Electrochemical impedance spectroscopy enabled CA125 detection; toward early ovarian cancer diagnosis using graphene biosensors

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Abstract
With current diagnostic methods detection of stage 1 or 2 ovarian cancer using CA125 is possible in only 75% of cases. The ability to detect CA125 at lower concentrations could significantly improve such early stage diagnosis. Here, the use of screen-printed graphene biosensors as a label-free detection platform for CA125 was evaluated. The sensor was fabricated through deposition of a polyaniline layer via electropolymerisation on to a graphene screen-printed electrode. The sensor surface was functionalised with anti-CA125 antibody via covalent cross linking to polyaniline. The fabrication process was characterised through cyclic voltammetry and electrochemical impedance spectroscopy. The limit of detection achieved was 0.923 ng/μL across a dynamic range of 0.92 pg/μL-15.20 ng/μL and represents the most sensitive CA125 detection reported to date. With sensitivity limits at this level, it will now be possible to conduct clinical trials using serum samples collected from early stage ovarian cancer patients and at risk individuals.

Introduction
Ovarian cancer (OC) is the seventh most common cancer type in females, with 7270 cases reported in the UK in 2015 and 239,000 reported worldwide, and has poor survival and prevention rates (35% and 21%, respectively) (Ovarian cancer statistics [Internet], 2017). The majority of OC patients are diagnosed with either stage 3 or 4 disease resulting in a 5-year survival rate of 27% and 16%, respectively. These poor outcomes have been the main driving force for the continued development of early detection strategies using the well-established serum biomarker CA125 (Menon et al., 2009).
With stage 1 survival rates at approximately 90%, detection of the early stage disease remains paramount (Cancer, 2017). Despite known OC risk factors include age, late menopause, early menarche and familial traits including a history of ovarian, breast, uterine, or colon cancer, 85% of OC cases are sporadic (Cancer, 2017). Currently, only 25% of OC patients are diagnosed with stage 1 or 2 disease, which involves repeated ultrasonography screenings to observe changes to the ovaries, and still requires the patients CA125 levels to be analysed ([Internet], 2017).

CA125 (MUC16) is expressed at higher concentrations in patients with epithelial OC. It was first described in 1981 through its detection by the OC125 monoclonal antibodies by Bast et al. (Teixeira et al., 2016a). Whilst CA125 remains the mainstay of OC diagnosis and treatment monitoring, it is limited in its utility as it does not allow the detection of all OCs. In stage 1, only 50% of patients have elevated CA125, which raise to between 75-90% in more advanced cases approximately, with false positives in both malignant and benign cases of the disease (Felder et al., 2017a). Despite this, in the absence of new or additional markers, CA125 remains the gold standard for OC diagnosis. MUC16 is a large 3-5 million Da glycoprotein composed of a tandem repeat region located between the N-terminal and C-terminal (membrane anchor) domains of the protein (Database G, 2017). MUC16 can be cleaved adjacent to the transmembrane region and released where it can be detected as a serum biomarker (Felder et al., 2017b). Recent studies have described the distribution of OC125 epitopes and differential antibody recognition throughout the tandem repeat region, which is dependant on conformational structure, and may influence recognition events (Gubbels et al., 2017).

Biosensors are devices that consist of a biological component (e.g., an antibody) that reacts with a target substance and to produce an output - the transducer (e.g., electrochemical) (Bressan, Bozzo, Maggi, & Binaschi, 2017). Point-of-care devices are at the forefront of biosensor development due to their potential to improve patient care through real time and remote (away from hospital) testing. Requirements for a successful point-of-care device include rapid label free detection, portability (Mclemore, Aouizerat, & Miaskowski, 2017), together with the ability to use only small volumes of sample (e.g., urine, blood, or serum), and, critically, a high level of specificity and sensitivity (Dorothee Grieshaber, 2017).

Graphene is sp2-hybridised carbon atoms arranged hexagonally in a one-layer thick sheet (Nichkova et al., 2017). It is an excellent material for use in sensors due to properties such as a high surface area, excellent conductivity, and high mechanical strength compared with similar materials (David & Armbruster, 2017). Graphene also has a low charge transfer analysis resistance compared with other carbon materials that enable well-defined redox peaks to be obtained when carrying out cyclic voltammetry (CV) analysis indicating fast electron transfer and diffusion controlled redox reactions (Song, 2017).

Polyaniline (PANI) has structural flexibility, chemical and electrochemical stability, controllable chemical and electrical properties, and low cost. PANI can be a conductive polymer due to the delocalised electron bonds and wide band gaps produced by oxidation of the molecule. In immunosensors, PANI can act as an electron transfer mediator improving the sensitivity of the device (Pumera, 2017).

We have developed a graphene sensor modified by electropolymerisation capable of detecting CA125 at low concentrations that could significantly improve early stage diagnosis. The immunosensor was produced via modification of a disposable graphene screen-printed electrode (SPE) with an amine layer that preserves the chemical structure of graphene and allows subsequent surface functionalisation with antibodies for the detection of CA125. The sensor showed a wide linear range from 0.92 pg/μL-15.2 ng/μL. A limit of detection (LOD) of 0.923 ng/μL was achieved that is lower than other current biosensing techniques and delivers the level of sensitivity suitable as early OC detection and screening of at risk individuals.

Material and Methods

Reagents and solutions

All chemicals used were of analytical grade and water was ultrapure grade. Aniline solution, sulphuric acid (H2SO4), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), N-hydroxysuccinimide (NHS), potassium hexacyanoferrate III (K3[Fe (CN)6]), potassium hexacyanoferrate II (K₄[Fe (CN)₆]₃) trihydrate, phosphate buffered saline solution (PBS), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (UK). CA125 protein and CA125 antibody were purchased from Bio-Teche and Thermofisher Scientific, respectively. PBS was prepared in a ratio of one tablet (1814.5-2005.5 mg/tab) of PBS in 200 mL of D.I. Water, pH 7.4. EDAC (25 mM), NHS (50 mM), BSA, and the electrolyte solutions were all prepared in PBS buffer.
Apparatus
Electrochemical measurements were conducted with a potentiostat/galvanostat (Autolab). The potentiostat/galvanostat was controlled with NOVA software and possessed a Frequency Response Analysis module. A switch box was used to provide an interface to connect the graphene screen-printed electrode (Gr-SPE) to the potentiostat/galvanostat. Gr-SPEs were purchased from DropSens/Metrohm (Asturias, Spain) (DRP110-GPH). 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 scanning electron microscope measurements were performed using a Hitachi High-Technologies S-4800.

Electrochemical assays
Cyclic voltammetry measurements were conducted in 5.0 mmol/L of [Fe(CN)6]3– and 5.0 mmol/L of [Fe(CN)6]4–, prepared in PBS buffer (pH 7.4). In CV assays, a potential sweep from −0.7 to +0.7 V at 50 mV/s was used. All assays were conducted in triplicate.

Electrochemical impedance spectroscopy (EIS) assays were also conducted in triplicate with the same redox couple [Fe(CN)6]3–/4– at a standard potential of +0.10 V, using a sinusoidal potential perturbation with amplitude of 100 mV and a frequency equal to 50 Hz, logarithmically distributed over a frequency range of 1000–0.05 Hz.

Surface modification
The PANI film was obtained according to our previous work (Teixeira et al., 2016b; Teixeira, Conlan, Guy, Sales, & Mater, 2014). In parallel, the antibody solution was mixed with 25-mmol/L EDAC, and 50 mmol/L of NHS, and incubated for 2 h at room temperature (RT). A 10 μL of this resulting solution was then deposited on the PANI/Gr-SPE surface. After 2 h at RT, the electrode was rinsed, BSA (0.5 mg/mL in PBS buffer) solution added and incubated for 30 min. The immunosensor was then washed three times with PBS buffer.

CA125 binding
CA125 binding to the antibody already immobilised on the immunosensor was achieved by placing 10-μL CA125 solution on the sensor surface. Different concentrations of CA125 solutions, ranging from 0.00096 to 15.2 ng/μL, were prepared by serial dilution of the 1 mg/mL standard CA125 solution in PBS. Samples were incubated at RT for 15 min for equilibration of antigen/antibody binding and then washed three times with PBS prior to redox probe EIS measurements.

Results and Discussion
Sensor validation
Chemical modifications at graphene surface were monitored using CV and EIS to validate each stage of the fabrication process (Fig. 1), characterising changes in electron transfer properties against the redox probe (Biosensors: Types and General Features of Biosensors [Internet], 2017). The EIS data were analysed by Nyquist plots to show the frequency response of the electrode/electrolyte system and area plot of the imaginary component (Z″) of the impedance against the real component (Z′). The charge-transfer resistance (Rct) at the electrode surface is given by the semicircle diameter obtained in EIS and can be used to define the interface properties of the electrode.

The CV of the PANI process can describe the formation of the PANI layer. With an increase in cycles being attributed to the thickness of the PANI on the SPE, and with current density increasing at the oxidation peak sites (Amaral et al., 2007). The increases in oxidation peaks indicate an increase in the conductivity of the PANI layer. Due to the lack of redox peaks in the latter stages of electrodeposition, the oxidation of the PANI is irreversible.

Following the PANI electropolymerisation process, a decrease in peak to peak current was observed (Fig. 1A). An initial insulating form of PANI (leucoemeraldine) can be oxidised into a conducting form (emeraldine base), however, further oxidation will revert the material to an insulating state (pernigraniline). We can assume this as current is still able to pass through the sensor. Due to the conductive properties of the emeraldine salt and the electrostatic interaction between the negatively charged electrolyte ions in the electrolyte and the positively charged amino groups on the PANI surface layer, the ion diffusion layer can be reduced (Linear Sweep and Cyclic Voltametry, 2017). Furthermore, with the introduction of CA125 antibody and BSA, current flow is reduced once again. This is due to electron transfer from the electrolyte to the electrode being obstructed by the introduction of these large non-conductive organic structures reducing current flow (Bonanni, Loo, & Pumera, 2012). Using CV analysis, chemical modifications of the active graphene electrode on the SPE sensor were monitored. The
electron transfer properties against the redox probe were characterised from the change in surface chemistry by these processes; with the addition of each layer to the sensor, the electron transfer capabilities of the SPE reduces. Rct values of 75.18 (PANI), 113.66 (AB), 165.56 (BSA) were recorded. It should be noted that the emeraldine PANI allows for a large current to flow through the sensor due to its conductive properties.

Immunosensor Raman surface analysis

Raman spectroscopy was used at each stage of development to characterise the surface of the sensor enabling optimisation of all steps of the process. The chemical nature of the surface, determined through molecular vibrational transitions, provides information on the structure and defects of graphene during the fabrication process (Fig. 2).

The Gr-SPE control shows three distinct Ramon peaks at 1350, 1600, and 2700 cm\(^{-1}\) that have been well described in literature (Elias et al., 2009). They are assigned to the D, G, and 2D peaks. To quantify the defect density on graphene, the intensity ratio of I\(_G\)/I\(_D\) bands can be used. The I\(_G\)/I\(_D\) ratio for the Gr-SPE control was 1.16 (Fig. 2, blue spectra) indicating structural defects. After PANI deposition, an increase in the I\(_G\)/I\(_D\) peak was observed when compared with the control showing an I\(_G\)/I\(_D\) ratio of 1.29 (Fig. 2, red spectra). Compared with Graphene, the sp\(^3\) peak of PANI showed a slight shift to higher frequencies, probably due to the electrostatic interaction between aniline monomers and Gr-SPE, both displaying aromatic features.

Overall, the obtained Raman spectra indicated the successful modification of the Gr-SPE, which considering the chemical modifications suggests the formation of a PANI film.
Scanning electron microscopy

The morphology of the SPE-graphene following each fabrication stage was also characterised using scanning electron microscope (Fig. 3).

Unmodified graphene exhibits a flat, smooth area with few significant topographical features (Fig. 3A). The addition of PANI by electropolymerisation resulted in the formation of a fibrous structure, observable at both the micro and nanoscale (Fig. 3B) and demonstrates that the polymer is in a crystalline form.

Analytical performance of the sensor

CA125 across a concentration range of 0.00096 to 15.3 ng/μL was used to determine the functionality and dynamic range of the anti-CA125 antibody functionalised PANI/Gr-SPE sensor. Sensor outputs are shown on Nyquist plots (Fig. 4A) and a corresponding EIS calibration curve, plotting \( R_{ct} \) Ab/PANI/Gr-SPE against the logarithm of CA125 concentration (Fig. 4B). No diffusion controlled effect was observed in the EIS spectrum, and the diameter of the semicircle increased with increasing CA125 concentrations.

The diameter of the semicircle increased with increasing CA125 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. This is as expected because protein structures bound to the surface of an electrode typically act as barrier to electrical transfer. Thus, the observed diameter increase is explained as the adsorption of CA125 onto anti-CA125 following an antigen-antibody reaction, where the adsorption of CA125 effectively blocks the \([Fe(CN)6]^{3-/4-}\) leading to an increase of \( R_{ct} \). The average slope of the \( R_{ct} \) versus \( \log [CA125] \) was 0.63 KΩ/[CA125, ng/L] with an \( R^2 \) correlation coefficient of 0.98. The LOD was determined to be 0.923 ng/μL.

Conclusion

The detection of CA125 provides an insight into the behaviour of OC. Monitoring the increase or decrease of glycoprotein level has been shown to have an 87% of accordance with the progression or regression of ovarian tumours, respectively. The aim of this work was to fabricate and characterise a Gr-SPE immunosensor for the detection of CA125 in PBS solutions. The device managed to achieve a LOD of 0.923 ng/μL across a dynamic range of 0.96 pg/μL to 15.2 ng/μL. The device afforded a more sensitive for CA125 than other devices that have been developed for this purpose. First generation immunoassays were based upon the use of OC125-like monoclonal antibodies as both capture and detection antibodies (Scholler & Urban, 2007). Currently, the quantification of serum CA125 levels is tackled using second-generation tests by means of antibodies from two distinct groups, OC125-like and M11-like (Scholler & Urban, 2007). CA125 II™ developed by Abbott is a second-generation chemiluminescent microparticle immunoassay for the detection of CA125. The assay has a sensitivity of 1.0 U/mL and specificity of 12%. A CA125 enzyme-linked immunoassays kit is also available from Phoenix Pharmaceuticals, which detects a minimum concentration of 5 U/mL using horseradish peroxidase. Assays from a variety of manufacturers have been compared by Davelaar et al. (Davelaar, Kamp, Verstraeten, & Kenemans, 1998). Commercially available multi-analyte tests include immunoradiometric assays, enzyme-linked immunoassays, and chemiluminescent microparticle immunoassay provided by a range of companies and have been extensively compared by Sharma

Figure 3. Scanning electron microscope images of (A) unmodified graphene; (B) graphene modified with polyaniline.
et al. (Kenemans, Verstraeten, Vankamp, & Vonnensdorff-Pouilly, 1995; Sharma, Raghav, O’Kennedy, & Srivastava, 2016). Whilst sensitivity has greatly improved with the second-generation assays, current commercial assays are long, expensive, and need to be handled by experts making them inadequate point-of-care tools. Furthermore, it is important to highlight that the current use of CA125 is focused on disease monitoring rather than early detection because the detection of biomarker is not sufficiently sensitive or specific (Duffy et al., 2005; Scholler & Urban, 2007). Currently, a large area of scientific interest concentrates on the use of CA125 and complementary biomarker quantification as tools for early cancer diagnosis. This study highlights the benefits of using semiconducting polymers and graphene electrodes for the detection of a CA125. Overall, the analytical performance of the biosensor suggests its potential for successful application as a point-of-care diagnostic device.

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Conflict of Interest

None declared.

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