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Highly Sensitive Electrochemical Detection of DNA Hybridisation by Coupling the Chemical Reduction of a Redox Label to the Electrode Reaction of a Solution Phase Mediator

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ABSTRACT

We have described a highly sensitive method for detecting DNA hybridisation, using a redox-labeled stem loop probe. The redox labels were poly(styrene-co-acrylic) (PSA) spheres of 454 nm diameter, modified by methylene blue (MB) deposited alternatively with poly(sodium 4-styrene sulphonate) (PSS) in a layer-by-layer process. Each PSA sphere carried approx. 3.7×10^5 molecules of MB, as determined optically. DIG-tagged stem loop probes were immobilised on screen printed electrodes bearing anti-DIG antibodies. Binding with target enabled straightening of the stem loop, which made attachement to the MB-coated PSA spheres possible. Measuring the current from the direct reduction of MB by differential pulse voltammetry, a 30 mer DNA target common to 70 strains of *Escherichia coli* was calibrated across the range 1.0 fM to 100 pM (gradient = 3.2×10^{-8} A $(\log \text{fM})^{-1}$, r^2 = 0.95, n = 60), with an LOD of ~ 58 fM. By using $Fe(CN)_6^{3-/4-}$ as a solution phase mediator for the MB reduction, we were able to lower the LOD to ~ 39 aM (gradient = 5.95×10^8 A $(\log \text{aM})^{-1}$, r^2 = 0.96, n = 30), which corresponds to the detection of 0.76 ag (~ 50 molecules) in the 2 µL analyte sample. We hypothesise that the lowering of the LOD was due to the fact that not all the MB labels were able to contact the electrode surface.

Keywords: Stem loop; differential pulse voltammetry; mediator; E. coli; layer-by-layer.

1. Introduction

There is currently great interest in sequence-specific DNA detection, due to potential for application in gene analysis, clinical diagnosis, forensic and environmental science and monitoring food safety.[1-5] Of the different detection methods available, electrochemistry has been intensively researched, due to the fact that electrochemical reactions produce an electrical signal without needing expensive transduction equipment, while having the potential for on-site, decentralized testing, and can be coupled with current minaturization technologies. To achieve sufficiently low detection limits with electrochemical systems, a number of different strategies have been attempted. DNA sequences have been amplified by enzymatic means, such as the polymerase chain reaction (PCR).[6] While this technique significantly lowers the detection limit, it increases the number of assay steps and therefore assay time, and requires both expensive equipment and trained personel. Other enzymatic methods have involved nuclease enzymes,[7,8] but these still require adding further steps to the assay.

If measurement is to be performed without sequence amplification, then the means of detecting hybridisation itself must be sensitive enough for the given application. Although label-free methods have been proposed,[9-11] generally speaking, detection via electrochemical labels has provided better sensitivities. Enzyme labels[12] have enabled very low detection limits but can suffer the problem of instability due to loss of enzyme activity over time. The alternative is to either use a nanometal or a redox label. Both require a reporter probe orientation such that the label is in contact with the electrode surface. In the nanometal case, if contact is not complete, the metal in question can still be measured by anodic stripping voltammetry, following acid dissolution.[13] However, this introduces further measurement steps. A redox molecule

can either be intercalated in the ssDNA or dsDNA structure,[14,15] or tagged to the end of the sequence. Intercalation means that the amount of redox charge delivered per DNA strand can only be increased by increasing the length of the strand. Where as in the case of an end-tagged DNA sequence, the quantity of charge delivered for a given sequence length can be increased by rational design of the redox label. Examples of DNA probes tagged with individual redox molecules include ferrocene attached to amino-modified DNA probes at one end,[16] and methylene blue (MB) attached to DNA probes at one or both ends.[17] Strategies to increase the number of redox molecules delivered per hybridisation event include: four ferrocene molecules bound linearly to a stem loop probe,[18] $Ru(NH_3)_6^{3+}$ molecules contained in a liposome label[19] and carbon nanotubes loaded with tris(2,2'-bipyridyl) ruthenium derivatives.[20] All of the aforementioned methods employed direct reaction of the mediator at the electrode surface. This may not always be the best strategy. Increasing the number of redox units per DNA probe will inevitably mean increasing the surface area/volume of the solid support. This raises the question of whether all redox molecules on the support can have adequate contact with the electrode surface. A further problem may arise from steric hindrance between neighbouring probes preventing a probe label actually reaching the electrode surface. A way to avoid these inefficiencies would be to use a second redox couple in solution, to mediate electron transfer from the label-bound species. To the best of our knowledge, this strategy has not so far been exmained. Hence, in this work we describe the construction of electrochemical labels bearing a high quantity of redox molecules (sub-micron size latex particles loaded with MB by layer-by-layer deposition). We used these labels to tag stem loop probes containing a 30 mer sequence common to 71 strains of *E. coli*. We show how a) the high quantity of MB provided a low detection limit and b) by mediating electron transfer from the

MB using a solution phase redox couple ($Fe(CN)_6^{3-/4-}$) we could lower that detection limit even further.

2. Experimental

2.1 Apparatus

UV-visible spectra were recorded using a Schott UV-Vis spectrometer model Uvikon XL. Electrochemical experiments were performed using an Autolab PGSTAT 10 computer-controlled potentiostat (Eco Chemie) with GPES software. Screen-printed electrodes (SPEs) were fabricated in-house exactly as described previously[21] and possessed a 1.5 mm × 3.5 mm carbon track working electrode and a 2 mm × 3.5 mm Ag/AgCl track combined reference/counter electrode.

2.2 Reagents and solutions

Anti-Digoxigenin (Anti-DIG) was purchased from Genway. The synthetic oliginucleotides were purchased from Thermoscience. Oligonucleotide stock solutions (100 μM) were prepared with sterile milliQ water and kept frozen. The probe and target oligonucleotides solutions were diluted using 10 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl. Poly(allylamine hydrochloride) (PAA) (molecular weight ≈ 70000 g mol^{-1}), poly(sodium 4-styrene-sulfonate) (PSS) (molecular weight ≈ 70000 g mol^{-1}), tween 20, poly-l-lysine (PLL) and avidin were purchased from Sigma-Aldrich. MilliQ water was used for all the solution preparations.

The DNA strands had the following sequences:

Stem loop probe: anti-DIG-5'-AAA GGC CGT CTT CCT GAG TAA TAA CTT CCT GAG TGA ATA ACG GCC AAA AA-3'-biotin

30 mer Target DNA: 5'-TAT TCA CTC AGG AAG TTA TTA CTC AGG AAG-3'

20 mer Target DNA: 5'- GGA AGT TAT TAC TCA GGA AG -3'

1 Mismatch DNA: 5'-TAT TCA CTC AGC AAG TTA TTA CTC AGG AAG-3'

3 Mismatch DNA: 5'-TAT TCA CTC AGC AAC TTA TTA CTC ACG AAG-3'

30 mer noncomplementary DNA: 5'-TCA TTT AGC TTT GTT AGC GTT AGG TAT ATC-3'

50 mer-noncomplementary DNA: 5'-AGT AAT GGA ACG GTT GCT CTT CAT TTA GCT TTG TTA GCG TTA GGT ATA TC-3'

2.3 Preparation of MB-Ball label

PSA particles were synthesised by reacting together styrene and acrylic acid in a nitrogen-purged aqueous solution in the presence of ammonium persulphate. The polymerisation time was 8 h. Full details are given in Pinijsuwan et al.[21] A suspension of 90.5 mg of PSA particles was sonicated in 10 mL of 95% ethanol until well dispersed. The ethanol was then removed after by centrifugation at 10,000 rpm for 15 min and the particles redispersed in 10 mL of milliQ water. After that the PSA particles were sequentially incubated in 20 mL of PAA, PSS, PAA and PSS solutions (1 mg mL^{-1} in 0.5 M NaCl). Each polyelectrolyte layer was allowed to adsorb for 30 min at room temperature, and then three centrifugations/redispersions steps were performed with sterile milliQ water before incubation in the next solution. The

polyelectrolyte-coated PSA spheres (PSA-PE_4) were stored in 20 mL of milliQ water (approx. PSA concentration = 42.27 mg mL^{-1}) at room temperature.

Methylene blue-loaded latex particles (MB-Ball) were prepared by adding 8 mL of 1 mM methylene blue to a 20 mL dispersion of 2.5 mg mL^{-1} PSA-PE_4 particles. The mixture was incubated for 30 min under stirring at room temperature and then triplicate centrifugation and redispersion cycles were performed using 8 mL of milliQ water, to remove unadsorbed methylene blue. Following this the PSA-PE_4 particles were incubated in a 20 mL solution of 0.5 M NaCl containing 1 mg mL^{-1} of PSS for 30min. These steps were repeated to give 3 layers of methylene blue on the PSA-PE_4. Finally the MB-ball particles were dispersed in 20 mL of sterile milliQ water at a concentration of approx. = 3.3 mg mL^{-1}.

2.4 Preparation of MB-Ball/Avidin Conjugate

A 1 ml suspension of MB-Ball particles was centrifuged and then redispersed in 990 µL of 10 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl. A 10 µL aliquot of avidin solution (21.14 mg mL^{-1}) was then added and the mixture was incubated under stirring at room temperature for 90 min. After that the solution was centrifuged and washed three times with 10 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl, to remove any free avidin. The MB-ball/avidin was stored in sterile 10 mM phosphate buffer, pH 7.0, at 4 ^{o}C prior to use.

2.5 Preparation of anti-Digoxigenin (anti-DIG) modified screen-printed electrode (SPE)

The SPE was washed with sterilized Milli-Q water and then dried under a nitrogen stream before use. A 10μL aliquot of poly-L-lysine (PLL) was deposited on the working area and incubated at room temperature for 20 min. The electrodes were then dried at 80 ^{o}C for 10 min, followed by dipping in 5 mL 10 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl, and then drying under nitrogen. The SPE surface was then exposed to a 5 μL aliquot of between 5 to 98 μg mL^{-1} of anti-DIG in 0.1 M phosphate buffer, pH 7.0, containing 1.0 M NaCl. The anti-DIG molecules were allowed to adsorb for 30 min at 25 ^{o}C, following which the SPE was rinsed twice (approx. 1 mL each rinse) with sterilized 10 mM phosphate buffer containing 0.1 M NaCl + 0.5% tween, then rinsed five times with the same buffer/electrolyte combination in the absence of tween, and then dried under nitrogen.

2.6 Detection of DNA hybridization using MB-Ball labels based on stem loop probe

Into each PCR tube were deposited 20 μL of 1 μM stem-loop DNA probe, 20 μL of target DNA and 60 μL 100 mM phosphate buffer, pH 7.0 + 1.0 M NaCl. The tube was incubated for 60 min, at 45 ^{o}C unless otherwise stated. A 10 μL aliquot was then removed from the tube and deposited on the surface of the SPE to allow binding between the surface-confined anti-DIG and DIG-tagged stem-loop DNA probe. The SPE was then rinsed twice with 10 mM phosphate buffer + 0.1 M NaCl + 0.5 % tween, and five times with 10 mM phosphate buffer, pH 7.0 + 0.1 M NaCl, both buffers being at 4 ^{o}C. The SPE was then dried under a nitrogen stream. The MB-

Ball/avidin suspension prepared as descibed in section 2.3 was diluted in a 1:1 ratio with 0.1 M phosphate buffer, pH 7.0, containing 1.0 M NaCl. This was used as the labeling solution. A 5 μL aliquot of this solution was deposited on the SPE, which was then left to stand for 30 min on a temperature-controlled mixing block (Bioer, model MB-102) set to 15 ^{o}C. This was followed by rinsing twice with 10 mM phosphate buffer + 0.1 M NaCl + 0.5 % tween, and five times with 10 mM phosphate buffer, pH 7.0 + 0.1 M NaCl, both solutions used at 4 ^{o}C. The SPE was then dried under a nitrogen stream. A 50 μL aliquot of 0.1 M phosphate buffer, pH 7.0 + 1.0 M NaCl (containing 0.25 mM potassium ferricyanide when mediated electron transfer was implemented) was then deposited over both electrodes (carbon working electrode and Ag/AgCl combined reference and counter). Differential pulse voltammetry (DPV) was performed using the conditions: step potential = 72 mV, amplitude = 50 mV, scan rate = 5 mV s^{-1}.

3. Results and Discussion

3.1 Construction of Redox Labels

Based on TEM measurement, the PSA spheres had a mean diameter of 454 nm and a relatively narrow size distribution (1 std. dev. = 3.0 nm, n = 100 particles). The PSA spheres posses a negative surface charge excess at neutral pH, due to deprotonation of the acid groups.[22] We used this fact to coat the spheres with two oppositely charged polyelectrolyte bilayers, i.e. $(PAA/PSS)_2$, in a layer-by-layer (l-b-l) process as

described previously.[21] TEM measurement of the coated spheres indicated an increase in diammeter to 461 nm (1 std. dev. = 2.6 nm, n = 100 particles). The main driving force for l-b-l deposition is electrostatic attraction, due to the fact that charge overcompensation occurs with the deposition of each layer, causing the zeta potential to oscillate symmetrically between positive and negative values.[23] Hence, the $(PAA/PSS)_2$ coating is expected to present an overall negative surface charge to the solution. Contributing factors to l-b-l deposition include Van der Waals forces, hydrogen bonding and hydrophobic interactions, as well as an increase in entropy due to the liberation of counter ions and solvent shell water molecules. For this reason, it has been found possible to replace one of the l-b-l polyelectrolytes with a smaller charged species, such as Eu^{3+}.[24] This allowed us to l-b-l deposit the positively charged redox molecule methylene blue (MB) between layers of negatively charged PSS. The completed structure was PSA-$(PAA/PSS)_2(MB/PSS)_3$. Building up the MB layers caused the PSA spheres to change from white to blue (see Supporting Information, Fig. S-1). The quantity of MB loaded was determined by dissolving the PSA in tetrahydrofuran (THF) and measuring the absorbance of the solution. As shown in Fig. 1, the absorbance spectra of a solution of dissolved spheres (peak at 622 nm, shoulder at 614 nm) was consistent with the spectra of pure MB in MilliQ water. Using the peak at 662 nm to plot a linear calibration curve ($Abs_{622\ nm} = 0.0078c$ (μM) - 0.0083, $r^2 = 0.9993$, $n = 11$), we determined the molar extinction coefficient of MB in THF to be 7.6×10^3 M^{-1} cm^{-1}. Based on the TEM radius we determined the mass of one PSA sphere to be 5.15×10^{-14} g, assuming a density of 1.05 g cm^{-3}.[25] The dry weight of an aliquot of known volume was then used to calculate the concentration of particles in the suspension, which was found to be 2.8×10^{10} PSA-$(PAA/PSS)_2(MB/PSS)_3$ particles mL^{-1}. Based on this concentration, the absorbance

from the dissolution of a known aliquot corresponded to a loading of 3.7×10^5 MB molecules per PSA sphere.

3.2 Detection of DNA Hybridisation by Direct Methylene Blue Reaction

The principle of hybridisation detection using redox-labelled stem loop DNA is illustrated in Scheme 1. The detection concept is based on the following sequence: 1) Homogeneous hybridisation between DIG-tagged stem loop probe and DNA target. 2) Binding of the DIG tag to electrode-confined anti-DIG antibodies. 3) Exposure of the immobilised DNA to cold buffer solution (~ 4 ^{o}C). Since this temperature is below the melting temperature, T_m, of the stem loop sequence (T_m stem loop = 30.87 ^{o}C (www.rnasoft.ca)) the stem loop reforms in the DNA strands that are not bound to target. Where probe-target binding occurs, the duplex remains unaffected due to probe-target formation possessing a more negative free energy than stem loop formation (T_m target-probe = 73.36 ^{o}C.[26] 4) Attachment between biotin tag at the 3' end of the hybridised probes and the avidin coating of the PSA-$(PAA/PSS)_2(MB/PSS)_3$ spheres. The unhybridised probes do not form the avidin-biotin bond due to the stem loop cauing steric hindrance to the approach of the relatively large PSA labels. 5) Voltammetric detection of the PSA-$(PAA/PSS)_2(MB/PSS)_3$ labels through either direct reduction or redox mediation using solution phase $Fe(CN_6)^{4-}$.

3.3 Optimisation of DNA Hybridisation Detection

Immobilisation of the anti-DIG antibodies on screen printed electrodes was performed by first coating the electrodes with poly-L-lysine, following the observation that antibodies can be stably bound to this surface and can retain the ability to capture their relevant antigens.[27] Since poly-L-lysine is positively charged, we expect the binding mechanism to be electrostatic. The concentration of anti-DIG exposed to the coated electrodes was varried in the range 5 to 98 μg mL^{-1} and the differential pulse voltammetric (DPV) peak height from direct MB reduction was recorded in the absence of target DNA (I_0) and in the presence of 1 pM of target DNA (I). Five electrodes were examined at each anti-DIG concentration. The result is shown in Fig. 2. It can be seen that the I/I_0 ratio reaches a maximum value at an immobilising solution concentration of 19.9 μg mL^{-1} and then decreases. We expect this is because a) at highly dense probe coverages steric hindrance lowers the efficiency of $PSA(PAA/PSS)_2(MB/PSS)_3$ attachment to the DNA probe, b) high density coverages may prevent horizontal alignment of DNA duplexes at the electrode surface. Such alignment is necessary for MB-electrode contact.

Using the 19.9 μg mL^{-1} stock for anti-DIG immobilisation we varried the hybridisation temperature from 30 ^{o}C to 60 ^{o}C. The effect of this on the previously defined I/I_0 ratio is shown in Fig. 3. It can be seen the optimum hybridisation temperature was 45 ^{o}C. As noted earlier, T_m of the probe-target duplex, (defined as the temperature where half the dsDNA in the sample is dehybridised) was calculated to be 73.36 ^{o}C. Hence, the optimum temperature found here is consistent with the general rule of thumb that the optimum hybridisation temperature is approx. 20 - 25 ^{o}C below T_m.[28]

3.4 Specificity and Sensitivity Utilising Direct MB Reduction

Under the optimised conditions for hybridisation detection using direct MB reduction, we examined the assay specificity as follows: The responses to 1 pM and 100 fM of complementary target (30 mer) were compared to the responses for (a) 1 pM of a 50 mer non-complementary target, (b) 1 pM of a 30 mer non-complementary target, (c) 100 fM of a 30 mer one base mismatch, (d) 100 fM of a three base mismatch. Five electrodes were used for each target. As shown in Fig. 4, the 50 mer non-complementary target produced a negligable response, while the 30 mer non-complementary target, one base and three base mismatches produced responses significantly lower (~ 25%) than the complementary strands. Based on a suggestion by one of the reviewers, we performed comparative assays on a one base mismatched target (100 fM) in which the washing of the screen printed electrode after target binding was performed a) using 10 mM phosphate buffer, pH 7.0 + 0.1 M KCl, as described in the Experimental section, and b) first using the same washing buffer at 45 ^{o}C prior to washing with the buffer at 0 ^{o}C. We found that washing with the buffer at 45 ^{o}C lowered the value of I/I_0 to almost zero for the one base mismatch, while a response to 100 fM complementary target could still be realised (see Supporting Information, Fig. S-2). It is likely that some probe-mismatch target opening occurred at the higher temperature. Fig. 5 shows a calibration of the complementary target under the optimised assay conditions, plotted on a log scale. It can be seen that the response was linear with the logarithm of concentration in the range 1.0 fM to 100 pM (gradient = 3.2×10^{-8} A (log fM)$^{-1}$, $r^2 = 0.95$, $n = 60$). We apply the commonly accepted definition of limit of detection (LOD) as being the concentration giving a response equal to $\dot{x} + 3\sigma$, where $\dot{x}$ is the mean response to a blank and σ is the standard deviation of that response.[29] Using the peak height to a 30 mer non-complementary

sequence as the blank, eight electrodes gave $\dot{x} = 1.30 \times 10^{-7}$ A, $\sigma = 6.52 \times 10^{-9}$ A, from which LOD ~ 58 fM. For the 2 μL aliquot dropped onto the electrode, this corresponds to the detection of 1.07 fg or 0.12 amol, i.e. ~ 7×10^4 DNA molecules. This detection limit could be lowered further as described below.

3.5 Sensitivity Enhancement Using a Solution Phase Mediator

As noted earlier, realisation of a voltammetric current from the PSA-$(PAA/PSS)_2(MB/PSS)_3$ label requires the DNA duplex to be essentially horizontal on the electrode surface, since the MB must be close enough to the electrode for electron transfer to occur. Given the large probability of steric hindrance from adjoining duplexes, it is likely that not all the MB labels will make adequate surface contact. A further consideration is whether charge percolation through the MB film is sufficient for all the redox charge in the $(MB/PSS)_3$ coating to be transported to the electrode. A method to circumvent these problems is to utilise redox cycling of the form:

solution: $MB + R \rightarrow LB + O$

electrode: $O \rightarrow R + ne^-$

Ostanta et al.[30] have detected DNA amination using the reactant system MB + $Fe(CN)_6^{3-}$. They showed that DPV peaks for $Fe(CN)_6^{3-}$ reduction increased in the presence of MB. Since both reactants are initially present in the oxidised form, the explanation must be that within the timescale of the voltammetric reduction peak the electrogenerated $Fe(CN)_6^{4-}$ is converted back to $Fe(CN)_6^{3-}$ by MB, thus raising the concentration of $Fe(CN)_6^{3-}$ at the electrode. We previously presented hydrodynamic

linear sweep voltammograms in support of this interpretation, and used $Fe(CN)_6^{3-}$ to mediate the reduction of MB adsorbed onto carbon nanotubes, for an immunoassay label.[31] Here we examined the use of $Fe(CN)_6^{3-/4-}$ to mediate charge from the PSA-$(PAA/PSS)_2(MB/PSS)_3$ spheres. We measured the direct and $Fe(CN)_6^{3-/4-}$ - mediated reduction current in the absence of of target DNA (I_0) and the presence of 1.0 pM of complementary target (I), while varying the quantity of $Fe(CN)_6^{3-}$. The inset to Fig. 6 shows the values of I/I_0 as determined from both direct and redox-mediated reduction, as a function of $Fe(CN)_6^{3-/4-}$ concentration. It can be seen that the I/I_0 ratio initially increased with mediator concentration, which can be attributed to the corresponding increase in the MB - $Fe(CN)_6^{3-}$ reaction rate. At very high $Fe(CN)_6^{3-}$ concentrations only a small proportion of the total $Fe(CN)_6^{3-}$ is turned over by the MB, and therefore the peak height eventually decreases. As seen in the inset to Fig. 6, the optimum $Fe(CN)_6^{3-}$ concentration was 0.25 mM. The main section of Fig. 6 shows the DPV recorded to 1.0 pM of target sequence, following hybridisation with the stem loop, in the absence and presence of 0.25 mM $Fe(CN)_6^{3-/4}$. We used this mediator concentration to calibrate target DNA across the range 0.1 fM to 25 fM as shown in Fig. 7 (gradient = 5.95×10^{-8} A $(\log \text{aM})^{-1}$, $r^2 = 0.96$, $n = 30$). Eight determinations of a 30 mer non-complementary sequence produced $\dot{x} = 4.46 \times 10^{-8}$ A, $\sigma = 7.59 \times 10^{-9}$ A. Applying the definition stated earlier produces LOD ~ 39 aM. For the 2 µL hybridisation solution this corresponds to the detection of ~ 0.72 ag or 0.1 zmol, i.e. ~ 50 DNA molecules. This is not as low as the LOD reported by Ferapontova et al.[32] who used a lipase label to cleave ferrocene derivatives bound to an electrode (20 aM). However, to the best of our knowledge it is the lowest reported LOD for a redox label used on its own, without the presence of an enzyme. It is also lower than some recent reports of chemiluminescent hybridisation detection.[33,34] For a given concentration of

target (100 fM) we noted some difference in the response to 20 mer and 30 mer targets using both direct and mediated detection (direct detection: $\dot{x}$ 20 mer = 2.85 × 10^{-8} A, σ = 1.94 × 10^{-9} A, $\dot{x}$ 30 mer = 3.81 × 10^{-8} A, σ = 9.68 × 10^{-9} A, i.e. 25 % difference. Mediated detection: $\dot{x}$ 20 mer = 1.19 × 10^{-6} A, σ = 8.21 × 10^{-8} A, $\dot{x}$ 30 mer = 1.11 × 10^{-6} A, σ = 1.00 × 10^{-7} A, i.e. 8 % difference. n = 5 for all determinations). Hence, although the use of mediated detection lowers the effect of target length, we can still expect this to be a factor affecting assay precision in the detection of real samples.

4. Conclusions

We have described a highly sensitive method of detecting DNA hybridisation based on mediated electron transfer from a redox-labeled stem loop probe. The redox label consisted of a PSA sphere loaded with a high quantity of MB. Two issues arose from the use of this label: 1) Whether steric hindrance from neighbouring probes would prevent adequate label - electrode contact. 2) Whether charge from all the MB on the PSA sphere would be able to precolate to the electrode. To avoid these problems we employed the $Fe(CN)_6^{3-/4-}$ redox couple in solution, to mediate charge transfer from the MB label. This relatively simple strategy enabled us to lower the LOD of the assay by three orders of magnitude, relative to direct MB reduction. To the best of our knowledge, this is the lowest reported DNA detection limit achieved by a redox label in the absence of an enzyme. Issues remaining with this method are the fact that a number of preparative steps are required before the analytical signal can be realised. These include the solution-phase hybridisation reaction, confinement of the stem loop

to the electrode surface and attachment of the MB ball label. The number of experimental steps are reflected in the error bars we reported for both calibrations. Therefore, further work should include the development of strategies which provide adequate detection limits while requiring less experimental steps. This will be very challenging. There may have to be a compromise between method simplicity and sensitivity.

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Legends For Figures

Figure 1 UV spectra of PSA-$(PAA/PSS)_2(MB/PSS)_3$ spheres following dissolution in THF (A), and a solution of 2 μM MB dissolved in MilliQ water (B).

Figure 2 Ratio of DPV peak heights in the absence (I_0) and presence (I) of 1 pM target DNA, shown as a function of anti-DIG concentration applied to the poly-L-lysine modified screen printed electrode. Error bars show $\pm$ 1 std. dev. (n = 5).

Figure 3 Response to target DNA as a function of hybridisation temperature using screen printed electrodes modified by a 19.9 μg mL^{-1} anti-DIG solution. I_0 and I are as defined in Fig. 1. Error bars show $\pm$ 1 std. dev. (n = 5).

Figure 4 Background-subtracted DPV peak current from MB reduction to different target sequences. The background value is taken as the response to buffer.

Figure 5 Calibration of 30 mer DNA target based on the DPV peak height from the direct reduction of MB. Error bars show $\pm$ 1 std. dev. (n = 5). Inset: Examples of DPVs recorded using 1 pM (□), 100 fM (■), 25 fM (▼), 10 fM (Δ), 2.5 fM (○), and 1.0 fM (●) of target.

Figure 6 DPV of 1.0 pM target following hybridisation procedure in the absence (●) and presence (○) of 0.25 mM $Fe(CN)_6^{3-}$. Inset: I/I_0 ratio as defined in Fig. 1 shown as a function of $Fe(CN)_6^{3-}$ concentration for the cathodic peak for direct MB reduction (○) and and $Fe(CN)_6^{3-}$-mediated reduction (●).

Figure 7 Calibration of 30 mer DNA target by DPV in the presence of 0.25 mM $Fe(CN)_6^{3-}$ as a solution phase mediator. Error bars show $\pm$ 1 std. dev. (n = 5).

Scheme 1 Schematic representation of the hybridisation assay using $Fe(CN)_6^{3-}$ mediation. The stem loop probes are attached to the surface of screen printed electrodes by DIG tags binding to immobilised anti-DIG antibodies. When hybridisation does not occur, steric hindrance prevents the MB ball label from attaching to the stem loop.

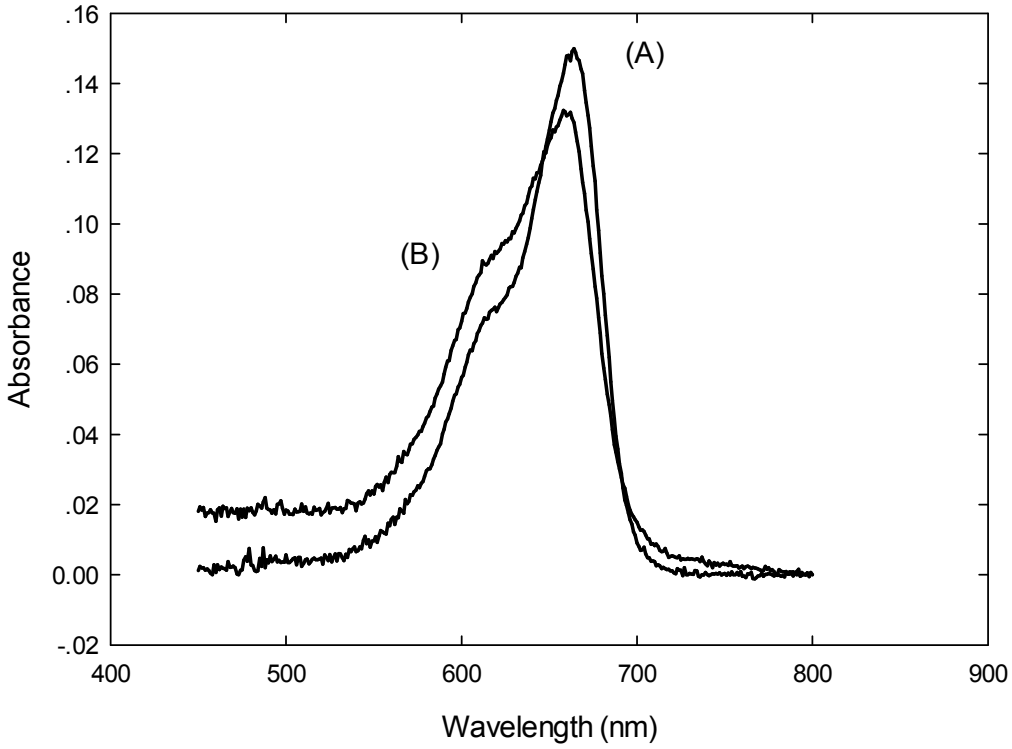


Figure 1

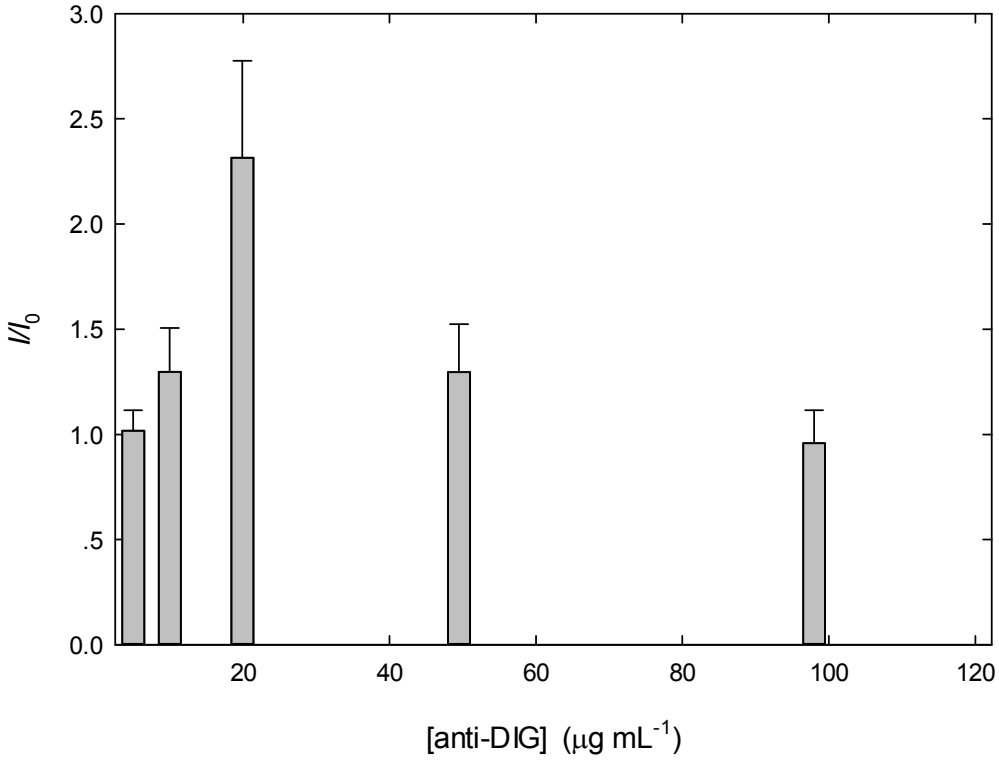


Figure 2

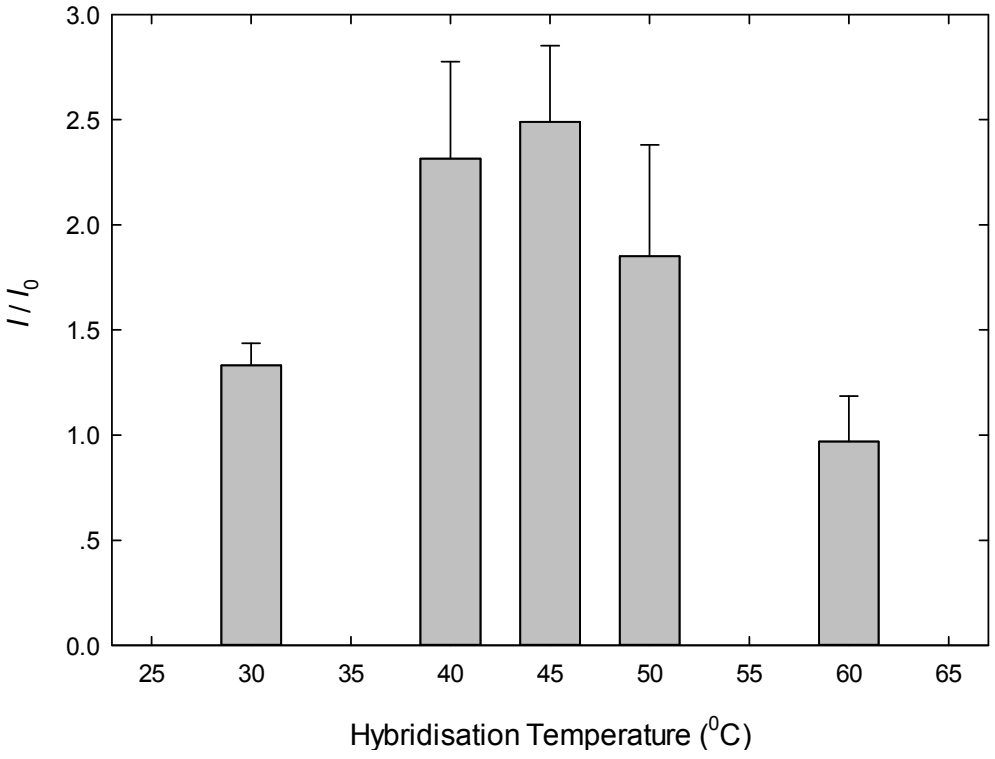


Figure 3

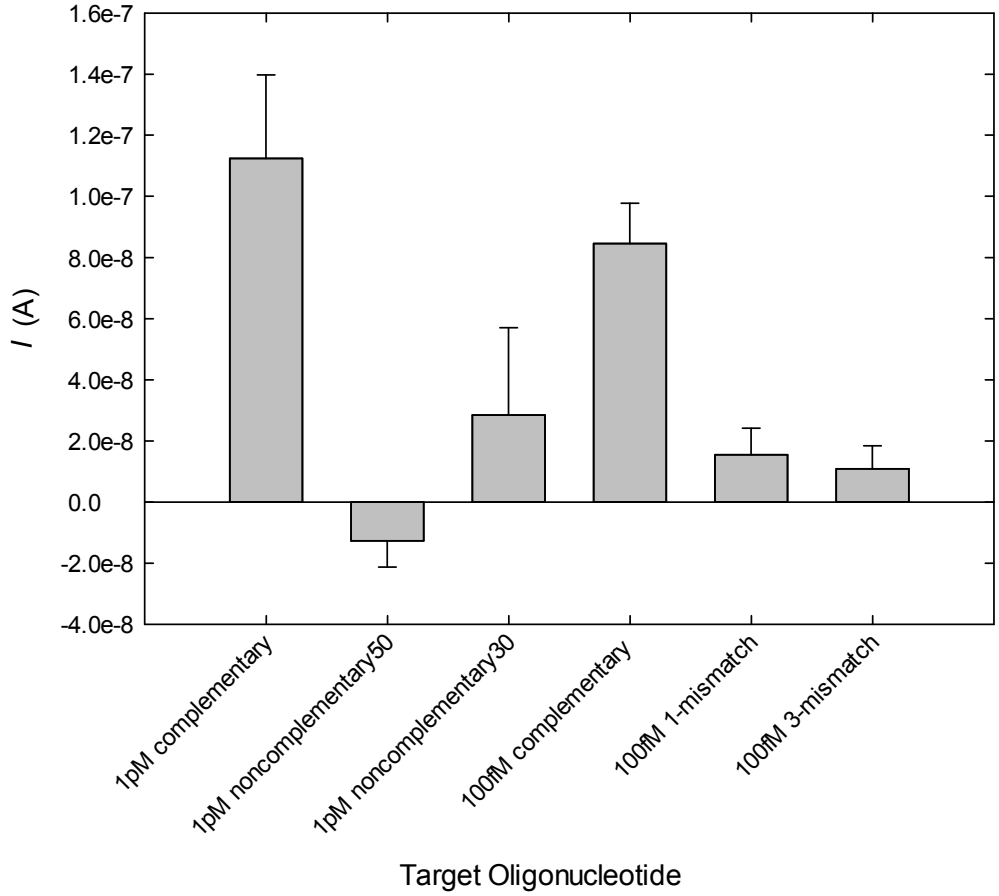


Figure 4

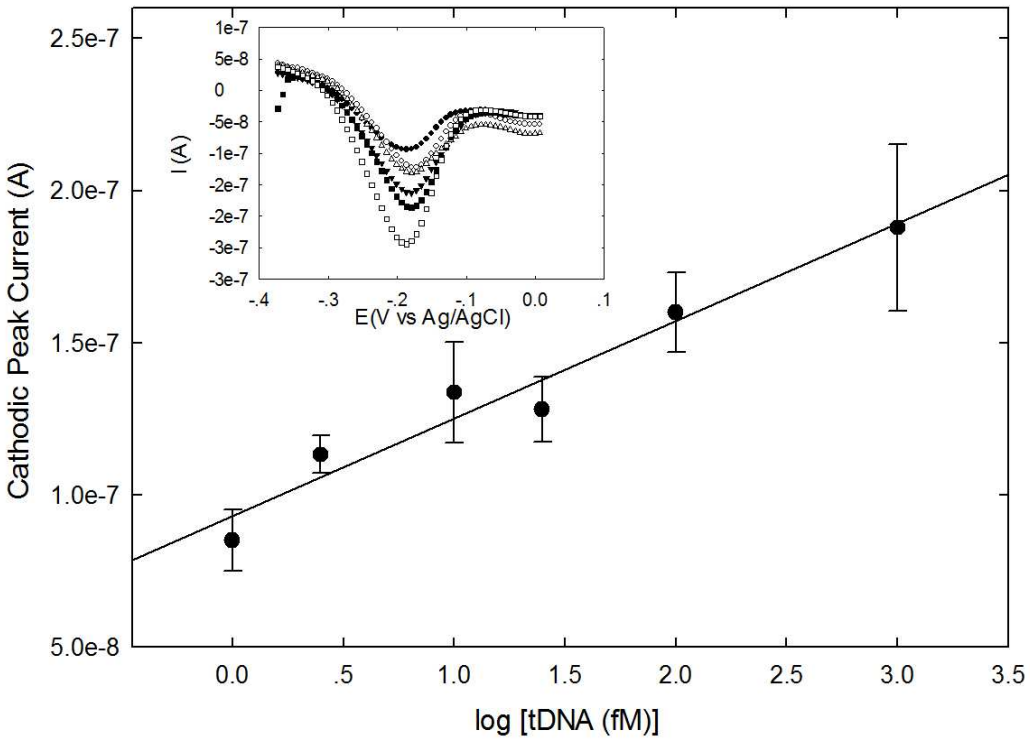


Figure 5

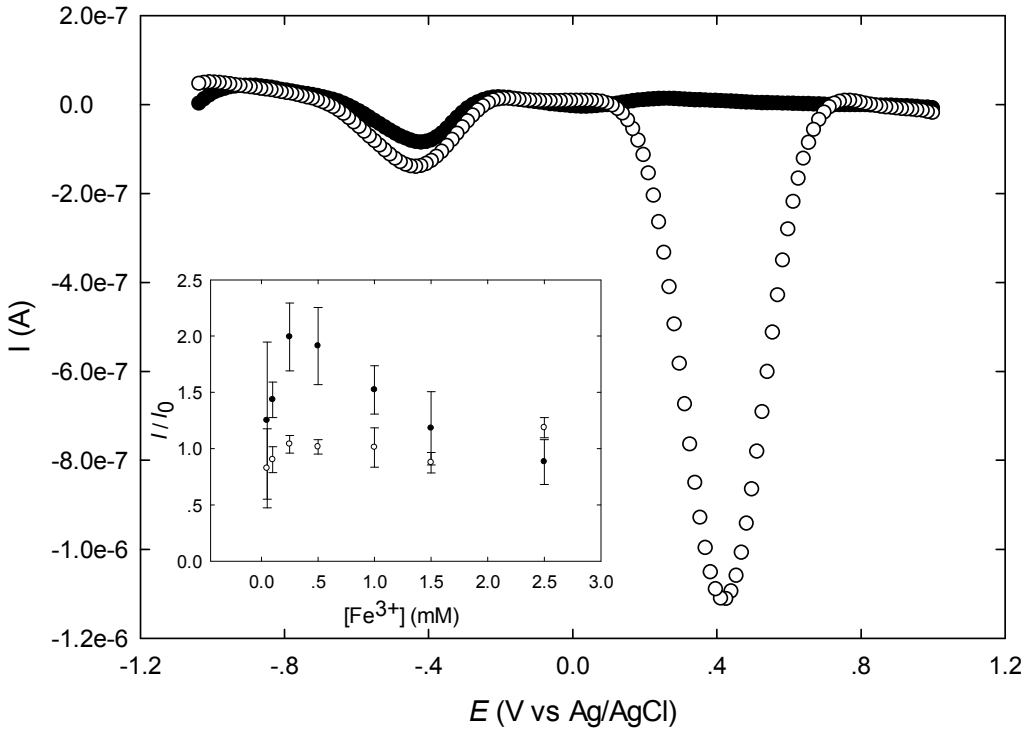


Figure 6

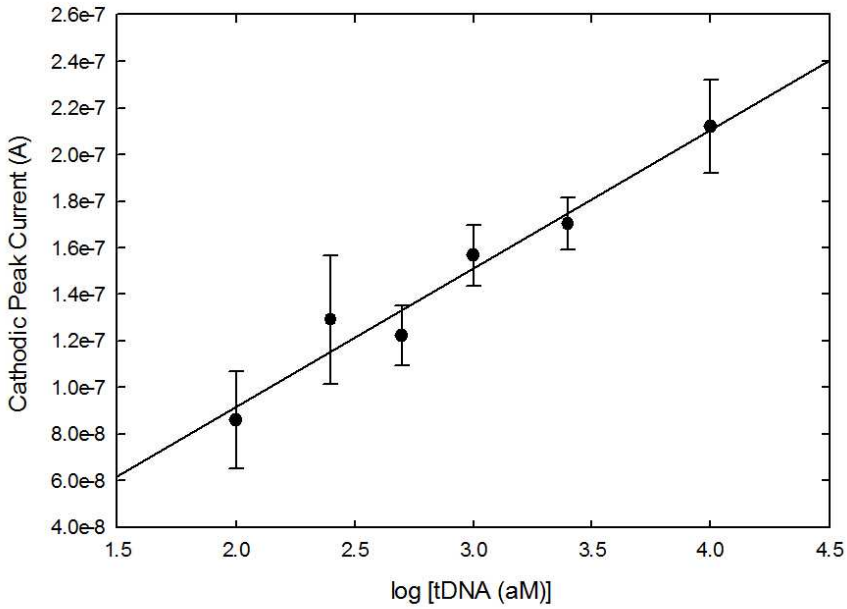
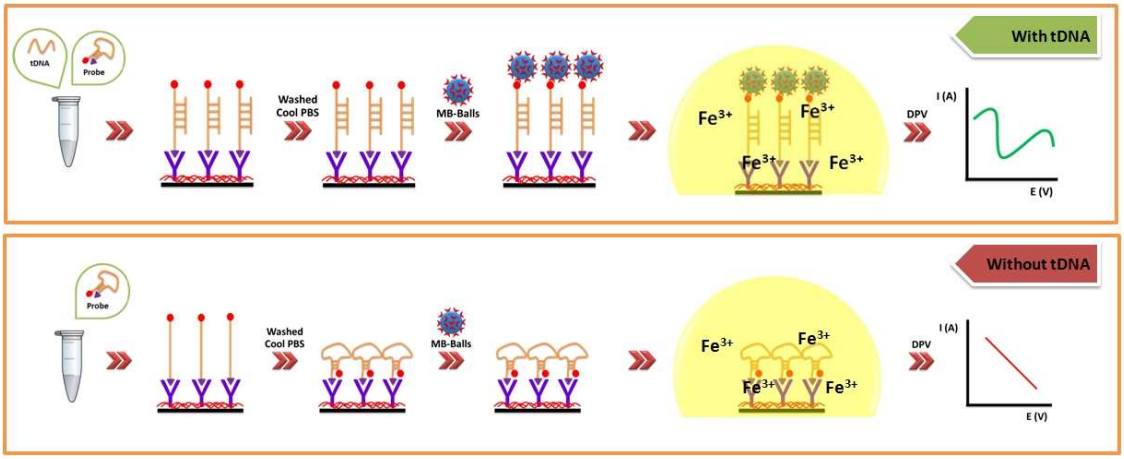


Figure 7



Scheme 1