were composed of a carbon counter electrode, an silver pseudo-reference electrode, and a printed graphene working electrode (4 mm $\varnothing$). Electrical characterization of SPEs was performed by connecting the SPEs to the potentiostat/galvanostat via a suitable switch box (DropSens).

Micro-Raman measurements were performed using a Renishaw InVia system with a 100 mW 532 nm excitation laser with approximately 10 mW of power on the sample. Ultra-high resolution SEM measurements were performed using a Hitachi High-Technologies S-4800 and AFM measurements were performed using a BioScope Catalyst™ BioAFM.

Atomic Force Microscopy (AFM) samples were imaged in PeakForce Tapping using a Bruker BioScope Catalyst. NanoWorld Arrow™ NCR cantilevers, with a nominal spring constant of 42 N/m, were used as probes. A total of three regions per sample were imaged, each region having an area of 25 μm^2 . The roughness subroutine in the Nanoscope Analysis software, v1.50, was used. For each 25 μm^2 region the RMS roughness R_q (Equation1) was measured on ten different areas of 1 μm^2 each, according to the procedure described in (Lewis et al., 2009):

$$R_q = \sqrt{\frac{\sum Z_i^2}{N}} \quad (1)$$

where, N is the number of height points in the analyzed area and Z_i is the vertical distance of data point i from the mean image data plane.

2.3. Single molecule force spectroscopy

All experiments were performed using a JPK Nanowizard II AFM (JPK, Berlin, Germany). A two-step chemical procedure was developed in order to link the amylase protein to the AFM probe surface. Briefly, after a 5-min wash in acetone, triangular silicon nitride cantilevers (DNP-10; Bruker-nano, Coventry, UK) were cleaned by immersion in piranha solution ($H_2SO_4:H_2O_2$; 3:1; v/v) for 30 min. The cantilevers were then incubated with 1 mL of 0.1% w/v of APTES pH 7.2 for 10 min, creating an amino-terminated tip surface, which were rinsed several times with PBS and water before incubation with LC-SPDP for 45 min to obtain a reactive pyridyl-disulfide surface. The amylase protein (Abcam, concentration: 300 $\mu g/mL$) was modified by reaction with SATP for 30 min in order to produce a free sulfhydryl group, followed by a series of

purification steps through a dextran column (Fischer scientific). LC-SPDP functionalized cantilevers were then washed and incubated in the presence of thiolated activated amylase, forming an Amylase functionalised probe through a disulfide exchange reaction with SPDP-activated protein (thiol chemistry is less sensitive to hydrolysis in aqueous solution compared to NHS chemistry, and thus enables the use of longer incubation times for efficient conjugation) (Chemical Functionalization and Bioconjugation Strategies for Atomic Force Microscope Cantilevers (Bergkvist and Cady, 2011)).

Set to operate in force spectroscopy mode, amylase functionalised DNP-10 (Bruker Nano, Coventry, UK) probes were used, with nominal spring constants of 0.065 N/m following functionalization (checked using the Thermal tune method). The maximum load force was set at 1.5 nN and the z speed for both extend and retract adjusted to 4 $\mu\text{m/s}$. A force delay of 200 ms was added to ensure contact time between the Amylase protein and the substrate surface. Data was collected from all channels and 768 force curves were collected as a series of 16×16 FV maps, from negative control, non-activated amylase and F_c region activated Amylase substrates to monitor alterations in the adhesions affinity of the device surface. A minimum of 3 biological repeats were used for this study and all data shown is representative of the binding rupture arrays observed.

2.4. Surface modification

The polyaniline (PANI) film was obtained according to our previous work (Teixeira et al., 2014). A drop of activated antibody solution was added to the PANI layer on the working electrode, and incubated for 2 h, at room temperature. The activated antibody solution had been pre-prepared, by incubating a 0.5 mg/mL antibody solution in 25 mmol/L EDAC and 50 mmol/L of NHS, for 2 h, at room temperature. Following exposure of the working electrode to the activated antibody solution the working electrode was then rinsed with PBS and incubated in BSA solution (0.5 mg/mL in PBS), for 30 min. The immunosensor was then washed (3 times) with PBS and kept at 4 °C until use.

2.5. Alpha-amylase binding

Alpha-amylase binding to the immobilized antibody was achieved by placing a drop of alpha-amylase solution on the immunosensor working electrode surface. Different concentrations of alpha-amylase solutions, ranging from 0.01 to 1000 U/L, were prepared by dilution of the 1000 U/mL standard alpha-amylase solutions in PBS or plasma. Alpha-amylase was also detected in human plasma samples and pus samples from patients. A period of 15 min was allowed for antigen/antibody binding. This was followed by PBS washing (3 times) prior to redox probe EIS measurements.

2.6. Electrochemical measurements

CV and EIS assays were conducted in triplicate. CV measurements were conducted in 5.0 mmol/L of $[\text{Fe}(\text{CN})_6]^{3-}$ and 5.0 mmol/L of $[\text{Fe}(\text{CN})_6]^{4-}$, prepared in PBS buffer, pH 7.4, using a potential scan from -0.7 to $+0.7$ V, at 50 mV/s. EIS assays were made using the same redox couple $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution, at a standard potential of $+0.10$ V, using a sinusoidal potential perturbation with amplitude of 100 mV and a frequency of 50 Hz, logarithmically distributed over a frequency range of 1000–0.01 Hz. Impedance data was fitted to a $[R(C[R(RC)])]$ circuit, using the Nova Software.

The immunosensor response to varying alpha-amylase concentrations was assessed by EIS measurements. The Limit of

Detection (LOD) was defined as the alpha-amylase concentration at which the calibration curve corresponded to a signal of 3σ , where σ is the standard deviation of EIS blank signals (obtained in the absence of the alpha-amylase).

3. Results and discussion

3.1. Coating of screen-printed graphene electrode with thin film polyaniline

A screen-printed graphene electrode was functionalized via polymerization with a thin film of polyaniline to provide amine groups on the SPE-graphene/ PANI (Fig. 1(B)). The PANI film was formed by coating the electrode in a solution of aniline and subsequent electropolymerization to form a conductive polymer layer over the graphene support, enabling the transport of electron carriers to the graphene SPE (Li et al., 2009; Bhadra et al., 2011; Zhixiang et al., 2012; Zi-Long et al., 2012).

Having deposited polyaniline, the sensor was then functionalized through covalently linking a α -amylase antibody to the PANI layer (Fig. 1(C)). A carbodiimide crosslinker chemistry EDAC/NHS was used for the specific activation of the $-\text{COOH}$ terminated amino acid chains in the antibody. This forms a highly reactive *O*-Acylisourea intermediate that rapidly reacts with NHS to produce a stable succinimide ester (Kuiyang et al., 2004). This ester then undergoes a nucleophilic substitution reaction with the amine groups on the PANI, leading to the formation of an orientated antibody grafted PANI layer (Fig. 1(C)). Due to exposure to the EDAC/NHS reagents, it is possible that each of $-\text{COOH}$ groups at the

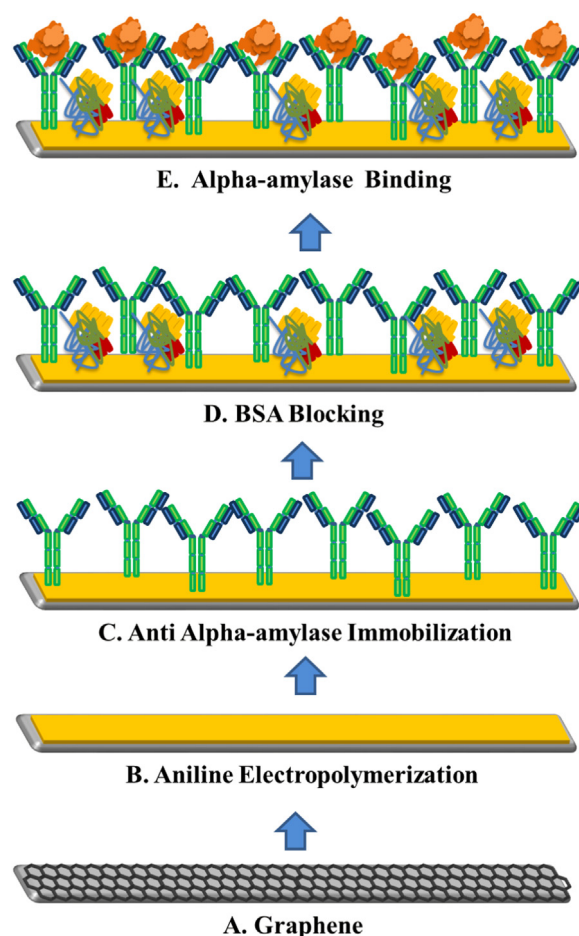


Fig. 1. Schematic diagram for the immunosensor assembly.

antibody may have been activated. It is not possible therefore to ensure exclusive antibody binding through the F_c region, only that orientation *via* the F_c region is significantly higher than with random antibody adsorption. Bovine serum albumin (BSA) was then added to the sensor surface randomly (Fig. 1(D)), and serves to prevent any non-specific interactions with the sensor surface thereby eliminating the possibility of the sensor generating any non-specific background signal.

3.2. Single molecule force spectroscopy

In order to demonstrate the stability of antigen–antibody binding during the device development process single molecule force spectroscopy (SMFS), using an amylase-functionalized silicon nitride cantilever was employed (Cao et al., 2007; Fuhrmann and Ros, 2010; Chunmei et al., 2014). A short LC-SPDP (sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate) linker molecule was used to permit free rotation of the immobilized amylase protein on the probe, mimicking amylase mobility at the substrate surface in solution (Ebner, 2007; Neundlinger et al., 2011). Following dynamic ramping of the α -amylase probes to the sensor surface, the force distance curve data was used to determine the stability of the covalent cross linking at the PANI antibody interface and its effect on sensor affinity for its target analyte. Fig. 2, shows that both the 'random adsorption (non-activated Ab)' and 'EDAC/NHS linker (F_c activated Ab coupling)' substrate surfaces resulted in a high number of specific force rupture events in the AFM retraction curve (47% and 53% of total curves respectively) demonstrating retention of antibody function on the PANI-graphene surface.

A significantly higher percentage (40%) of rupture events occurred at a force above 500 pN with the 'EDAC/NHS linker (F_c activated Ab coupling)' sample compared to just 16% for the random adsorption (non-activated samples) (Fig. 2). This indicates that EDAC/NHS coupling achieves an increased percentage of F_c immobilized antibodies compared to the random adsorption method.

3.3. Qualitative analysis of the immunosensor surface

Throughout the sensor development process a series of surface characterization techniques were used to monitor the step-wise assembly of the device to inform process optimization.

Raman Spectroscopy (RS) – Raman spectra indicate the chemical nature of the surface through molecular vibrational transitions. RS was used here to provide information on the structure, carbon atom hybridization state, defects, functionalization and graphene layer depth during the fabrication stages (Fig. 3) (Nafie, 2001). Control graphene-SPE spectra showed three Raman bands at 1350, 1600 and 2700 cm^{-1} that are assigned to the well-documented D, G and 2D bands in standard graphene (Elias et al., 2009). The G

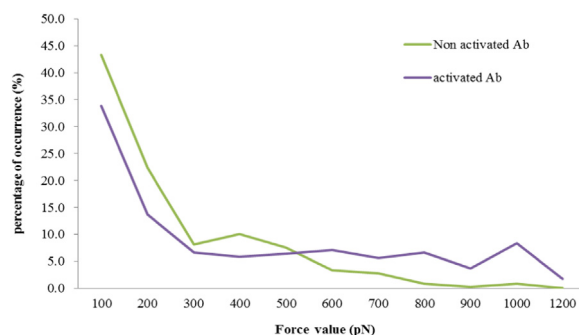


Fig. 2. Percentage of occurrence of steps according to their force values for the activated antibody (Ab) compare to the non-activated antibody.

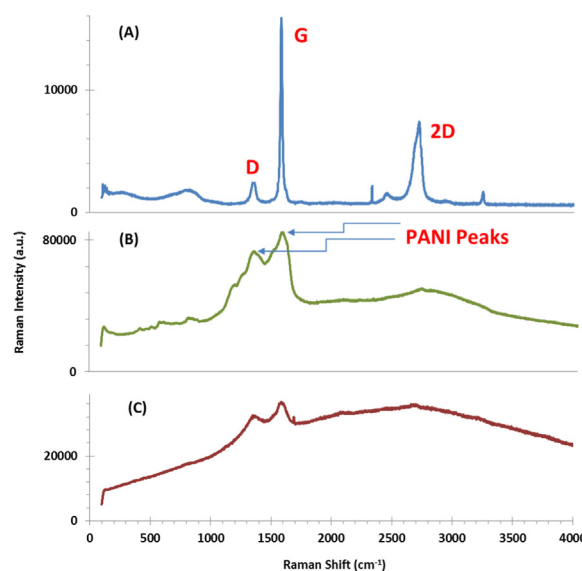


Fig. 3. Raman spectra of (A) graphene-SPE, (B) PANI/graphene-SPE and (C) anti- α amylase/PANI/graphene-SPE.

band represents the in-plane bond-stretching vibrations of sp^2 -hybridized carbon atoms, while band D is related to the vibrations of the carbon atoms of sp^3 -hybridized carbon atoms of disordered and/or defected graphite (Elias et al., 2009). The intensity ratio of I_G/I_D bands can be used to quantify the defect density in graphene. The I_G/I_D ratio measured on the sensor surface for graphene was 1.15 and 1.18 respectively (Fig. 3(A) and (B)) indicating significant disorder as a result of structural defects (Ding et al., 2013).

Similar spectra were obtained from both SPE-Graphene/PANI (Fig. 3(B)) and SPE-Graphene/PANI/Ab (Fig. 3(C)) surfaces, showing an increased intensity of D peak, corresponding to an I_G/I_D ratio equal to 1.16 when compared to the control. After electrochemical polymerization, all of the Raman spectra exhibited distinct bands attributable to PANI, (Da-Wei et al., 2009) which overlap the characteristic bands of standard graphene (at 1300 and 1600 cm^{-1} ; Fig. 3(B)). The sp^3 graphene peak became more intense and broad following PANI deposition, (Liu et al., 2012) resulting in a significant increase in the intensity ratio of bands D/G. Antibody binding therefore introduced an additional increase in the Raman intensity (Fig. 3(C)).

3.4. Scanning Electron (SEM) and Atomic Force Microscopy (AFM)

The morphology, topography and structure of the SPE-graphene following each fabrication stage were also characterized using both SEM and AFM (Fig. 4).

Unmodified graphene exhibits a flat, smooth area with few significant topographical features (Fig. 4(A)). The addition of PANI results in the introduction of a fibrous structure following the electropolymerization process was observed at both the micro and nanoscale (Fig. 4(B)) which demonstrates that the polymer is in a crystalline form (Kavitha et al., 2012). Following the functionalization with the anti- α -amylase antibody, the fibrous structure becomes uniformly coated with globular antibody clusters (Fig. 4(C)).

Surface roughness (R_q) analysis using AFM further demonstrated the reproducibility of each stage of the fabrication process. (Fig. 4(D)). Non-functionalized graphene exhibited lower R_q values of 13.5 ± 6.36 nm. The addition of PANI resulted in a significant increased R_q of 81.9 ± 14.8 nm ($P < 0.001$), and following antibody addition, the surface R_q values significantly decreased to

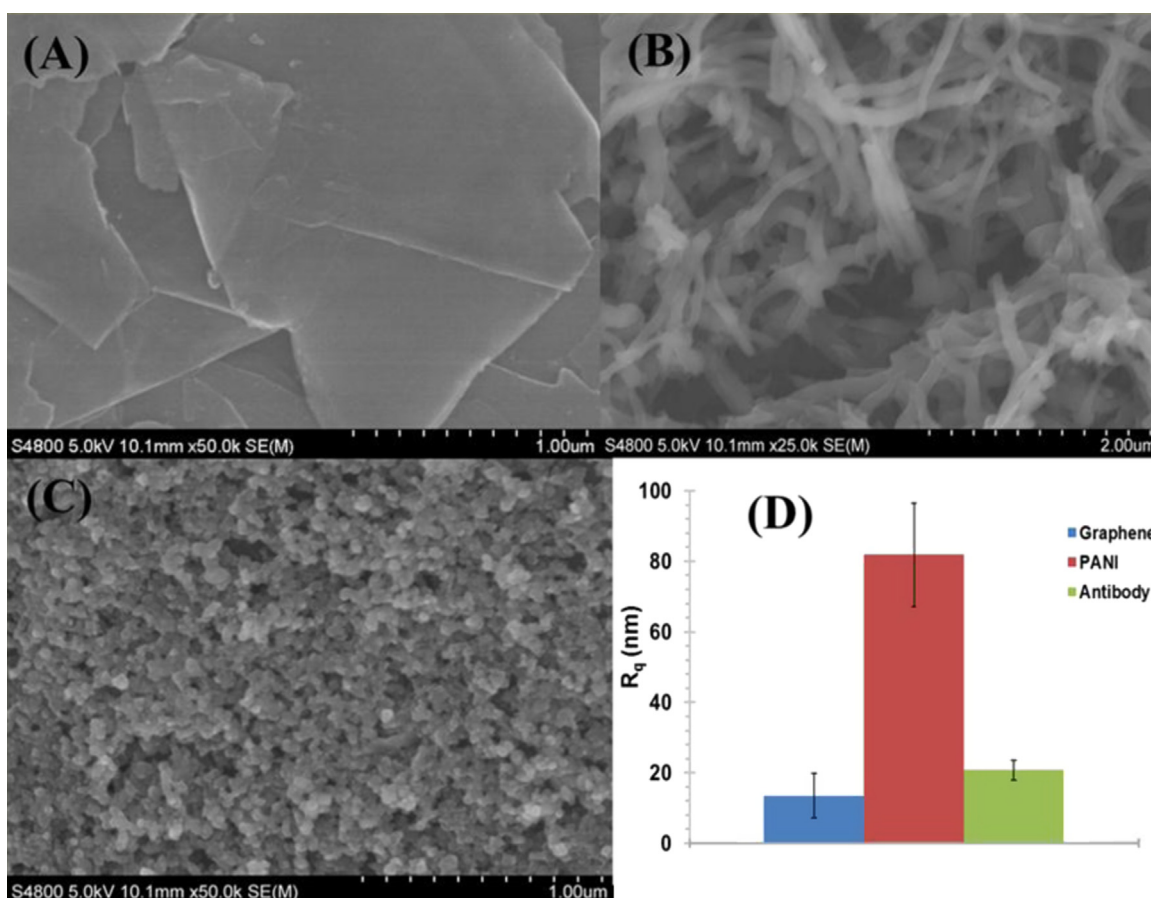


Fig. 4. SEM images of: (A) unmodified graphene; (B) graphene modified with PANI (C); Antibody attached to the PANI layer and (D) Plot of the roughness R_q for the different samples: Graphene $R_q = 13.5 \pm 6.36$ nm; PANI $R_q = 81.9 \pm 14.8$ nm; Antibody $R_q = 20.8 \pm 2.86$ nm.

20.8 ± 2.86 nm ($p < 0.001$).

3.5. Electrochemical characterization

The effects of modifications at the graphene surface were monitored using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) respectively, in order to characterise changes in electron transfer properties against the redox probe (Jonathan and Nader, 2007). Nyquist plots were used to show the frequency response of the electrode/electrolyte system and an area plot of the imaginary component (Z'') of the impedance against the real component (Z') (Fig. 5). The charge-transfer resistance (R_{ct}) at the electrode surface is depicted by a semi-circular output plot obtained from EIS that can be used to define the interface properties of the electrode.

As shown in Fig. 5(A) a peak-to-peak potential difference (ΔE_p) and peak-to-peak current difference (ΔI_p) of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple show the changes at each step of the surface modification of graphene-SPE. The unmodified graphene-SPE shows a quasi-reversible electrochemical response for the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple with ΔE_p of 0.164 V and ΔI_p of 0.248 mA. The modification of graphene-SPE surface with PANI in a ΔI_p increase of 0.139 mA and a ΔE_p decrease of 0.241 V. This result is attributed that a positively charged amino group of the PANI molecule which attracts the negative charge of $[\text{Fe}(\text{CN})_6]^{3-/4-}$, causing an easy electron transfer reaction on the electrode surface (Erhan et al., 2013).

A cyclic voltammogram of the SPE-graphene/PANI/Ab electrode showed a decrease peak-to-peak potential separation (ΔE_p of 0.088 V). Further, addition of the BSA blocking agent to the SPE-

graphene/PANI/Ab electrode surface gave rise to a change on the electrochemical behavior of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ couple, leading to ΔE_p increase of 0.03 V and decreased ΔI_p value of 0.067 mA. BSA molecules cause masking of the electrode surface for oxidation/reduction of the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (Heli et al., 2007).

The unmodified graphene surface shows a very fast electron-transfer process ($R_{ct} = 572.5 \Omega$, Fig. 5(B)). Following the electrochemical deposition of PANI onto the graphene-SPE a similar resistance was obtained (lilac curve, $R_{ct} = 624.5 \Omega$), indicating that the PANI is an excellent electric conducting material which accelerated the electron transfer process. After covalent attachment of anti- α -amylase antibody, the R_{ct} increased to 866.2Ω (green curve), demonstrating that anti-alpha amylase was successfully immobilized on the PANI surface and blocked the electron exchange between the redox probe and the electrode. The R_{ct} increased further when BSA was added to the SPE-graphene/PANI/Ab (red curve, $R_{ct} = 1454.7 \Omega$), due to the nonconductive nature of the protein.

3.6. α -amylase measurements in phosphate buffered saline (PBS), mouse and human plasma

PBS – Having determined the electrochemical properties of the functionalized sensor, the sensor was tested for its ability to measure amylase concentrations in a phosphate buffered solution that was used as a blood plasma mimic. Increasing amylase concentrations, from 1 to 500 U/L, which covers the clinically relevant range of amylase levels in the human body, were applied to individual sensors. Both Nyquist plots (Fig. 5(C)) and the resulting EIS calibration curve (Fig. 5(D)) clearly demonstrate the effective

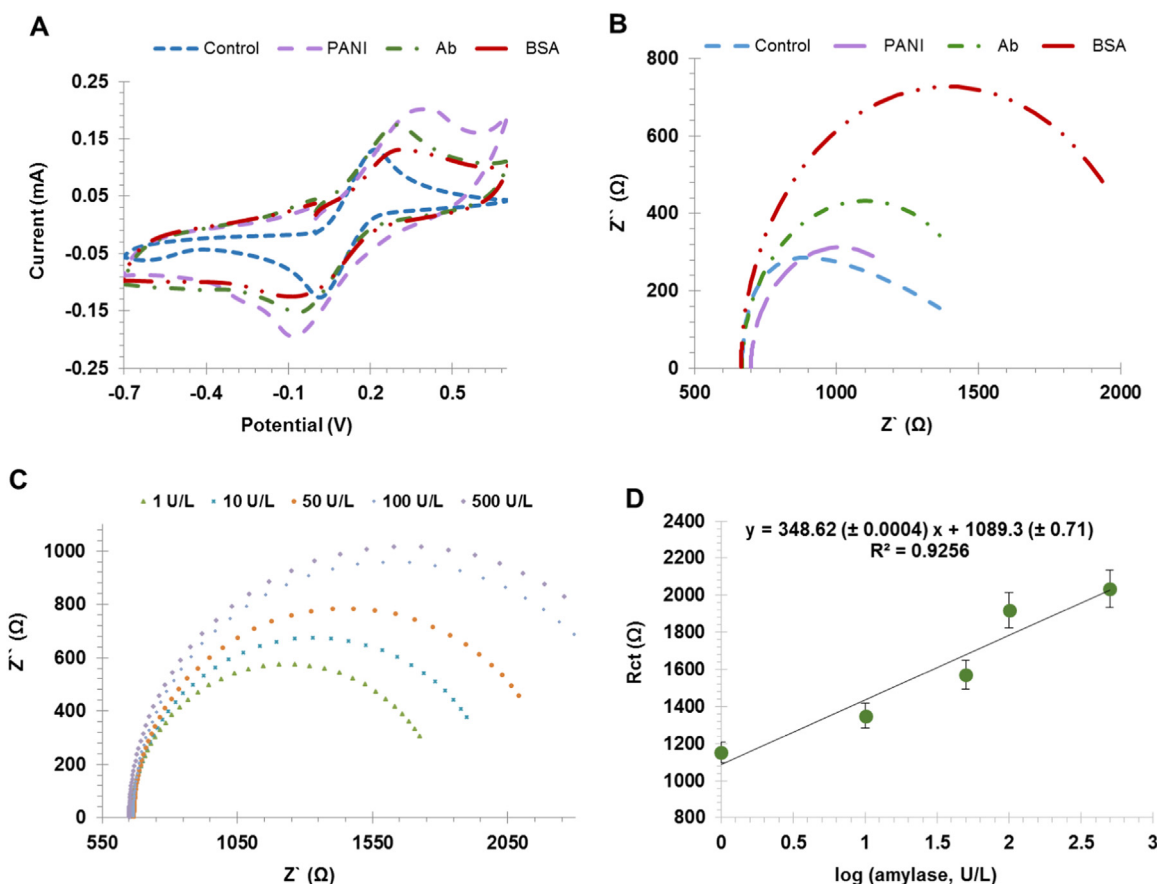


Fig. 5. CV data taken at each assembly stage of the immunosensor (A), EIS spectra of each stage (B). (A) CV record after modification of PANI/graphene-SPE with antibody and BSA. (B) Nyquist plots of BSA/anti- α -amylase/PANI/graphene-SPE sensor, obtained in 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ PBS buffer pH 7.4. EIS spectra of calibration along with its calibration plot (C) and (D). (C) Nyquist plots of the BSA/anti- α -amylase/PANI/graphene-SPE sensor, previously incubated in increasing concentrations of amylase, obtained in 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ PBS buffer pH 7.4; (D) the R_{ct} values of the previous calibration plotted against $\log$ amylase concentration, with a standard deviation of 13.929%. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

response of the immunosensor to increasing amylase concentrations.

The diameter of the semicircle increased with increasing amylase concentrations demonstrating an increased resistance as a result of increased analyte concentration at the sensor surface. In general, the change in the semicircle diameter is a result of the change in the interfacial charge transfer resistance (R_{ct}); that is, the resistance corresponding to the carrier transfer from the modified electrode to the ferricyanide in the solution. Thus, the observed diameter increase is explained as the adsorption of plasma onto anti- α -amylase following an antigen–antibody reaction, where the adsorption of plasma effectively blocks the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ leading to an increase of R_{ct} . The R_{ct} in the Nyquist plot increased linearly with the amylase concentrations. This is as expected because protein structures bound to the surface of an electrode typically act as barriers to electric transfer. The average slope of the R_{ct} versus $\log[\text{amylase, U/L}]$ was 0.348 $\text{K}\Omega/[\text{amylase, U/L}]$ with an R^2 coefficient of determination of 0.93. The limit of detection (LOD) was 0.025 U/L. This was as expected as protein structures bound to the surface of an electrode typically act as barriers to electric transfer.

3.7. Mouse plasma

In order to determine whether sensor function was effective when exposed to a complex biological fluid, mouse plasma containing known quantities of amylase were used. α -amylase concentrations were measured in un-spiked and plasma samples with

purified human- α -amylase. The linear response ranged from 1 to 1000 U/L and the average slope was of 0.472 $\text{K}\Omega/[\text{amylase, U/L}]$. The EIS calibration curve of the immunosensor in response to increasing concentrations of amylase in mouse plasma showed no adverse effect on sensor performance in the presence of a more complex fluid compared to the PBS (Fig. 6(A)).

3.8. Human plasma

To provide an initial demonstration of the potential clinical utility of the device, human plasma samples obtained from a patient suffering with clinical infection were analyzed. By spiking the human plasma with known concentrations of amylase we were able to determine the effectiveness and LOD of the sensor at physiologically relevant analyte concentrations.

Nyquist plots (Fig. 6(B)) of EIS spectra show the dynamic range detectable when the sensor was exposed to various human plasma concentrations (9.9–1009.9 U/L).

In the development model used for this pilot study, the maximum interval to obtain a result was 300 s. In clinical practice, turnaround times (TAT) is a key indicator of laboratory performance (Chauhan et al., 2014). The current TAT for clinical assays in current practice is 45 min (Fei et al., 2015). Such studies, however, invariably omit the time taken for phlebotomy, transport of samples, and the interval from when samples are available to when they are picked up and interpreted by the attending physician. This is important when considering diseases whose mortality and morbidity increases with delayed diagnosis and clinical decision

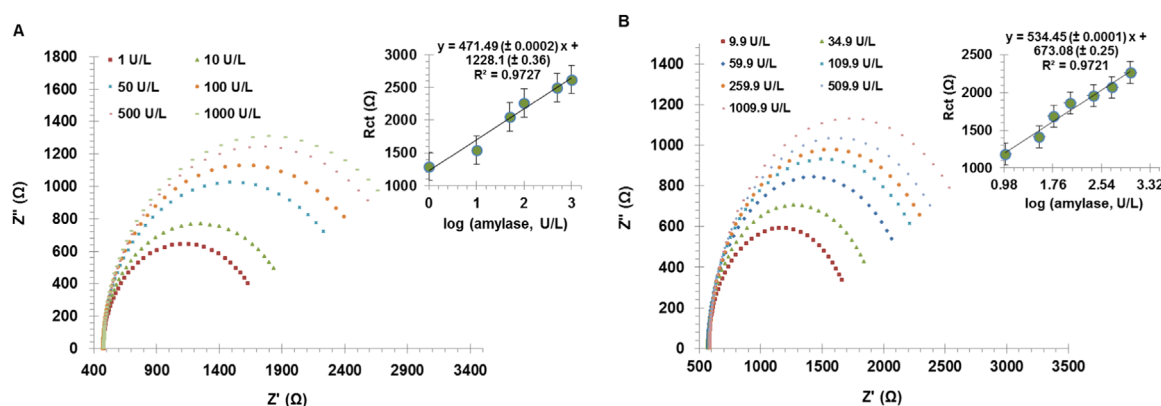


Fig. 6. (A) Nyquist plots of BSA/anti- α amylase/PANI/graphene-SPE sensor, in 5.0 mM [Fe(CN)₆]^{3-/4-} mouse plasma, previously incubated in increasing concentrations of amylase. Inset (A): corresponding calibration curve, plotting log(amylase) against R_{ct}, with a standard deviation of 3.42%. (B) Nyquist plots corresponding to the calibration of the immunosensor in human plasma with amylase standards in U/L. Inset (B): corresponding calibration curve, plotting log(amylase) against R_{ct}, with a standard deviation of 12.987%.

times (Debi et al., 2013). With optimization and miniaturization of the developmental model used in these studies, it is expected that the 300 s interval from loading a sample to obtaining a result will be substantially shortened. Moreover hemolysis, sample inactivation, and presence of contaminants interfering with colorimetric assays commonly lead to assay failure with current clinical assay systems. Since the system described in this study is label-free, and does not require either colorimetric change, it is reasonable to support the notion that the new technology will not be affected.

Finally, it is worth noting that the results indicate a three-log fold expansion in the lower limit of quantification when compared to current clinical assay systems (Bowling and Katayev, 2010; Federal Drug Agency (FDA), 2015). This is of interest to forensic medicine where detection of amylase can lead to a DNA profile from saliva, semen or vaginal secretions (Casey and Price, 2010).

4. Conclusion

The graphene-based label-free immune sensor we describe in this study represents a step change in the quantification of α -amylase. Quantitative, highly sensitive amylase sensing is achieved, with a wide limit of detection (0.025–1000 IU/L using spiked human blood plasma samples. Unlike current clinical assays, this technology is not based on enzymatic activity and/or colorimetric change, and would therefore not be affected by hemolysis, structural or functional inactivation of the amylase enzyme itself. The sensor design is compatible with miniaturization to a bedside diagnostic, avoiding the requirement for laboratory set-up, and the associated turnaround times, enabling the potential for a clinically compatible technology with a log fold expansion in the usable limits of quantification. The potential for a substantially more cost-effective, fully portable test that can be administered by the bedside, and the scalability for this diagnostic technology that can be achieved through standard semiconductor manufacturing techniques, would also expand the remit of such diagnostics beyond the secondary care sector, whilst avoiding the need for formal phlebotomy. Whilst retaining the potential for substantial cost-saving, and the possibility for expansion to multiplex and re-usable systems, the manufacture process is straightforward and easily applicable to any target molecule for which an antibody can be manufactured.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2016.05.034>.

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