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An optimised electrochemical biosensor for the label-free detection of C-reactive protein in blood

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ABSTRACT

C-reactive protein (CRP) is an acute phase protein whose levels are increased in many disorders. There exists, in particular, a great deal of interest in the correlation between blood serum levels and the severity of risk for cardiovascular disease. A sensitive, label-free, non-amplified and reusable electrochemical impedimetric biosensor for the detection of CRP in blood serum was developed herein based on controlled and coverage optimised antibody immobilization on standard polycrystalline gold electrodes. Charge transfer resistance changes were highly target specific, linear with log CRP concentration across a 0.5–50 nM range and associated with a limit of detection of 176 pM. Significantly, the detection limits are better than those of current CRP clinical methods and the assays are potentially cheap, relatively automated, reusable, multiplexed and highly portable. The generated interfaces were capable not only of comfortably quantifying CRP across a clinically relevant range of concentrations but also of doing this in whole blood serum with interfaces that were, subsequently, reusable. The importance of optimising receptor layer resistance in maximising assay sensitivity is also detailed.

1. Introduction

C-reactive protein (CRP) is an acute-phase protein synthesized by the liver, widely accepted as a biomarker for cardiovascular disease and inflammation (May and Wang, 2007; Miller et al., 2007; Mygind et al., 2011; Pai et al., 2008). Generally, levels in plasma are less than 2.0 mg/L for healthy individuals (Vikholm-Lundin and Albers, 2006), but increase up to 1000 fold during an acute phase of inflammation (Gabay and Kushner, 1999). The American Heart Association and the United States Centre for Disease Control have suggested three categories of CRP concentration for the evaluation of cardiovascular disease risk: a CRP concentration below 1.0 mg/L representing low risk, a 1.0–3.0 mg/L range average risk, and levels above 3.0 mg/L representing high risk (Kushner and Sehgal, 2002; Lee et al., 2011). The reliable and early quantification of this target is often then, cited as a means of improving the outcome of cardiovascular or inflammatory disease through appropriate intervention or treatment.

Currently, a number of CRP testing methods are available in clinical laboratories using turbidimetric and nephelometric technologies (Roberts et al., 2000, 2001), or human CRP enzyme-linked immunosorbent assay (ELISA) kits. However, these methods are generally not suitable for the clinical practice as they are either not sensitive enough, time-consuming, prone to false negatives or cost-ineffective (Pearson et al., 2004). CRP quantification methods based on surface plasmon resonance (SPR) (Hu et al., 2006; Meyer et al., 2006), piezoelectric microcantilevers (Wee et al., 2005), quartz crystal microbalance technology (Kim et al., 2009), microfluidics (Lee et al., 2011) and electrochemistry (Buch and Rishpon, 2008; Centi et al., 2009), have been developed during the past few years. Among these, electrochemical assays promise most in terms of low cost, flexibility and sensitivity. Electrochemical impedance spectroscopy (EIS), in particular, can sensitively monitor the changes in capacitance or charge-transfer resistance associated with material binding at specifically prepared receptive electrode surfaces and requires no prior labelling (Bogomolova et al., 2009, Rodriguez et al., 2005). In recent years a number of CRP assays by EIS have been reported. To date, however, these have been either of limited sensitivity (Vermeeren et al., 2011), not demonstrably specific (Hennessey et al., 2009), or to not encompass a clinically relevant range (Chen et al., 2008; Qureshi et al., 2010).

We report herein, the development of a robust and highly sensitive assay for this marker in whole and dilute blood serum across the entire clinically relevant range. The interfaces are

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readily prepared, exhibit very good selectivity and are re-useable after assay with no apparent loss of sensitivity. We have, additionally, considered the importance of receptive layer initial resistance in subsequently observed sensitivity and demonstrated not only a clear correlation but also an ability to tune, and therefore optimise, receptive film characteristics.

2. Materials and methods

2.1. Chemicals and reagents

Human CRP, human blood serum and bovine serum albumin (BSA) were purchased from Sigma Aldrich. The goat anti-human CRP polyclonal antibody was purchased from AbD Serotec. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich. Polyethylene glycol (PEG)-thiol HS-C_{11}-$(EG)_3$-OCH_2-COOH was purchased from Prochimia Surfaces, Poland. Ultrapure water (18.2 MΩ/cm) was obtained from a Milli-Q system and used throughout. Phosphate buffered saline (PBS) with Tween-20 (PBST, 10 mM, pH 7.4) was prepared by dissolving PBS tablets (Sigma Aldrich) in water with 0.2% v/v Tween-20 added, and filtered using a 0.22 μm membrane filter. All other chemicals were of analytical grade.

2.2. Apparatus

Electrochemical experiments were performed on an Autolab Potentiostat 12 equipped with a FRA2 module (Metrohm Autolab B.V.). A conventional three-electrode system with a gold disk working electrode (1.6 mm diameter, BASi), platinum wire counter electrode and a silver/silver chloride (Ag/AgCl) reference electrode (CH Instruments) were used. All potentials are reported relative to this reference. CRP stock solution concentrations were calculated via the UV absorbance at 280 nm (Motie et al., 1996) using a Shimadzu UV spectrometer (Shimadzu Scientific Instruments).

2.3. Surface preparation

Gold electrodes were initially polished with 3.0, 1.0 and 0.1 μm diamond spray (Kemet International Ltd) in sequence and ultrasonically washed in water for about 5 min prior to immersion in freshly prepared piranha solution (concentrated H_2SO_4:H_2O_2, v/v 3:1; caution: this must be handled with extreme care!) for 15 min. Electrodes were then electrochemically polished by potential cycling (CV) between −0.1 and 1.25 V until a stable reduction peak was obtained. The effective surface area of the gold electrode can be calculated at this point (Hoogvliet et al., 2000), and the reported charge transfer resistance (R_{ct}) normalised by this.

Pre-treated gold electrodes were then dried in a flow of nitrogen gas and immediately immersed in a 10 mM solution of HS-C_{11}-$(EG)_3$-OCH_2-COOH in ethanol for 16 h at room temperature. The biocompatible (Cho et al., 2011; Klapshina et al., 2010) and antifouling properties (Harder et al., 1998; Schilp et al., 2009) of such films are sufficient to enable specific assessments to be made in complex biological fluids. After SAM formation, gold surfaces were rinsed with ethanol, then water, and dried in a flow of nitrogen gas prior to incubation in a solution containing 0.4 M EDC and 0.1 M NHS for 15 min (terminal carboxyl group activation) and then 10 μM CRP antibody solution (PBST, pH 7.4) for 1 h (Scheme S1, supporting information), unless stated otherwise.

2.4. Electrochemical impedance spectroscopy

EIS spectra were recorded across a 0.05 Hz–10 kHz frequency range. The amplitude of the applied potential sine wave was 10 mV with the direct current potential set at 0.25 V (the E_0 of the redox probe used, 1.0 mM $[Fe(CN)_6]^{3-/4-}$). Data was acquired in 10 mM PBST solution, plotted in the form of complex plane diagrams (Nyquist plots), and fitted through an ideal Randles equivalent circuit (Vyas et al., 2010).

Assays were carried out by electrode incubation in CRP spiked PBST, specific dilutions of blood serum or whole blood serum at room temperature for 30 min each. In the first two cases PBST was pre-doped with a redox probe. EIS responses were normally recorded in the same incubation solution, with the exception of the whole (undiluted) blood serum tests, where the electrodes were rinsed with PBST after incubation prior to assessment in PBST containing 1.0 mM $[Fe(CN)_6]^{3-/4-}$. To initially evaluate interfacial selectivity, BSA was used. Used surfaces were regenerated using 6 mM NaOH and 0.6% ethanol for 5 min (Albrecht et al., 2008) prior to PBST washing.

3. Results and discussion

3.1. Biosensor fabrication and initial impedance

EIS presents a useful means of characterising the stepwise fabrication of a receptive surface. In Nyquist plots, the semicircles at low sampling frequency report on charge transfer restrictions imposed sterically or electrostatically as films are constructed. Predictably, there are sharp increases in R_{ct} as the receptor layer is fabricated. R_{ct} specifically increases from less than 4 kΩ/cm^2 to ~45 MΩ/cm^2 after the formation of the PEG SAM and further upwards on antibody immobilisation (Fig. 1). This significant increase in R_{ct} is fully consistent with an increase in interfacial steric bulk associated with successful antibody immobilisation. The resistance of the interface thereafter responds in a calibratable manner to target protein binding.

Significantly, we have considered the possibility that the initial resistance plays a role in subsequently observed sensitivity; since the assay is based on increases in charge transfer resistance, one might logically expect sharper increases, and greater assay

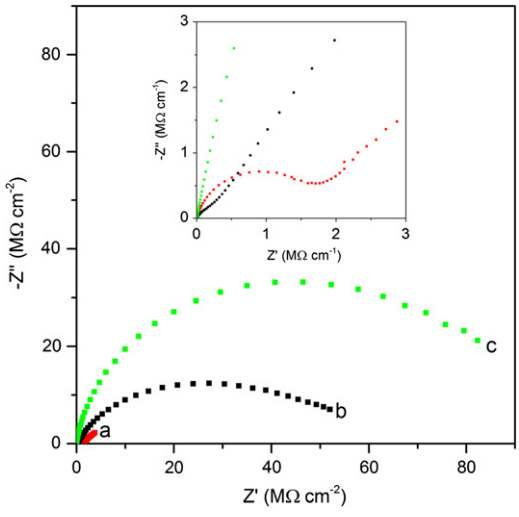

Fig. 1. Nyquist plots of different electrodes recorded in PBST (10 mM, pH 7.4) solution containing 1.0 mM $[Fe(CN)_6]^{3-/4-}$: (a) bare gold electrode; (b) gold electrode modified with the self-assembled monolayer; and (c) gold electrode with CRP antibody immobilized on the monolayer. The inset magnifies the high frequency region.

sensitivity, if the receptor layer is initially of comparatively low resistance. This initial resistance is directly tuneable through the antibody surface density. This is itself controllable through either incubation time or incubation concentration as the layer is constructed (Fig. S1). Fig. 2 summarises the observed trend in assay sensitivity with initial layer charge transfer resistance. Notably, as the antibody surface coverage decreases (as the immobilization timeframe is reduced from 70 min to 10 min), assay sensitivity initially increases in magnitude by up to 400% (and the limit of detection decreases consequently) before falling presumably as the density of surface bound and functional antibodies falls below the point where specific target binding is effective (in terms of probability). These observations are robust and reproducible across numerous assays. We believe them to be general and, from a practical perspective, to have considerable value in the construction of optimally responsive interfaces.

3.2. Detection of CRP in buffer

The prepared interfaces were subsequently used to screen CRP in PBST. From the progressive increase in (and ultimately saturation of) R_{ct} with concentration (Fig. S2 and Fig. 3), a dissociation constant K_D of 1.1 ± 0.11 nM can be derived, a value in excellent agreement with a previous differential pulse voltammetry determination with the same polyclonal antibody (Hennessey et al., 2009).

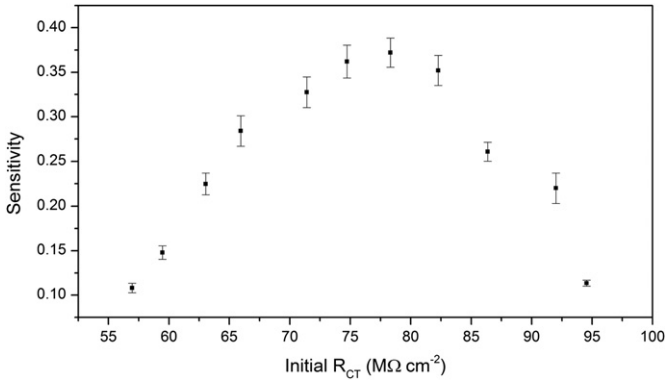

Fig. 2. Relationship between initial R_{ct} and assay sensitivity of the CRP biosensor. Sensitivity is defined as the slope of the sensor calibration curve. The CRP concentrations for these tests were 0.5–50 nM (each specific concentration assayed 5 times).

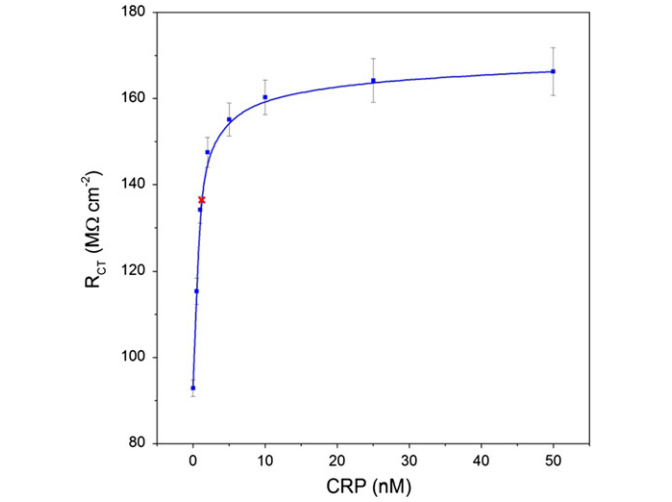

Fig. 3. Recorded charge-transfer resistance (R_{ct}) of the biosensor after the incubation with different concentrations of CRP protein in PBST (10 mM, pH 7.4) containing 1.0 mM $[Fe(CN)_6]^{3-/4-}$. The red cross indicates the CRP concentration equivalent to the K_D (measurement repetition $n=3$).

Prior to saturation, R_{ct} increases linearly with logarithmic sensitivity on CRP concentration across a 0.5–50 nM range (equivalent to 60 μg/L–6.0 mg/L) with a limit of detection (LOD) of 176 ± 18 pM (calculated by an extrapolation of the assay linear range to a response equivalent to three times the standard deviation of the noise, or zero target response). This low detection limit (equivalent to ~19 μg/L) confirms the sensitivity to be comfortably sufficient for practical application and is married to a assay range encompassing that which is clinically relevant. This range, taken with its associated detection limit, exceeds the clinical relevance of any prior reported CRP assay to the best of our knowledge. The prepared interfaces are, additionally, unresponsive ($<3\%$ change in signal) to BSA levels of up to 100 nM, an observation we ascribe to the excellent antifouling properties of carefully prepared hydrophilic pegylated support layers (Alcantar et al., 2000).

3.3. Detection of CRP in the blood serum

From a point of care perspective, the direct and facile assessment of CRP in blood serum is necessary. In any label-free assay, however, this is exceedingly demanding. Though a number of amperometric or sandwich based EIS immunoassay methods have been demonstrated (Balkenhohl and Lisdat, 2007a, b; Pan et al., 2010; Rosales-Rivera et al., 2011; Tran et al., 2011), to the best of our knowledge, there exists no prior report of a non-amplified and label free impedance assay that has been reported as being effective, and linearly calibratable in undiluted complex biological media.

Being confident about the degree of control we had over our electrode interfaces and in the light of the low levels of response to even high levels of BSA, we screened here for CRP in blood serum in two ways. In the first instance, *in situ* assessments were made with CRP spiked blood serum at controllable dilution in PBST (Fig. S3). Under such circumstances reliable linear assessments were possible across the clinically relevant range with LOD of 262 ± 28 pM at serum concentrations up to 20% v/v (Fig. 4).

Subsequent *ex situ* analyses were carried out with CRP spiked undiluted blood serum. Resistance optimized sensor electrodes were incubated in these solutions for 15 min and then measured after rinsing with PBST. As is evident in Fig. 5, the sensor response in such analyses is markedly close (in the higher concentration range of most clinical relevance, 10–50 nM, the differences are $<3\%$) to that observed in spiked PBST, with LOD of 322 pM. This enables analysis to be performed in whole blood serum across the CRP concentration range required for useful cardiovascular

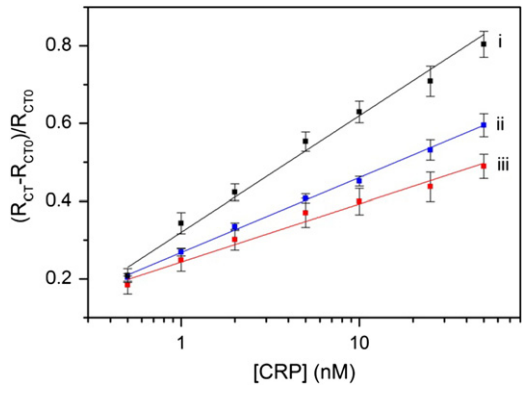

Fig. 4. Calibration curves for the CRP biosensors performed in different solutions: (i) PBST (10 mM, pH 7.4) with 1.0 mM $[Fe(CN)_6]^{3-/4-}$; (ii) PBST (10 mM, pH 7.4) with 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 10% human blood serum; and (iii) PBST (10 mM, pH 7.4) with 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 20% human blood serum (measurement repetition $n=3$).

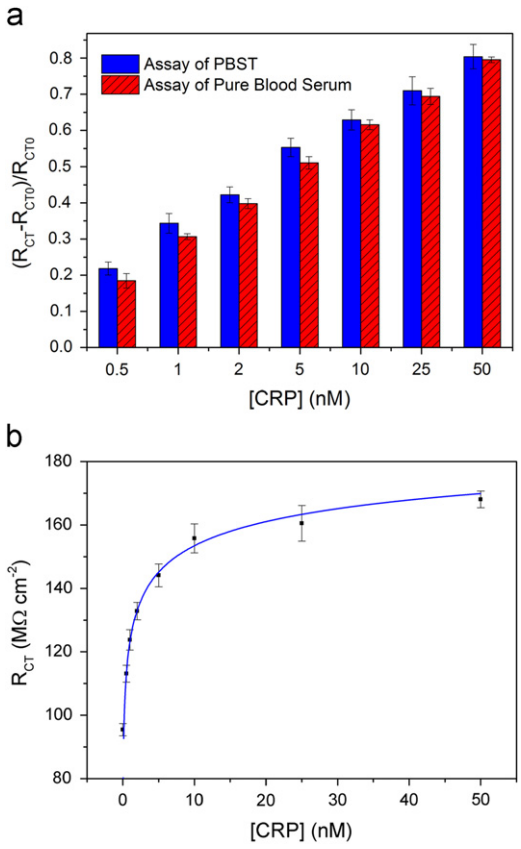

Fig. 5. (A) Comparison of biosensor assays of CRP in buffer solution and pure blood serum and (B) The *ex situ* sensor response to CRP in undiluted blood serum. Biosensors were incubated in CRP spiked buffer solution or neat blood serum and impedance analyses carried out in PBST solution containing 1.0 mM $[Fe(CN)_6]^{3-/4-}$. Impedance levels of CRP free buffer or pure blood serum were taken as background (measurement repetition $n=3$).

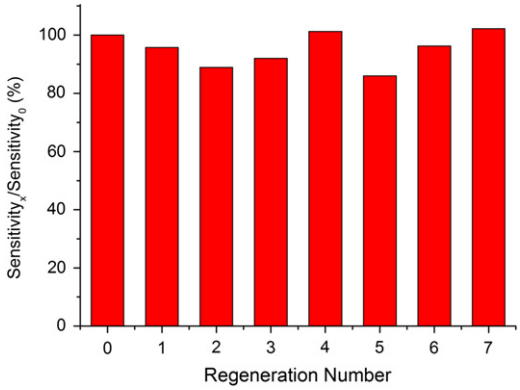

Fig. 6. Regeneration of the CRP biosensor interface by electrode immersion in 6 mM NaOH. This was achieved by immersion of the electrode in 6 mM NaOH and 0.6% ethanol for 5 min prior to washing with PBST. Subsequent impedance analyses, here, were undertaken in solutions of PBST (10 mM, pH 7.4), 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 10% human blood serum.

disease risk assessment. From a practical perspective, these assays can be carried out with as little as 5 μL of undiluted blood serum and report quantitatively within 10–15 min. The utilised electrode interfaces are also, subsequently, reusable (see below).

3.4. Biosensor regeneration

Surface regeneration was achieved with high fidelity (see Fig. 6) by immersion of used surfaces in 6 mM NaOH and 0.6% ethanol for 5 min (to disassociate the CRP antibody–antigen complex) and then washing with PBST. We believe the robust regeneration is possible partially because of the absence of the BSA passivation commonly employed at such immunoassaying surfaces. In such circumstances, this passivation may also be displaced from the electrode surface during regeneration–negating any further specific assaying. The interfaces herein could be reused without significant detriment of the assay (97% of the original interface response retained over 7 regenerations). This regeneration is effective for assays carried out in PBS, diluted serum or whole serum.

4. Conclusions

An optimised and reusable electrochemical label free biosensor capable of the reliable detection of CRP across the clinically relevant range in dilute or whole blood serum is presented. The *in situ* determined polyclonal antibody binding affinity maps very well onto previous determinations at comparable interfaces. In addition to facilitating high assay sensitivity and selectivity, the prepared biosensor interfaces also exhibit satisfying reusability. In optimising the initial interfacial resistance through antibody surface density, assay sensitivity can be markedly increased. It is notable that this, initially counterintuitive, observation is, we believe, applicable within any faradaic EIS assay.

Assays such as these are easily integrated into portable and multiplexed formats capable of sampling just a few μL of biological fluid within minutes. We believe the presented results serve as an important basis for the development of convenient point of care analysis of a marker long considered as a sensitive reporter of infection, trauma, inflammation and cardiac risk.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2012.06.051.

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