Improving performance of a rapid electrochemical MRSA assay: optimisation of assay conditions to achieve enhanced discrimination of clinically important DNA sequences under ambient conditions.

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Abstract

Electrochemical impedance spectroscopy (EIS) is a highly useful approach for the label free measurement of DNA hybridisation at functionalised electrode surfaces. Since label free detection relies upon a change in the electrochemical signal arising directly from the presence of target oligonucleotide or DNA/RNA sequences it is necessary to improve understanding of the conditions which produce a stable baseline value, promote optimal hybridisation of complementary sequences and can reduce non-specific binding effects. This study investigates both artificial DNA oligonucleotide sequences and clinical samples of MRSA genomic DNA, initially demonstrating that the use of tris(2-carboxyethyl)phosphine (TCEP) during probe layer formation improves both the initial baseline signal reproducibility and also the magnitude of the response upon hybridisation with a complementary target. Having demonstrated enhanced performance from TCEP modified electrodes, the assay is then used to detect clinical samples of MRSA. It is shown that improved performance is achieved both in terms of signal magnitude and discrimination against negative controls. Finally, formamide is added to the EIS measurement buffer and it is demonstrated that EIS measurement is possible in the presence of high formamide concentrations and that non-specific binding is also reduced under such conditions. The importance of these findings lies in the design of future electrochemical assays for nucleic acid biomarkers which are capable of functioning under ambient conditions but still have discriminatory power. A diagnostic device which does not have to operate at elevated temperatures will lead to increased simplicity and substantial battery and time savings which will further the widespread realisation of portable clinical diagnostic devices.

Introduction

Chemical and electrochemical sensors have found widespread application in health[1,2] and industrial/environmental monitoring[3–6]. For medical sensing, DNA hybridisation assays typically rely on the base-pair complementarity of two opposing strands of DNA. Upon recognition and hybridisation a signal is provided by e.g. fluorescence-labelled target which reports the binding event. Collection of a threshold signal allows detection of binding and confirmation of target recognition. This approach is common to many diagnostic tools such as microarrays and allows for high throughput screening of genetic sequences[7–9]. The hybridisation conditions necessary to promote specific sequence recognition are relatively well studied and due to the nature of the assays can often involve elevated temperatures and stringency washing. The prospect of transferring DNA hybridisation assays to electrochemical platforms has long been acknowledged[10–12] and studies have been carried out which allow the transfer of microarray sequences to electrode immobilised forms for electrochemical detection[13]. EIS is a powerful tool for monitoring interfacial phenomena and is particularly advantageous when implemented in nucleic acid detection because it permits label free monitoring of DNA hybridisation[14]. A number of studies have been carried out which report the use of EIS for DNA-DNA recognition[15–21], PNA-DNA recognition[22–25] morpholino-DNA recognition[26] and conformational change of the recognition interface[27]. In general, the scheme for detection by EIS involves measurement of the interfacial charge transfer resistance (R_{CT}) at a probe modified electrode in the absence and presence of
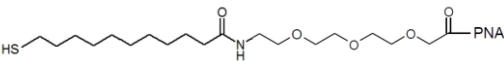
the target sequence. Depending on the charge of the redox mediator employed, increases and decreases in R_{CT} can be measured as hybridisation takes place. More specifically, the voltage on the working electrode in a three electrode cell is subjected to a series of small magnitude sinusoidal peturbations which decrease in frequency. From the measured response and using an equivalent circuit, various physical parameters such as the solution resistance (R_S), double layer capacitance (C_{DL}), charge transfer resistance (R_{CT}) and the Warburg Impedance (Z_W) can be extracted. A number of studies have been published which report a series of modifications and improvements to EIS based DNA assays which lead to improved sensitivity and performance [15,19–21] including the use of dendrimers, nanoparticles and micro/nano electrodes. One significant disadvantage with the majority of these studies is the introduction of additional complexities which make such tests difficult to implement in clinical scenarios where simplicity of engineering and operation are major design concerns. Some recent papers have examined the possibility of making straight forward design alterations to realise the possibility of sensorial application and these involve simple considerations in the form of relative position of the binding sequences [28–30] This paper reports some new relatively simple optimisations to an established EIS assay for the clinically important antibiotic resistant bacteria MRSA. The assay has low limits of detection and high sensitivity (~500 fM) and the optimisations result in improved assay performance through increased reproducibility of electrode starting value, increased reproducibility of detection signal and make the test better able to run at ambient temperatures and therefore more amenable to implementation in routine clinical testing.

Materials and Methods

Reagents

DNA oligonucleotides were purchased from Metabion (Martinsried, Germany). PNA oligonucleotides were ordered via Cambridge Research Biochemicals (Cleveland, UK) from Panagene (Daejeon, South Korea). PCR kit and DNeasy blood and tissue kit were purchased from Qiagen (Crawley, UK). Potassium ferricyanide, potassium ferrocyanide, sodium saline citrate (SSC), monosodium phosphate, disodium phosphate, dimethyl sulfoxide (DMSO) and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma Aldrich (Poole, UK).

Table 1. Sequences and structures of oligonucleotides used during the study.

	Oligo name		5' Modif.	Sequence 5'-3'
1	PNA 48_02	PNA	Thiol-C11-AEEEA-PNA	ACTAGGTGTTG GTGAAGATATAC
2	DNA 48_c	DNA	-	GTATATCTTCAC CAACACCTAGT
3	DNA_nc	DNA	-	ACCTTTGCTCAT TGA

DNA Extraction from *S. aureus*

MRSA and MSSA bacteria were sub cultured onto Columbia blood agar and incubated overnight at 37°C in a 5% CO_2 atmosphere. Cells were inoculated into saline and the optical density measured using a Densicheck (bioMerieux). This gave values in McFarland units, proportional to the cellular concentration of bacteria in the suspension. A bacterial cell suspension of approximately 10^8 cells/mL

was produced in this way and ten-fold dilutions ranging down to 10^2 cells/mL prepared from this suspension. Real time PCR was performed to characterise the DNA yields from the dilution series. The bacterial cells were pelleted by centrifuging 1 mL of the suspension at 5000 x g for 10 mins. The supernatant was discarded and the bacterial pellet resuspended in 200 μL of enzymatic lysis buffer (2 x TE buffer, 1.2% Triton X, 50 μg/mL Lysostaphin), before incubating for 30 mins at 37°C. 200 μL of bacterial lysate was added to 20 μL Proteinase K and DNA extracted using the bioMerieux NucliSens easyMAG automated platform. Guanidine thiocyanate was the active chaotropic agent in the lysis buffer, acting as a protein denaturant in the purification and extraction of nucleic acids from cellular material. The purified nucleic acid solution was then removed from the vessel without dislodging the magnetic silica pellet - DNA was eluted in 100 μL of water.

Electrochemical Impedance Spectroscopy (EIS)

Gold disk electrodes (2 mm diameter) were purchased from IJ Cambria Scientific (Carms, UK). Each solid gold working electrode was thoroughly pre-cleaned by mechanical polishing with 0.05 μm alumina powder (IJ Cambria Scientific (Carms, UK) for 1 min, rinsing with water and immersing in an ultrasonic water bath for 1 min (to eliminate any residual alumina) and finally cleaning for 10 min in piranha solution (6 mL concentrated H_2SO_4 + 2 mL 30% (v/v) H_2O_2 solution). Then the electrodes were thoroughly washed with water and dried under a stream of nitrogen. After cleaning, the gold disk electrodes were incubated with a solution of 1.5 μM thiol-modified PNA solution + 30 μM mercaptohexanol in 50 % (v/v) DMSO for 16 h at 30 °C. Electrodes were rinsed in 50 % (v/v) DMSO and incubated in 1 mM mercaptohexanol in 50 % (v/v) DMSO for 1 h at 30 °C. Then the electrodes were washed with 50 % (v/v) DMSO and the EIS measurement buffer (0.1 mM $K_3[Fe(CN)_6]$ + 0.1 mM $K_4[Fe(CN)_6]$ + 10 mM phosphate buffer) for 2h and 1 h respectively. Long buffering and equilibriation times were employed because previous work found such rinsing times to be superior for ensuring a stable baseline behaviour when measurements were recorded. For electrodes where the effect of tris (2-carboxyethyl)phosphine (TCEP) was under investigation, 5 mM TCEP was added to the probe and blocking solutions.

EIS measurements in batch end point assays were performed using a three electrode system with an Ag/AgCl/3M KCL reference electrode and a platinum wire counter electrode (both from Metrohm (Runcorn, UK) connected to an Autolab potentiostat running FRA software (Metrohm, Runcorn, UK). EIS measurements were performed at a DC potential of 0.24 V with an amplitude of 10 mV rms using a frequency range between 100 000 Hz – 0.1 Hz (15 frequencies) in 0.1 mM $K_3[Fe(CN)_6]$ + 0.1 mM $K_4[Fe(CN)_6]$ + 10 mM phosphate buffer. The DNA sample was prepared by mixing 45 μL of sample with 5 μL of 20xSSC and then heating at 95°C for 5 mins, storing on ice for 2 mins and heating at 30°C for 5 mins. The electrode was incubated with the sample for 2h at 55°C with shaking (650 rpm). Following incubation with sample, electrodes were washed with 2xSSC, 0.2xSSC and EIS measurement buffer for 10 mins in each. EIS measurements were performed pre and post hybridisation.

The online assay was performed by recording continuous EIS measurements with gold screen printed electrodes (SPEs) (DRP-C223BT) with an integrated Ag pseudo reference electrode and a gold counter electrode (Dropsens, Oviedo, Spain). A single well from a Schott Nexterion 16-well self-adhesive superstructure (Stafford, UK) was cut out and fitted around the electrode in which 50 μL of EIS measurement buffer was present. The well was sealed with an adhesive lid from the Schott Nexterion 16-well self-adhesive superstructure kit (Stafford, UK). 45 μL of sample was mixed with 5 μL of 10x EIS measurement buffer and pretreated by heating at 95°C for 5 mins, storing on ice for 2 mins and heating at 30°C for 5 mins. Once the sample was prepared the EIS measurement buffer was removed from the electrode surface and replaced with the 50 μL sample + measurement buffer solution. The adhesive lid was resealed and EIS measurements continued. The measurements were performed at open circuit using the on chip Ag reference electrode.

Results and Discussion

EIS based DNA hybridisation tests were run in either batch mode or kinetic mode. Batch mode involved the use of a macrodisc electrode where the EIS measurement was taken pre and post incubation with a sample. The result was expressed as a "Signal Ratio" which was calculated by dividing the R_{CT} value obtained post hybridisation by the prehybridisation value. The method of presenting EIS DNA detection data has been used extensively in the literature[22]. This form of experiment was useful for understanding the roles of different parameters in assay performance before transferring to a real time "kinetic mode" measurement which would be akin to the clinical test. Kinetic mode involved the use of SPEs and a small volume sample well into which target solutions were added and the EIS response measured continuously during hybridisation. We have previously demonstrated the development of a rapid MRSA test using this kinetic measurment[22]. Figure 1 shows an example of typical Nyquist plots obtained pre and post hybridisation using a gold macrodisc electrode and also contains a diagram of the Randles' equivalent circuit used to fit and obtain R_{CT} values. Initially, after probe film immobilisation and equilibration a measurement was recorded in order to establish a baseline value for R_{CT} at the working electrode. Across the course of this study the starting values for R_{CT} were collected and the mean and S.D. and relative standard deviation (R.S.D) calculated. Figure 2 shows the mean and S.D. for electrodes prepared with and without the use of TCEP. This compound has been shown to be extremely useful in the reduction of dithiol bonds in solution when preparing SAMs[31], in the controlled assembly of dithiol containing SAMs for orientated enzyme attachment[32] and for the selective production of mono and bi-coordinated dithiol layers[33]. The probe film immobilisation procedure was the same in both cases except that TCEP treatment involved the addition of 5 mM TCEP to the immobilisation and blocking/back filling solutions. The use of TCEP in the backfilling solution has been shown as a particularly effective method for ensuring removal of dithiol attachments at the surface[33]. It can be seen that R_{CT} values were larger and less reproducible using the standard immobilisation protocol and that the use of TCEP reduced the variability in starting values and reduced R_{CT} values overall. Large R_{CT} values are observed from electrodes prepared with MCH only and the reduction in the mean R_{CT} value as observed here points towards incorporation of increased amounts of PNA probe sequence into the film resulting from the use of TCEP during sensor preparation. The likelihood of disulfide containing molecules being present in the SAM is greatly reduced through the use of TCEP in sensor preparation and this may explain the observation of increased monolayer reproducibility for TCEP treated SAM preparations [31–33].
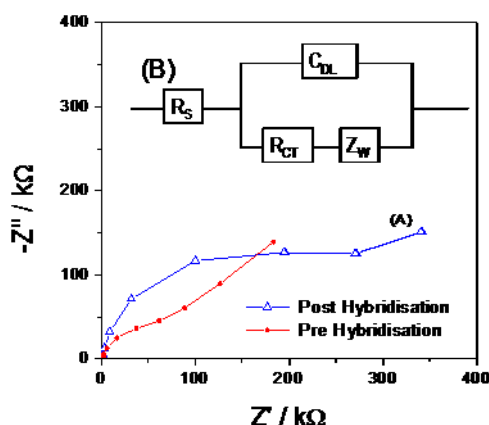

Figure 1. (A) Nyquist plot showing the EIS response pre and post incubation with a complementary DNA sequence (incubation conditions – 2h at 50°C in 2xSSC). (B) Randles' equivalent circuit used to fit impedance data in this study.
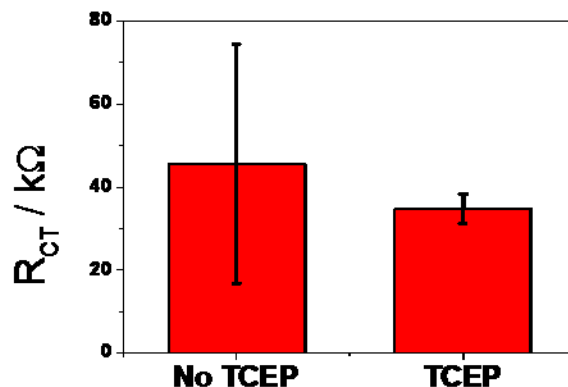
Figure 2. Mean initial R_{CT} values obtained from electrodes prepared using the standard protocol (no TCEP – n = 26) and electrodes prepared using a modified protocol involving TCEP (n=11). Bars represent S.D.

Having shown that TCEP use during probe immobilisation improved the reproducibility of starting R_{CT} values it was necessary to test the response of the electrodes following incubation with complementary target. In this test, electrodes prepared with and without the use of TCEP were used to detect an MRSA oligonucleotide target. This enabled the effect of TCEP pre-treatment to be judged in terms of the electrode response following DNA hybridisation. From figure 3 it can be seen that when incubated with fully complementary artificial target the electrodes prepared using TCEP produced a "Signal Ratio" approximately double that of those prepared without the use of TCEP (Signal Ratio of 37.4 vs 20.2). Variation in the form of relative standard deviation (R.S.D.) was also lower in the case of TCEP modified electrodes with R.S.D. representing 22% of the mean vs 34% for non TCEP modified electrodes demonstrating that a more reproducible response was observed for TCEP modified electrodes when binding DNA target. Again the increase in signal can be explained through the greater incorporation of PNA into the sensing film at the density set by the composition of the immobilisation solution and the resulting increased levels of DNA-PNA hybridisation causing a greater electrostatic barrier to the redox species at the electrode surface[24].
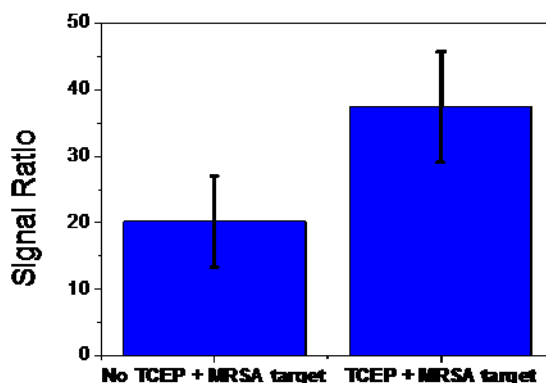

Figure 3. Effect of use of TCEP during probe immobilisation on Signal Ratio following incubation with a fully complementary oligonucleotide sequence. (n=4). Bars represent R.S.D.

Binding genomic DNA from clinical MRSA samples

Having established that TCEP provided a beneficial effect in terms of reproducibility for initial R_{CT} values and in terms of reproducibility and magnitude of response to incubation with artificial target it was then necessary to test the response to incubation with bacterial genomic DNA (gDNA). In previous work we have demonstrated the successful and quantitative detection of bacterial gDNA with detection limits (L.O.D) of 10 pM for artificial oligonucleotides and 500 fM for bacterial gDNA and one thing to note of particular importance is the variable length of the gDNA targets produced by cell lysis and DNA recovery[22]. In this case a probe for the *mecA* sequence of MRSA gDNA was used with gDNA from the antibiotic susceptible MSSA strain employed as the negative control experiment. gDNA was extracted from 10^7 cells/mL MRSA and MSSA samples respectively, which, were sufficiently concentrated to readily achieve successful detection and thus evaluate the effect of the electrode preparation step since the assay was running comfortabley above the established L.O.D. The electrode responses can be seen in figure 4 which shows that the largest response in terms of increased "Signal Ratio" came from the incubation of MRSA gDNA with TCEP modified electrodes. In comparison to electrodes prepared without TCEP the increase in signal was approximately 5.5 times greater. It is also important to note the increased variability associated with the binding of gDNA compared to a fully complementary artificial oligonucleotide sequence, which, can be attributed to the irreproducible nature of thermal DNA fragmentation[34] and also the random positioning of the complementary sequence within the fragments[28,29]. Finally, it can also be noted that non specific binding in the case of MSSA incubated electrodes was reduced for those prepared with 5 mM TCEP present in the immobilisation solution. These findings demonstrate that using TCEP prepared electrodes for the detection of gDNA samples of MRSA led to enhanced performance both in terms of magnitude of the signal increase and discrimination against the negative control (MSSA).
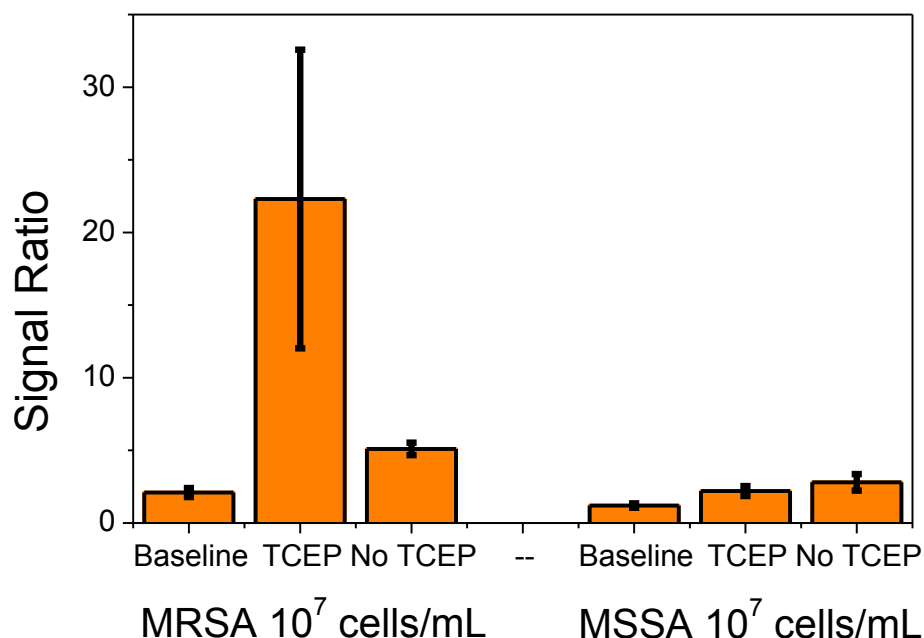

Figure 4. Incubation of MRSA and MSSA gDNA (10^7 cells/mL) using electrodes with immobilised *mecA* PNA probes prepared with and without TCEP and with shaking at 650 rpm. Bars represent S.D (n=7).

Online detection in ambient conditions

For application as a point of care test it is necessary to give consideration to detection in ambient conditions. Having established that the use of TCEP during probe film immobilisation was beneficial in terms of reproducibility of starting R_{CT} values and the magnitude of the "Signal Ratio" response to incubation with complementary target it was necessary to test in the "online" format where DNA target is simply added to the electrode during continuous EIS measurement and result times of 15 mins are possible[22]. It is well known that for DNA hybridisation assays such as microarrays it is easily possible to modulate the incubation conditions, i.e. carry out incubation at elevated temperature in order to ensure only specific binding takes place. In this study, consideration was given to clinical diagnosis in short times (<15 mins) with a simplified test involving minimal power consumption, ease of engineering and operation with point of care use. Whilst it is possible to design oligonucleotide probes for discrimination at room temperature it is still a challenging task to ensure no non-specific binding takes place from other nucleic acid sequences present in sample volumes e.g. from human DNA collected on the swab or chromosomal DNA from the bacteria. A possible strategy to improve detection at ambient conditions is to add formamide to the measurement buffer. With this in mind it was decided to attempt electrochemical detection in a measurement buffer containing formamide. Aqueous solutions of formamide are well known for their ability to reduce the thermal melting temperature and disrupt the kinetics of DNA association[35]. Often, solutions of 30-70% formamide are used in DNA hybridisation tests to remove single stranded target DNA bound to a surface tethered probe. The online assay developed for MRSA [22,28] depends on the ability to discriminate between specific and non-specific DNA sequences at ambient conditions and in the presence of interfering nucleic acid sequences from clinical samples. Therefore, it is highly desirable to design a system with maximum discriminatory power and through the use of 40% formamide in the EIS buffer it is possible to in effect reduce the thermal melting point of the strands by ~ 30°C[35]. Figure 5 shows the effect of adding 40% formamide into the measurement buffer on the resulting cyclic voltammogram at a PNA probe modified electrode. The ferri-ferrocyanide reaction is normally a one electron transfer reaction at 298 K and using the formula:

$$E_P = \frac{2.3RT}{nF} \quad (1)$$

peak separation can be calculated as 59 mV for an unmodified electrode. Peak separations at probe modified electrodes were 176 mV in EIS buffer and 188 mV in formamide modified EIS buffer. This large degree of separation results from coverage of the electrode surface with thiolated probe and MCH molecules and the relatively dilute nature of the measurement buffer. The open circuit potential was measured as 49 mV in EIS buffer and found to shift to 0 mV in 40% formamide EIS buffer. Since the reference electrode is metallic silver the expected formal potential for the couple would be 0 V as observed in the case of the formamide buffer. For the ordinary measurement buffer the shifted formal potential may have arisen through some chlorination of the metallic silver reference electrode, particularly since the measurements were recorded with SPEs. An on chip silver electrode was employed as a pseudo reference electrode in this experiment because it was easily accommodated into the small sample volume. Both anodic and cathodic peak currents were found to reduce by 26% in formamide modified EIS buffer but it was still possible to observe redox behaviour from the ferri-ferrocyanide couple which was present at a relatively low concentration of 0.1 mM. Having established the possibility of making EIS measurements in 40% formamide modified EIS measurement buffer, an online assay was performed which involved the addition of fully complementary and non-complementary targets in the presence and absence of 40% formamide in the EIS measurement buffer (see Figure 5). It can be seen from figure 6 that the use of formamide containing EIS measurement buffer reduced the amount of non-specific binding from non-complementary DNA sequences whilst still giving rise to significant binding of the fully complementary target. This elimination of a non-specific binding response will make room temperature sequence discrimination much easier from complex clinical samples because an unambiguous result will be produced. It will not be necessary to fit the binding curves and perform analysis to determine

whether binding is of a specific or non-specific nature. The use of TCEP pre-treatment of the electrode along with the 40% formamide buffer increased the discriminatory power of the assay.
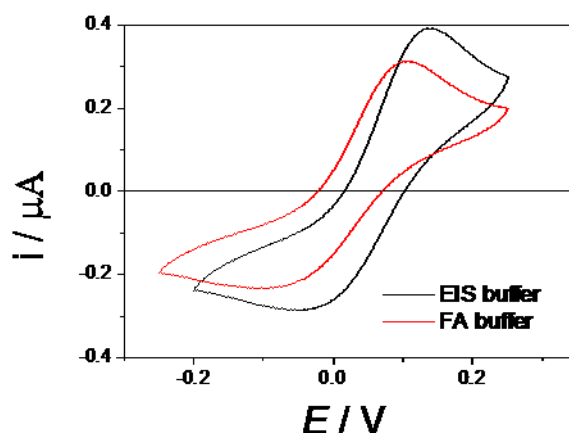

Figure 5. Cyclic voltammetry in EIS buffer and EIS buffer containing 40% formamide. The reference electrode was silver and EIS measurements were carried out at OCP.
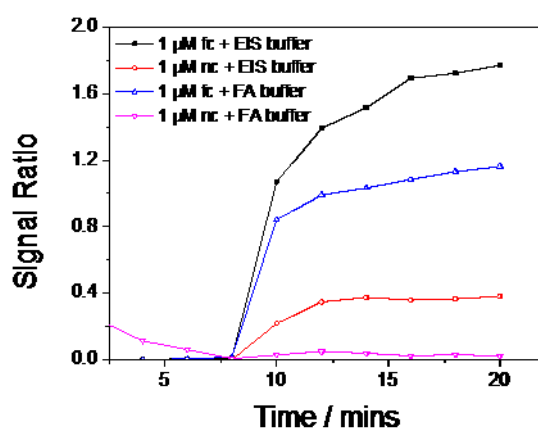

Figure 6. Signal Ratio response (baseline corrected) to the addition of fully complementary and non-complemtary artificial oligonucleotides in EIS measurement buffer (EIS buffer) and EIS measurement buffer + 40% formamide (FA buffer).

Conclusions

It has been possible to improve the reproducibility of initial R_{CT} values by including TCEP in the probe immobilisation protocol. Larger increases in signal were also observed from electrodes prepared using this method. The TCEP modified electrodes were applied to the detection of MRSA gDNA from clinical isolates and it was also found that electrodes produced using TCEP modified preparation protocols showed enhanced performance in terms of signal increase and non-specific binding effects. Finally, with the aim of demonstrating the possibility of improved discrimination under ambient conditions, formamide was successfully added into the EIS measurement buffer and reduced non-specific binding from non-complementary sequences. These findings together demonstrate that relatively straight forward optimisations can be made to an electrochemical biosensor system which improves reproducibility, sensitivity and specificity thus making the system more suitable for deployment into clinical settings. In

this case, the improvements will allow improved detection of MRSA in hospital and clinical settings under ambient conditions.

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