Towards Simple, Rapid Point of Care Testing for Clinically Important Protein Biomarkers of Sepsis

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Abstract

Sepsis is characterised by a dysregulated immune response following infection with a micro-organism. Treating and managing sepsis relies on fast and accurate diagnosis followed by introduction of the correct medications and supportive measures. Biosensor measurements for sepsis typically take the route of identification of the infectious agent and/or monitoring of a clinically relevant biomarker such as lactic acid or C Reactive Protein (CRP). In this work, we have used low cost Screen Printed Electrodes (SPEs) in conjunction with antibodies for interleukin-6 and Enterotoxin A to demonstrate the possibility of measuring these two sepsis related biomarkers in 10 minutes at clinically relevant concentrations (pg/mL). The clinical utility in this approach lies in the time to result and the relative simplicity of the assay. Current biomarker testing, especially in near real time, is absent from many intensive care wards and these results demonstrate the possibility of realising such measurements. The method of sensor production employed in this study is generic and therefore can be applied to a panel of similar sepsis biomarkers on a wide variety of electrode substrates. These results demonstrate a clear direction towards a simple multi-marker assay for sepsis which can assist with diagnosis, triaging and clinical management of the condition as it progresses and recedes.

Introduction

Sepsis is a major cause of morbidity and mortality in the UK. Recently, it was publicised by the National Health Service that on an annual basis the condition results in the death of 37,000 individuals from a total of 150,000 cases and that it is responsible for more deaths than lung, breast and colon cancer combined [1]. In 2016, the definition of sepsis was changed by consensus in order to take into account advances in understanding of the pathobiology, management and epidemiology of the condition [2]. At present, no gold standard diagnostic test exists and it is acknowledged that sepsis is more accurately described as a syndrome with a still unconfirmed pathobiology, meaning there is a high degree of uncertainty surrounding clinical testing for the condition. It is becoming clear that in addition to the now recognised early activation of pro and anti-inflammatory pathways [2] a range of cardiovascular, neuronal, autonomic, hormonal, bio-energetic, and metabolic and coagulation pathways play a role in the development of the condition [2]. The widened perspective offered in the 2016 definition of sepsis [2] also takes into account the high importance of clinical heterogeneity, underlying co-morbidities and source of infection as important factors in determining the overall outcome. The condition known as sepsis is defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection”. To put this definition into perspective, even a moderate degree of organ dysfunction is associated with an in hospital mortality rate > 10% [2]. Full details on the recent consensus definition of sepsis included clinical criteria have been described elsewhere [2]. Improved diagnostic testing and patient management are required to enhance survival rates and patient outcomes.
In current practice, there is a reliance on standard laboratory techniques to diagnose sepsis, with time lags of 12-72 hours. For example, a delay in sepsis diagnosis of one hour results in a 6-10% increase in the chance of death [3]. It is therefore clear that the development of sensor technologies with fast analysis times has the potential to revolutionise sepsis diagnosis and management.

A number of biomarkers have been reported in the literature as being potentially useful for the diagnosis and monitoring of sepsis, in fact the total reported is greater than 100. Enterotoxin A is a member of the family of proteins known as Staphylococcal and Streptococcal Enterotoxins, of which there are approximately 20 proteins which share a common phylogenetic link and thus have similar structures, functions etc. [4]. At present, 23 proteins have been identified, including Enterotoxin A and they have amino acid sequences of 220-240 amino acids and similar molecular weights (25-30 kDa) [5]. Enterotoxin A and B are the most commonly encountered, often in cases of sepsis [6] and food poisoning [4]. In addition, Enterotoxin B has been implicated as an agent for bio-terrorism and weaponisation [4]. What is significant for this work on assay development, is that Enterotoxin A is serving as an example of a commonly secreted virulence factor in cases of sepsis where the pathogen is Staphylococcus aureus [4,5,7].

Interleukin-6 (IL-6) is a 21 kDa glycoprotein secreted by leukocytes, which means it is defined as a cytokine: A substance which is known to be released by cells of the immune system. It is also secreted by a number of other cells, including: Fibroblasts, osteoblasts, keratinocytes, endothelial cells, mesangial cells and tumour cells [8]. It is present at elevated levels in times of fever and plays an important role in the differentiation of B cells, which are antibody-producing white blood cells. This explains why IL-6 is referred to as a pro-and anti-inflammatory cytokine; it has functions which act in both pathways. It is also an important substance for many different physiological functions, for instance bone homeostasis, due to the induction of osteoclast formation, and control of the body’s metabolism. Most crucially for this work, IL-6 is a commonly mentioned biomarker, potentially of high importance for sepsis diagnosis and monitoring [9,10]. Clinically relevant levels of IL-6 have recently been reported as being in the range of 5-20 pg/mL for physiologically normal situations and up to 1000 pg/mL in the cases of patients with sepsis [11,12].

Sensor systems have found application in a number of fields including industrial process monitoring [13,14], drug purity [15], environmental monitoring [16] and medical diagnostics [17] and can be developed using any number of approaches including optical, piezoelectric and electrochemical. Electrochemical sensors utilise the two or three electrode systems, with the working electrode always being the electrode under test. A scalable, low cost source of three electrode systems are screen printed sensors, which can be used in a disposable manner (Figure 1A). Here, Electrochemical Impedance Spectroscopy (EIS) is used as the detection approach because it is a powerful technique for the study of interfacial phenomena at electrified interfaces (Figure 1B). By applying a small sinusoidal excitation potential and measuring the corresponding current response it is possible to probe several physical phenomena, which include: The Solution Resistance ($R_s$), the Double Layer Capacitance ($C_{DL}$), the Charge Transfer Resistance ($R_{ct}$) and the diffusion impedance (Warburg impedance-W) (Figure 1C). Due to the fact that EIS is so sensitive to changes at the electrode surface it is possible to monitor the binding of biological molecules in real time and in a label free manner. A common approach for the detection of protein biomarkers is to immobilise a sensing film composed of antibodies for the target analytes on the electrode and measure the increase in charge transfer resistance ($R_{ct}$) when the target is bound. A number of electrochemical sensors have been developed where this approach was employed and assays developed include: TREM-1 [18], procalcitonin [19], ochratoxin [20], IL-8 [21] and interferon gamma [22]. Previous point of care style assays have been developed for IL-6 but have required the use of additional reagents such as sandwich assays and optical detection techniques [23,24] or complex electrode modifications [25]. The advantage of the system we
The report is its relative simplicity in terms of electrode preparation, time to result and introduction of the sample onto the electrode surface.

In this paper, we develop EIS based measurements for two protein biomarkers commonly elevated in sepsis patients. We demonstrate the possibility of measuring IL-6 and Enterotoxin A at clinically relevant levels in ten minutes. The approach to immobilising the antibodies is simple and generic meaning similar tests can be developed for a whole range of sepsis biomarkers. Furthermore, no physical or chemical modifications are made to the electrode surface, e.g. use of nanoparticles, graphene etc. to enhance electron transfer. This is because clinically relevant detection levels (pg/mL) were achieved, demonstrating that the approach is suitable for widespread implementation in the detection and monitoring of sepsis.

Methods and Materials

Gold macro disk electrodes \( (r = 0.8 \text{ mm}) \) were employed in order to perform benchmark cyclic voltammetry and Electrochemical Impedance Spectroscopy (EIS) experiments. An Autolab PGSTAT208 (Metrohm-Autolab B.V., Utrecht, Netherlands) under PC control was employed for electrochemical measurements. When not using a screen printed electrode the reference electrode employed was a silver/silver chloride electrode and the solution of potassium chloride employed was 3 M. The counter electrode was a platinum foil. Drop Sens (Oviedo, Spain) Au C233BT Screen Printed Electrodes (SPEs) were employed as the sensor chip for experiments involving development of rapid measurements for Enterotoxin A or IL-6 (Figure 1A). The on chip counter (Au) and reference (Ag) electrodes were employed to make the three electrode system for measurement of target binding.

The electrode surface was functionalised by reacting equal volumes of the antibody stock solutions (Abcam, Cambridge, UK) with 40 mM Sulfo-LC-SPDP (Sigma, Aldrich, UK) in 1× PBS for 40 mins. The mixture was then incubated with an equal volume of 150 mM DTT (Sigma Aldrich, UK) for 1 hour on a mixing plate to provide agitation. 10 µL of the solution was then drop coated onto the working electrode. The electrodes were stored in a humidity chamber for 16 hours to allow immobilisation and SAM formation to take place and also the high humidity prevented evaporation of the drop. Following this step, electrodes were rinsed with 1x PBS and then incubated for 1 hour in 1 mM 6-mercaptop-1-hexanol to backfill any unoccupied sites on the electrode surface. Following this, electrodes were left to equilibrate in 1x PBS for 1 hour prior to beginning detection of the corresponding antigen.

Electrochemical Impedance Spectroscopy (EIS) measurements were carried out at open circuit potential and using a measurement buffer consisting of 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide and 1× PBS. For the AC excitation signal, a total of 50 frequencies were employed ranging from 100,000 Hz to 0.1 Hz.
(spaced logarithmically) using an amplitude of 10 mV rms. The measurement is summarised in Figure 1B. Data were fitted using the Randles’ equivalent circuit as shown in Figure 1C. For some EIS responses it was necessary to fit with a modified version of the circuit (e.g. use of a constant phase element instead of a double layer capacitor).

Results and Discussion

First of all, it was necessary to prepare electrodes which provided credible evidence of satisfactory levels of electron transfer. Measurements on untreated electrodes did not provide satisfactory results and it was not possible to immobilise functional antibody layers onto these devices. In order to prepare a surface suitable for immobilisation of the antibody film it was necessary to condition the electrode by imposing an anodic voltage (2 V) for 120 s followed by 10 cyclic voltammograms in 0.1 M sulfuric acid. The rationale is that holding the electrode potential at 2 V for 120 s evolves oxygen bubbles at the working electrode, which has the effect of cleaning the surface. Performing cyclic voltammery between 0 and 1.5 V for 10 cycles (Figure 2A) has the effect of progressively cycling the electrode surface through the production of a gold oxide surface, oxygen evolution and the subsequent gold oxide reduction process upon reversal of the scan direction. The peaks associated with these surface processes are highlight in Figure 2A. To verify the surface had been satisfactorily cleaned, EIS measurements were performed in 1 mM potassium ferri/ferrocyanide + 1x PBS. When displayed in a Nyquist Plot, EIS measurements of clean macroelectrode surfaces recorded in a solution buffer containing redox active species produces a typical semi-circular response for high to medium frequencies with a 45° line at low frequencies. The diameter of the semi-circle gives the “charge transfer resistance” $R_{CT}$, which can be thought of as a measurement of the ease with which electrons are transferred across the interface. In this instance $R_{CT}$ is low (2.2 kΩ ± 0.01 kΩ) and a better equivalent circuit fit was achieved by replacing the Double Layer Capacitor ($C_{DL}$) circuit element with a constant phase element (Q) [26] in order to more accurately model the heterogeneous nature of the electrode surface. Having obtained a response which produced the features typically associated with a clean gold surface, it was then possible to immobilise antibody films onto the devices and measure antigen binding.

For initial testing, electrodes were prepared with antibody sensing layers for detection of either Enterotoxin A or IL-6. In these experiments, the electrode was covered with 100 µL of potassium ferri-ferrocyanide + 1x PBS as the measurement buffer and was allowed to equilibrate. An initial measurement was taken in order to determine $R_{CT}$ prior to binding of the antigen. Having established a baseline value for $R_{CT}$, an addition of 1 µL of antigen solution was made and the impedance re-measured after 10 minutes. Figure 3A shows the initial EIS measurement from an Enterotoxin A antibody coated electrode and following 10 minutes of incubation in 1 ng/mL Enterotoxin A solution. Figure 3B shows the same experiment but for an IL-6 antibody coated electrode and following incubation in 50 pg/mL IL-6 solution. Clear increases in the charge transfer semi-circle are evident.
in both cases following addition of 1 ng/mL Enterotoxin A to the Enterotoxin A antibody modified electrode and 50 pg/mL IL-6 to the IL-6 antibody modified electrode. The EIS responses shown in Figure 3A and Figure 3B have different profiles and it was necessary to modify the approach to equivalent circuit fitting in order to obtain acceptable fits (i.e. lower $\chi^2$ values for overall goodness of fit). In the case of Figure 3A it was necessary to fit with a simple Randles’ circuit by omitting the Warburg element and using a constant phase element to take account of the rough surface of the screen printed electrode. For this electrode, the cleaning process and modification with the Enterotoxin A antibody resulted in an obvious reduction of the heterogeneous electron transfer constant (since Warburg behaviour is no longer evident). For the IL-6 antibody coated electrode the need to fit with a Warburg element was evident from the shape of the response (i.e. presence of a 45° line at low frequencies) and so this element was retained in the equivalent circuit. Finally, to express increases in $R_{CT}$, a number of methods have been suggested in the literature. One common approach is to divide the baseline $R_{CT}$ value by the $R_{CT}$ value following incubation with target to gain a “Signal Increase Ratio” [17]. This method was employed in this study and results are displayed in Figure 4. What is clear from Figure 3A and Figure 3B is that specific antibody-antigen interactions for Enterotoxin A and IL-6 were measurable using the screen printed electrodes and EIS.

Table 1: For indicative purposes-blood concentration of IL-6 and correlation with survival rate [8].

<table>
<thead>
<tr>
<th>Blood concentration IL-6 (pg/mL)</th>
<th>Survival rate (%)</th>
</tr>
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<tbody>
<tr>
<td>20-40</td>
<td>100</td>
</tr>
<tr>
<td>40-7, 500</td>
<td>37</td>
</tr>
<tr>
<td>7500+</td>
<td>11</td>
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At this point in the study, it was decided to concentrate on the detection of IL-6 because it is a potential inflammatory biomarker for sepsis whose levels correlate very strongly with recovery from the condition (Table 1). Normal levels were identified as 20-40 pg/mL [11,12] and the selected low concentration of 50 pg/mL is designed to be just above this range. Developing a rapid test for this biomarker could be very useful for rapidly admitting into hospital and also for triaging patients. The measurements showed that it was possible to detect IL-6 at 50 pg/mL in 10 minutes following incubation of the antigen on the electrode (Figure 4A) with much higher increases noted for 500 and 5000 pg/mL solutions of IL-6. At such high concentrations it is quite possible that the antibody binding sites present on the electrode surface were saturated with antigen, making quantification at such high concentrations difficult. What is clear for higher concentrations (Table 1) is that elevated IL-6 levels correlate with unfavourable survival rates and the prototype test reported herein clearly detects IL-6 elevation above clinically normal levels. Further work is required to establish the linearity of the assay and its dynamic range. In addition to specific interactions, the IL-6 modified antibody electrode was incubated with 100 ng/mL Enterotoxin A solution to establish if any cross reactivity/non-specific binding had
occurred in the presence of a different antigen. Figure 4B shows this was not the case with the signal increase ratio remaining at approximately 1 for the 100 ng/mL solution of Enterotoxin A but increasing to > 2 in 10 minutes for the specific interaction between the IL-6 coated antibody and the 50 pg/mL IL-6 solution.

The assay performed in a highly satisfactory manner with the fast and sensitive detection of IL-6 identified as having potential for aiding in the rapid diagnosis and real time monitoring of sepsis. One issue that was identified through the period of experimentation was electrode variation with significant differences in $R_{CT}$ being noted from one electrode to another. Figure 5 summarises this variation by showing a series of Scanning Electron Microscope (SEM) images (A-D) recorded from different electrodes after cleaning. Each image reveals a markedly different surface profile with the presence of blemishes and aggregates on the electrode surface. Figure 5E shows the mean starting impedance and standard deviation following antibody immobilisation on 16 electrodes and again demonstrates a high degree of variation. This inconsistent behaviour of the electrodes can be attributed to variable surface areas (impedance is inversely proportional to surface area), inconsistencies introduced by the cleaning protocol, inconsistencies introduced in the screen printing process and batch to batch variation between screen printed electrodes. In taking this work forward towards a point of care test, it is our intention to develop an electrode substrate with improved consistency of initial measurement. Whilst the approach is in effect subject to a normalisation through use of the “Signal Increase Ratio”, achieving consistent starting values will aid with quantification and improving detection at or around the clinical threshold.

**Conclusions**

It was possible to use screen printed electrodes to develop a rapid and simple assay for the interaction between antibody and antigen for sepsis biomarkers. Com-
monly employed strategies from the literature which improve electron transfer and sensitivity (e.g. electrode decoration with nanoparticles, graphene and orientated antibody immobilisation etc.) were not necessary to achieve clinically relevant detection levels in ten minutes. This means the approach reported is far more amenable to mass manufacturing since it involves drop coating antibody solutions onto the clean electrode surface. Measurements involving Enterotoxin A and IL-6 were developed. As a key sepsis marker, the IL-6 measurement was evaluated and expanded further to show that detection was possible at clinically relevant levels (50 pg/mL) in ten minutes. The assay protocol was very straightforward and involved transferring solutions of antigen onto the electrode much like a blood sample would be transferred in a clinical situation. Inconsistent behaviour was observed from the screen printed electrode substrates with starting values for the impedance varying significantly. A potential route to improving assay reproducibility will be through use of improved electrode substrates (i.e. reduced inter electrode variation) or developing more sophisticated surface pre-treatment techniques. Possible strategies for improvement of electrodes include; gold sputtering techniques and production of microelectrodes which have been shown to offer analytical advantages over larger electrodes for detection of biomarkers [27].

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References

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