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Label-free electrochemical biosensors for clinical diagnostic

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Abstract—We present the development of a high sensitivity, label-free, biosensor platform suitable for multiplexed point-of-care diagnostics. A sensor surface based on a carboxy-terminated oligo ethylene-glycol (OEG) self-assembled monolayer (SAM) was developed and fully characterised. Optimal conditions for antibody immobilisation were found for a buffer pH approximately one unit below the pI of the antibody, which yielded both higher antibody density on the sensor surface as well as higher sensor response to the antigen. At the same time the surface showed good resistance to non-specific adsorption of high concentrations of proteins. A non-faradaic electrochemical impedance spectroscopy biosensor to detect human chorionic gonadotropin (hCG) in full serum was demonstrated as a proof of concept. By using the phase of the impedance at 100 mHz as the sensor response, a linear relationship of the phase shift vs the logarithm of hCG concentration was established between 26 fM and 0.26 nM with a sensitivity of 0.6 degree per decade, which is a significant improvement over current state-of-the-art biosensor systems.

Keywords—Biosensors; Label-free; Impedance Spectroscopy; hCG.

I. Introduction

The monitoring of human health through sophisticated in vitro diagnostic devices is becoming pivotal in a society placing increasing importance on both disease prevention, and on stratified and individualised patient care. However, current diagnostic services are under intense pressure to meet these emerging healthcare requirements. In recent years, the demand of clinical analysis and monitoring of biological samples has been moved from large clinical laboratories to point-of-care (POC), i.e. less equipped locations, such as bed-side care in hospitals, nursing homes, or the patient's home itself [1]. As a consequence POC tests are usually carried out by personnel with little or no specific training [1]. Therefore, it is critical for the instrumentation to be self-contained, portable and easy-to-use. At the same time, as demands for early diagnosis keep rising, the demand for devices that can assist with routine check-up procedures and that can be used to screen for a multitude of risk-factor indicators is increasing. To achieve these goals, the time-to-result and sample manipulation procedures need to be reduced and simplified, respectively, and innovative diagnostic technologies are required to meet these emerging healthcare needs.

A biosensor is an analytical device where a biological event, for example the binding of an analyte, or biomarker, is converted into a quantifiable and processable signal [2]. A biosensor typically comprises a molecular recognition system, also referred to as bioreceptor, that binds specifically to the target; and an interface architecture, where a the binding event takes place and gives rise to a signal which is picked up by the transducer element. The transducer signal is converted to a meaningful quantity and displayed for the user to read.

Many currently available, highly sensitive biosensors rely on indirect detection, i.e. by means of a secondary probe or label which is introduced in a separate step and binds to the receptor-bound target [3]. Two advantages of these systems are the potential for signal amplification, e.g. by using enzymatic labels [3], and high resistance to non-specific binding [1] because the detection of the target relies on two independent binding events. However, this type of sensor is expensive, as it requires two recognition systems, time-consuming and makes real-time measurements a significant challenge. In addition, it requires multiple steps and hence can usually be carried out only by highly trained personnel to ensure quality of the analysis and interpretation of the results.

Biosensors that can detect the target directly (label-free) instead offer the possibility of fast analyte detection from a sample with no, or very little, sample preparation, enabling real-time measurements [1,4]. However, such label-free biosensors lack an amplification mechanism and rely on a single binding event, and hence require both a high affinity and specificity bioreceptor for the target, and a very sensitive transducer. Therefore, highly sensitive biosensors with low limit of detection are potentially more difficult to achieve.

A very promising class of label-free biosensors is based on electrochemical transducers, and different types of electro-chemical biosensors have been developed. For example, electrochemical impedance biosensors measure the impedance of the electrode/solution interface without disrupting the initial state of the interface. This is possible by applying a small alternating (AC) excitation voltage around a steady bias (DC) and measuring the resulting current. Electrochemical impedance spectroscopy (EIS) sensors measure the impedance of the electrode/solution interface across a frequency range. If the impedance of the electrode/solution interface changes upon binding of a molecule of interest to a recognition element immobilised on the biosensor surface, EIS can be used to detect this binding event. In general, the number of target molecules bound to the surface-immobilised bioreceptors is a very good measure of the concentration of the target in solution. Impedance biosensors are intrinsically label-free, and once optimised, the same system can be employed to detect virtually any target simply by changing the bioreceptor attached to the surface.

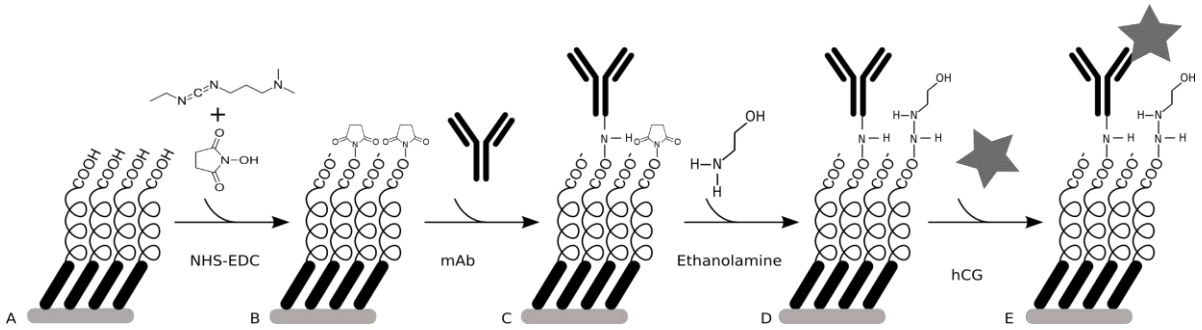

Fig. 1 Schematic illustration of the functionalisation of the sensor surface. A) SAM formation. B) Activation of COOH groups by means of EDC–NHS. C) Covalent binding of antibodies to the SAM. D) Deactivation of residual NHS activated sites by means of ethanolamine. E) Binding of hCG to sensor surface.

When a solid electrode is exposed to an electrolyte solution, charge is accumulated on the surface, which leads to a capacitor-like behaviour of the interface. Therefore, a widely employed approach for impedance biosensors is to model the sensor/solution interface as a capacitor and to estimate the change in capacitance of the sensor upon binding of the target to the surface-immobilized receptor, in absence of redox probes [4]. In previous work, the total capacitance of the sensor/solution interface, C_{tot}, has been evaluated by measuring the transient current at the sensor electrode upon application of a potentiostatic step [4]. With this method, Berggren et al. developed biosensors for antibodies, antigens, proteins, DNA fragments, and heavy metal ions [4]. For example, human chorionic gonadotropin (hCG) and Interleukine-6 (IL-6) were detected in low molarity phosphate buffer (PB) at concentration as little as 15 fM and 0.5 fM, respectively [4]. Although only in buffer, this is still one of the most sensitive biosensors for hCG reported to date.

Impedance biosensors can be produced very cost effectively by inexpensive mass production technologies. The required electrodes are fabricated by means of photo-lithography or screen printing, and the required instrumentation such as lock-in amplifiers is simple and readily available. Provided that adding additional sensors to the same device only requires the addition of an extra electrode they are ideally suited for multiplexing [5]. Furthermore, they are easy to use as they require minimal, or no, sample manipulation and the time-to-result is much shorter than in labelled biosensor systems. Hence, impedimetric biosensors are promising candidate for POC diagnostics. To reach this goal it is essential that sensors with high specificity and low limits of detection in complex solution such as blood serum, are developed.

In this paper a high sensitivity, label-free, multiplexed, electrochemical biosensor platform suitable for POC diagnostics is presented. The human hormone hCG was used as an exemplar system in a redox-probe-free EIS sensor, and by monitoring the phase of the impedance at 100 mHz, a limit of detection of 26 fM hCG in full serum is demonstrated.

II. Materials and Methods

A. Materials

Acetone, absolute ethanol, methanol, tris, Decon 90, platinum wire and Ag/AgCl reference electrode (662-1795) were purchased from VWR International Ltd (UK). NaOH, glacial acetic acid, HCl, ethanol 200 proof, sodium phosphate monobasic monohydrate, sodium phosphate dibasic, sodium acetate trihydrate, 2-(N-morpholino)-ethanesulfonic acid (MES), hCG and bovine serum albumin (BSA) were sourced from Sigma-Aldrich Co (USA). EDC, NHS and ethanolamine-HCl were purchased from GE Healthcare UK Ltd (UK). Bare gold disks for the AUTOLAB ESPRIT surface plasmon resonance (SPR) system were sourced from Metrohm Autolab BV (Netherlands). Acid capped esa(ethylene glycol)-undecanethiol (OEG) and monoclonal anti β-hCG antibodies (mAb) were supplied from ProChimia Surfaces Sp.zo.o. (Poland) and Abbott Laboratories (USA), respectively.

B. Surface plasmon resonance

The mAb were immobilised to the surface in different coupling buffers, namely 50 mM acetate buffer (AcB) with pH 4.5 – 6 and PB with pH 5.5 – 8, as detailed below. The SPR disks were cleaned by sonication in 100mM NaOH and 0.1% Triton X100 aqueous solution, and in ethanol 200 proof for 10 minutes each as per manufacturer's instructions. The OEG SAM was formed by immersion of the substrates for 40 hours in 0.5 mM OEG solution of ethanol 200 and 5% acetic acid (Fig. 1A) to avoid the formation of dimers and the esterification of the COOHs [6]. The substrates were rinsed in ethanol, dried under a N_2 stream and mounted in the SPR instrument. mAbB were bound to the surface using standard protocols [7]. After rinsing, the surface was prepared for functionalisation by injecting freshly prepared 1:1 mixture of 400 mM EDC:200 mM NHS in MES buffer pH 5.6 for 10 minutes (Fig. 1B). The surface was then quickly rinsed with MES and the appropriate coupling buffer. Solutions of 0.02 mg/ml mAb in the respective coupling buffer were injected on the surface for 45 minutes (Fig. 1C). The surface was then rinsed three times. Ethanolamine 100 mM pH 8 was injected for 10 minutes (Fig. 1D) to block any free NHS ester. To verify the responsiveness and specificity of the modified surface, after stabilisation in PB pH 7.2 the mAB were challenged with hCG (Fig. 1E) at different concentrations, and BSA was used as negative control.

C. Electrochemistry

For electrochemical measurements, Ti/Au (15 nm/80 nm) was evaporated by electron-beam evaporation at a base pressure of 10^{-7} mbar on to Si/SiO_2 wafers. Prior to

evaporation the Si/SiO_2 wafers were cleaned by 5 minutes sonication in acetone, methanol and DI water. The Au coated wafer was cut in devices of appropriate size and cleaned by sonication in acetone, ethanol and isopropanol for 10 minutes each. The samples were then dried under a N_2 stream and cleaned by freshly prepared piranha solution (70% H_2SO_4, 30% H_2O_2) for 5 minutes. The samples were sonicated in ultrapure water for 10 minutes and in ethanol 200 prior to immersion in OEG solution, prepared as mentioned above. The sensor surface was functionalised with mAb in AcB pH 5.5 following the protocol described above.

The working electrode area (9.13 mm^2) was defined by means of a square-cut rubber gasket. Cyclic voltammetry was performed between −0.5 V and 0.6 V in PB (100 mM) containing 2 mM of the redox couple ($Fe(CN)_6^{3-/4-}$) at 62 mV/s, for five cycles. Nonfaradaic EIS measurements were performed in 100 mM PB (pH 7.2) applying a 10 mV AC voltage, with no DC bias, for frequencies between 100 kHz and 50 mHz. Faradaic EIS measurements were acquired in 100 mM PB (pH 7.2) with 2 mM redox couple, applying a DC bias of 0.215 V vs reference electrode.

For calibration of the sensor after antibody immobilisation, the devices were incubated in serum for 20 minutes prior to nonfaradaic EIS measurement. After each measurement the cell was emptied and fresh serum injected. The incubation step and measurement was repeated for three more cycles. Serum samples spiked with increasing hCG concentration (26 fM to 0.26 nM) were injected and measured sequentially. The phase at 0.1 Hz was extracted from the EIS scans and the average of multiple scans was used as the sensor output.

III. Results and Discussion

The sensor devices were characterised by means of both SPR and electrochemistry. First, the SAM was characterised electrochemically by means of cyclic voltammetry, faradaic and nonfaradaic EIS. Electron transfer was found to be blocked effectively by the SAM, as evidenced by CV scans which showed the absence of redox peaks, and EIS measurements performed in presence of redox probes which showed the absence of faradaic processes (data not shown). This is indicative of the formation of a well packed, electrically blocking, SAM [8].

Second, the immobilisation process of the antibodies was characterised by means of SPR to establish the adsorption conditions, i.e. coupling buffer pH, that maximise the sensor response. It was found that for buffer pH ≤ 5.5 the amount of adsorbed mAbB was equal to 4.1 ± 0.4 ng/mm^2, which indicates full coverage, provided that the theoretically calculated values of maximum surface coverage is 3.77 ng/mm^2 [9], when the antibodies are considered to be homogeneous spheres, and 5.5 ng/mm^2, when they are assumed to be uniformly oriented and in their most compact configuration. In contrast, adsorption density was negligible (≤0.45 ng/mm^2) for pH ≥ 6. This can be explained by considering the electrostatic forces between the mAb and the surface [9]. At pH smaller than the isoelectric point (pI) the mAb has a net positive charge. The mAb pI is ≈ 6.5, hence at

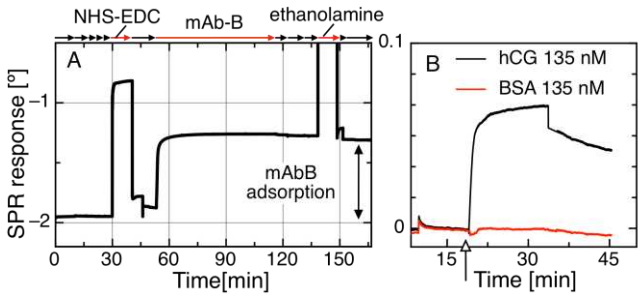

Fig. 2 Example SPR sensogram of (A) functionalisation of surface with mAb in AcB pH 5.5 by NHS-EDC chemistry and (B) surface response to hCG (black line) and BSA (red line), both at a concentration of 135 nM in 100 mM phosphate buffer pH 7.2. The arrow indicates the time of injection.

pH ≤ 5.5 the mAb are positively charged while the residual carboxylic acids on the sensor surface are negatively charged. This results in an electrostatic attraction that drives the adsorption of antibodies to the surface so that covalent binding can occur. It was found that for AcB pH 5.5, i.e. pH ≈ pI – 1, the sensor yields both the highest adsorption of mAb and at the same time the highest response of the sensor to hCG.

The specificity of the sensor to hCG and the resistance to nonspecific binding of the OEG SAM [10] was verified by means of SPR using BSA as negative control. BSA was chosen as it is known to bind non-specifically to surfaces [3] and serum albumin is present in high concentration in blood serum (41-45 mg/ml). Therefore, investigating the selectivity of the sensor to BSA is important to gain insight into the sensor's behaviour when exposed to complex solutions. The response of the sensor to 135 nM hCG, established over three separate experiments under the same conditions, is equal to 75 ± 8 m° (0.62 ± 0.06 ng/mm^2) compared to the very small response to the same concentration of BSA (Fig. 2).

Subsequently, the immobilisation of mAb in AcB pH 5.5 was also characterised by non-faradaic EIS. A decrease in the impedance phase at low frequencies is noted in the spectra after mAbs are adsorbed on the surface. A decrease of the phase at 100 mHz measured in PB 100 mM pH 7.2 equal to 2.4° ± 0.8° was observed across six samples (data not shown).

The sensor/solution interface can be described by a network of electronic circuit elements with overall impedance equivalent to that of the interface. The phase of the impedance is close to −90° for a highly insulating, defect-free, sensor/solution interface, and it increases towards zero for a non-blocking interface [12,13]. Therefore, the observed decrease of the phase towards −90°, indicates a decrease in leakage of the coating layer [12,13], suggesting that the antibodies form a layer that further hinders the access to the gold surface by currents of ions.

Fresh sensors were then made and characterised by means of EIS in horse serum (Fig. 3), to test both the selectivity of the sensor for hCG, and the behaviour of the sensor in clinically relevant samples. Fig. 3 shows the phase spectra obtained by means of EIS in blank and spiked serum. Although it was possible to fit the EIS spectra with an

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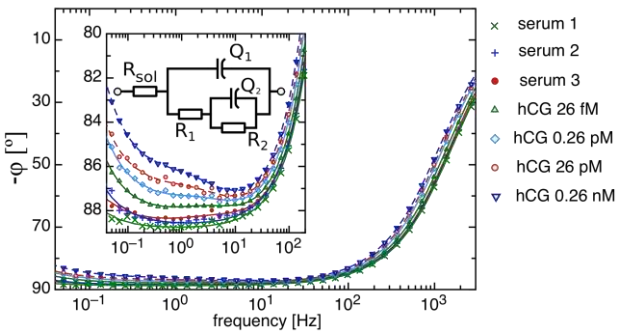

Fig. 3 Typical Impedance phase (φ) spectra recorded by means of EIS (symbols) for successive non-spiked serum injections and increasing concentration of hCG between 26 fM and 0.26 nM in serum. Each spectrum was obtained after 20 minutes of incubation. Lines represent fits to EIS measurements according to the equivalent circuit presented in the inset.

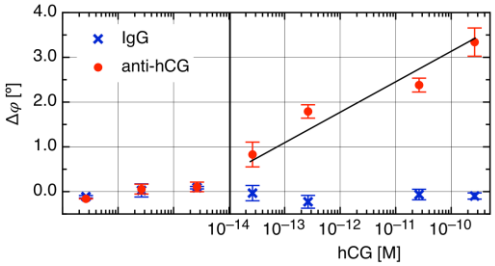

Fig. 4 Impedance phase-shift at 100 mHz (Δφ) measured by means of EIS for non-spiked serum injections (left) and spiked serum with increasing concentration of hCG (right), for sensor surface functionalised with anti-hCG monoclonal antibody (red dots) and anti-mouse antibodies (blue x). Error bars represent ±SD between samples. The line represents a linear fit of the phase shift vs log[hCG] for hCG response from 26 fM to 0.26 nM.

equivalent circuit (Fig. 3) the simplest circuit that was found to fit the data accurately comprised five circuit elements with seven free parameters.

Owing to the complexity of the interpretation of this equivalent circuit system, the calibration curve of the sensor (Fig. 4) was obtained by monitoring the shift in phase, Δφ, at a single frequency of 100 mHz. This frequency was chosen as representative of the phase of the system at low frequencies [12], where the signal to noise ratio is maximised. The phase-shift Δφ increases linearly with the logarithm of hCG concentration over 5 decades, showing a sensitivity of 0.6° ± 0.1° per decade for hCG in full horse serum. The curve shown is the average over 4 measurements and the error bars indicate the standard deviations. The lowest concentration detected for hCG in serum was 26 fM. We note that the detection limit obtained in full unprocessed serum for this sensor is comparable to the one obtained for the capacitive sensor discussed in the introduction, but the latter used in 10 mM PB [4], which is significantly less challenging than serum.

Considering that the sensor shows no response to blank injections of serum (Fig. 4, left), i.e. serum not spiked with hCG, helps in correlating the response of the sensor to the concentration of hCG when the sensor surface is exposed to spiked buffer. Moreover, on the same graph, the response of a sensor modified with anti-mouse antibodies to both blank and hCG-spiked serum is reported as negative control.

The phase of the impedance is independent of the surface area of the sensor, in contrast to other frequently employed quantities such as the magnitude of the impedance, the charge transfer resistance, or the capacitance of the layer. This is a highly desirable characteristic for a biosensor as it makes it robust against manufacturing tolerances typical for miniaturisation and therefore multiplexing.

IV. Conclusions

A label-free EIS biosensors with high sensitivity for the model target hCG in full serum was demonstrated. Optimal binding conditions for the capture antibodies to the sensor surface were established by SPR and found for binding buffers with pH ≈ pI – 1. The phase shift of the impedance at a fixed frequency of 100 mHz was found to be a good measure for the amount of binding observed, and the EIS sensor was calibrated for a range of different hCG concentrations in horse serum. A linear relationship of the phase shift vs the logarithm of hCG concentration was found between 26 fM and 0.26 nM with a sensitivity of 0.6 degree per decade. The sensor did not show any response to consecutive injections of serum, and sensors modified with antibodies not specific to hCG showed no response. Moreover the phase at a single frequency is an easy-to-measure physical quantity that does not require post-processing or fitting of complex equivalent circuits.

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