A New Analytical Approach to Detecting Single Nucleotide Polymorphisms in DNA

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Executive Summary

- Self assembled monolayers (SAMs) are a convenient, simple way to functionalize gold surfaces.

- However strict protocols must be followed during preparation of the gold substrate in order to achieve a high quality surface.

- An electrochemical response can be detected from a ferrocenyl hexanethiol (short chain redox mediator) SAM modified gold electrode.

- Short chain double stranded DNA can be attached to a gold surface via the SAM method:

    - Thiol modified single stranded DNA will form a SAM on gold.
    - Methylene Blue (redox mediator) modified complementary target strand DNA will hybridise to this probe strand.

- An electrochemical response can be detected from this DNA modified gold electrode.

Aims

A Single Nucleotide Polymorphism (SNP) is a DNA sequence variation where one nucleotide base has been substituted for another. Detecting SNPs in DNA is important for genomic research, since they are often implicated in various genetic disorders, as well as heart disease, diabetes, and cancer.

Current methods of detecting SNPs rely on the fact that the melting temperature of SNP-containing DNA is lower than the corresponding SNP-free DNA, due to the instability of the mismatched base pairs. However, one of the main drawbacks is that these methods are fairly slow and inefficient.

Use of a laser heated conducting diamond electrode could provide an alternative method to interrogating DNA melting electrochemically.

The aim of this project was to develop a strategy to covalently tether DNA molecules, which have been tagged with a redox mediator, to the surface of an electrode and identify an electrochemical response.

Results

Self Assembled Monolayers (SAMs) provide a convenient and simple way to tailor the interfacial properties of metals and semiconductors. Historically, the most studied SAMs have been those formed from simple alkanethiols on gold. Indeed, gold is now considered the standard substrate for forming SAMs, since gold binds thiols with very high affinity, and it is reasonably inert.[1]

Gold substrates are prepared by physical vapour deposition methods – thermal evaporation or sputtering. A thin film of metal is deposited onto a glass or quartz support. Firstly, an adhesion layer of titanium (for sputtering) or chromium (for evaporation) is deposited, followed by a layer of gold.

The adhesion layer is important for improving the adhesion of metals that do not form oxides readily to substrates with an oxidised surface.[1] In fact, a major difficulty throughout the project was to ensure that the gold remained on the support and did not scratch or peel off. This was particularly problematic for gold that was sputtered. In order to improve adhesion, different procedures

[1] JC Love, LA Estroff, JK Kriebel, RG Nuzzo, GM Whitesides, *Chem. Rev.*, 2005, **105**, 1103-1169.

were used to clean the glass/quartz slides before deposition. That procedure which gave the best adhesion is detailed in the experimental section.

Evaporation is preferred over sputtering because (1) it was observed that gold remained better adhered to the support; (2) the gold surface produced is smoother, resulting in a better quality SAM formation.

The idea of redox active electrodes, where a redox active group is held at a fixed distance from a metal electrode surface, has been around for some time[2] but only recently has it led to more studies into electrodes modified with SAMs.[3]

Before attempting to use a relatively complex procedure to attach a large biomolecule to a gold electrode, the concept of surface bound electrochemistry was trialled by attempting to attach 6-(ferrocenyl)hexanethiol to a gold electrode. This chemical comprises ferrocene, a redox mediator, covalently bound to a short 6-carbon chain functionalized with a thiol at the other end. An illustration of the resulting SAM is shown in Figure 1.

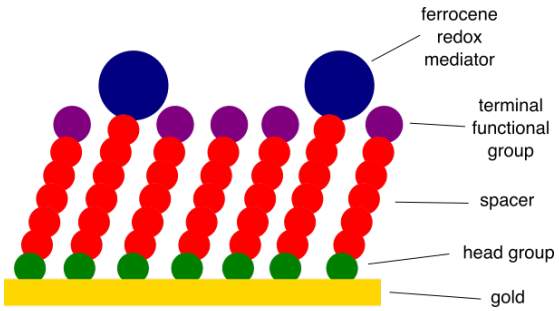

Fig. 1 Schematic of a SAM of ferrocenyl hexanethiol and pentanethiol diluent on a gold surface. The anatomy of the SAM is labelled. Note this is illustrative only and not indicative of the actual ratio of ferrocene to diluent molecules. Adapted from ref. 1.

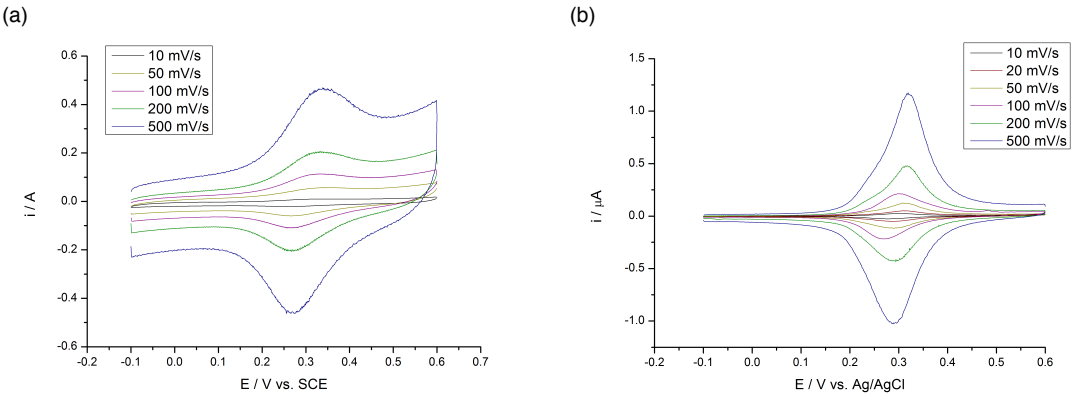

Fig. 2 Cyclic voltammograms of ferrocene linked via a hexane chain to (a) commercially available gold electrode (diameter 2 mm) and (b) evaporated gold electrode (diameter 1 mm). Both run in 0.1 M $HClO_4$ electrolyte solution at 25 °C, varying scan rate.

[2] K Weber, SE Creager, *Anal. Chem.*, 1994, **66**, 3164-3172.
[3] AL Eckermann, DJ Feld, JA Shaw, TJ Meade, *Coord. Chem. Rev.*, 2010, **254**, 1769-1802.

The experiment was done on both a commercially available gold electrode and an evaporated gold electrode made by the procedure outlined. Results from the cyclic voltammetry obtained are shown in Figure 2.

In the case of surface bound electrochemistry, where both the oxidised and reduced forms of a chemical species are strongly adsorbed to the electrode surface, the peak current, i_p, is directly proportional to the scan rate, v, as described by Eq. 1. The surface coverage, Γ, can be determined from the slope of the plot of i_p vs. v. [3,4]

$$i_p = \frac{n^2F^2}{4RT} vA_{SUR}\Gamma \qquad (1)$$

n is the number of electrons transferred during the redox process (for the redox mediator ferrocene, $n = 1$), F is Faraday's constant (96485 C/mol), R is the gas constant (8.314 J K^{-1} mol^{-1}), T is the temperature (298 K throughout), and A_{SUR} is the electrode surface area (0.00785 cm^2 for the evaporated gold electrodes; 0.0314 cm^2 for the commercial macro gold electrode).

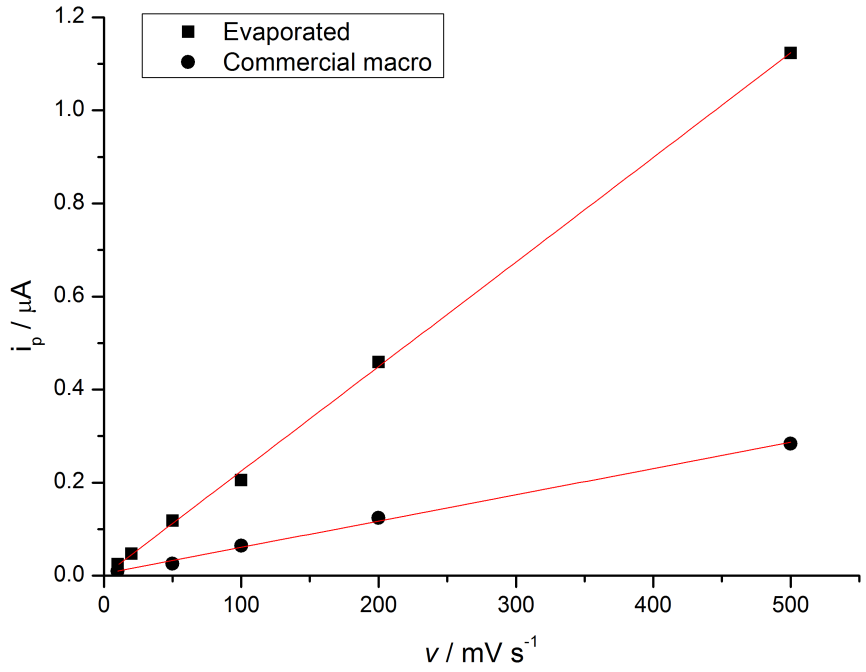

Fig. 3 Plot of the peak current, i_p versus scan rate, v, for the ferrocenyl hexanethiol modified evaporated and commercial macro gold electrodes.

[4] E Laviron, *J. Electroanal. Chem.*, 1979, **101**, 19-28.

Electrode	Slope	Γ / molecules cm^{-2}
Evaporated	2.25×10^{-9}	1.84×10^{11}
Commercial macro	5.64×10^{-10}	1.15×10^{10}

Table 1 For the two ferrocenyl hexanethiol modified gold electrodes used, the slope of the plot of peak current versus scan rate, and the surface coverage Γ as calculated from Eq. 1.

Contrary to what is expected, the evaporated electrodes, which have a four times larger surface area than the commercial macro electrode, actually obtain a ten times greater surface coverage of ferrocene molecules (Table 1). Whilst no surface characterisation has been done of either electrode, it is postulated that the commercial gold electrode is rougher than the evaporated; hence a better quality SAM formation can be achieved.

Additionally, this result can be seen in the cyclic voltammetry (Figure 2) where the peak current of the evaporated electrode is around 1.2 μA whereas the peak current of the commercial macro electrode is lower – around 0.5 μA. If both electrodes had equal surface coverage it would be expected that a larger area electrode would give a larger peak current. Nevertheless, DNA surface coverages of $10^{10} – 10^{11}$ molecules cm^{-2} are acceptable, since other literature reported values are in the order of 10^{12} molecules cm^{-2}.[5]

The signals observed in the cyclic voltammograms in Figure 1 show that it is possible to tether redox active moieties a fixed distance away from an electrode surface.

In order to apply this technique to double stranded DNA, rather than a relatively simple organometallic compound, the DNA needs to be functionalized with a redox active group. Methylene Blue (MB) is often chosen for this role, because it intercalates well with the base stack of DNA and hence forms a good interaction.[6]

Firstly, it is important to understand the electrochemistry of MB. Cyclic voltammetry was performed on bulk solutions of MB (Figure 4), where the reaction is limited by diffusion of MB molecules to the electrode surface. Note that this is different to the situation with surface bound electrochemistry, where the MB is bonded to a DNA strand and also to the electrode, and diffusion does not play a part.

[5] R Johnson, N Gale, JA Richardson, T Brown, PN Bartlett, *Chem. Sci.*, 2013, **4**, 1625-1632.
[6] CG Pheeney, JK Barton, *Langmuir*, 2012, **28**, 7063-7070.

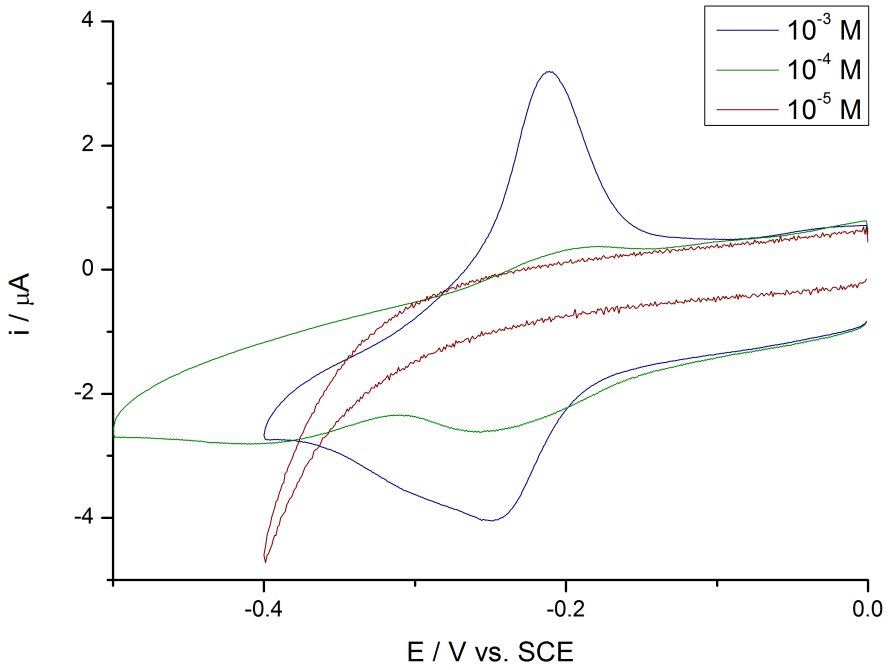

Fig. 4 Cyclic voltammograms of methylene blue of varying concentrations in phosphate buffer solution, $v = 100$ mV/s, $T = 25$ °C.

As expected, the peak current decreases proportionally with decreasing concentration of MB.

The experimental section details the structure of the MB labelled double stranded DNA along with a schematic of the preparation of the DNA modified gold electrode. Cyclic voltammograms of bare gold, and gold modified with a SAM of probe DNA only (before hybridisation) (Figure 5) show no signal, since neither contain MB.

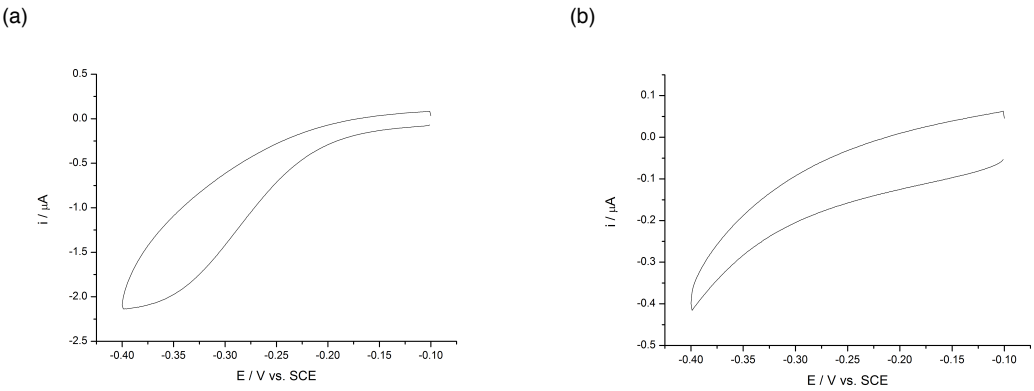

Fig. 5 Cyclic voltammograms of evaporated gold electrodes (a) not functionalized *i.e.* bare gold, and (b) functionalized with probe DNA only, in phosphate buffer solution, $v = 100$ mV/s, $T = 25$°C.

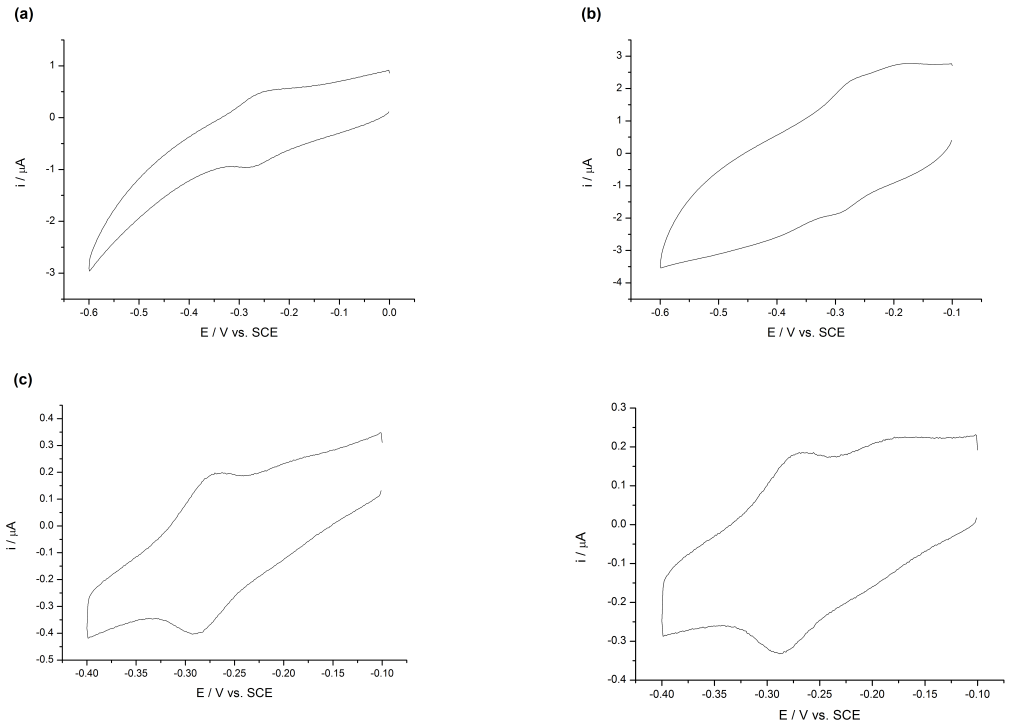

Fig. 6 Cyclic voltammograms of evaporated gold electrodes modified with methylene blue labelled double stranded DNA and mercaptohexanol (MCH) diluent prepared in a solution of (a) 1:0 ratio DNA:MCH, (b) 1:1 ratio, (c) 1:5 ratio, (d) 1:10 ratio. All are run in phosphate buffer solution, v = 100 mV/s, T = 25 °C.

Results from gold electrodes modified with MB labelled double stranded DNA are shown in Figure 6. Those electrodes prepared in solutions 1 and 2 can be seen to give a weaker signal than those prepared in solutions 3 and 4. Therefore it is considered that the diluent molecule is important and impacts the SAM formed. DNA molecules will pack close together but their large size means some of the gold surface is left exposed. Subsequent hybridisation may mean that the target DNA adsorbs to the gold surface instead of hybridising with the probe DNA strand.

Notice the very large background current in all electrodes. This can be correlated with (1) the thickness of the SAM, since a SAM formed from DNA strands is relatively large, and (2) defects in the structure of the SAM, which may allow electrolyte to reach the electrode surface.[1]

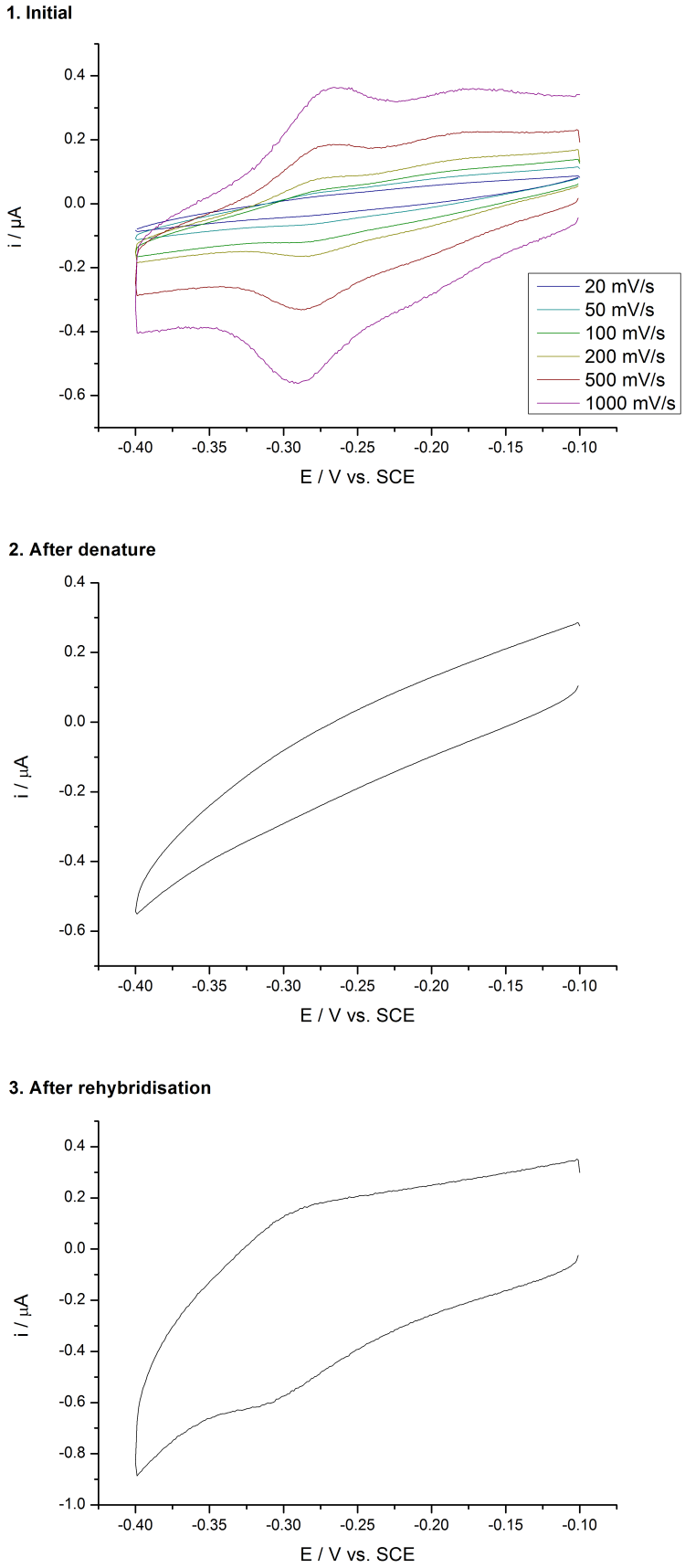

Fig. 7 Cyclic voltammograms of gold electrode modified (1) initially with methylene blue labelled double stranded DNA; (2) after denature by heating; (3) after rehybridisation by slow cooling process. All run in phosphate buffer solution, varying scan rate for (1), v = 100 mV/s for (2) and (3), T = 25 °C.

Figure 7 shows results from a stability test. Firstly are the initial results from the DNA modified electrode prepared in solution 4. Next the electrode was heated to separate the DNA strands, resulting in no signal, since it contains the probe DNA only. Finally the electrode was added back to the target DNA and slowly cooled to allow rehybridisation to occur. The signal returns, albeit somewhat weaker.

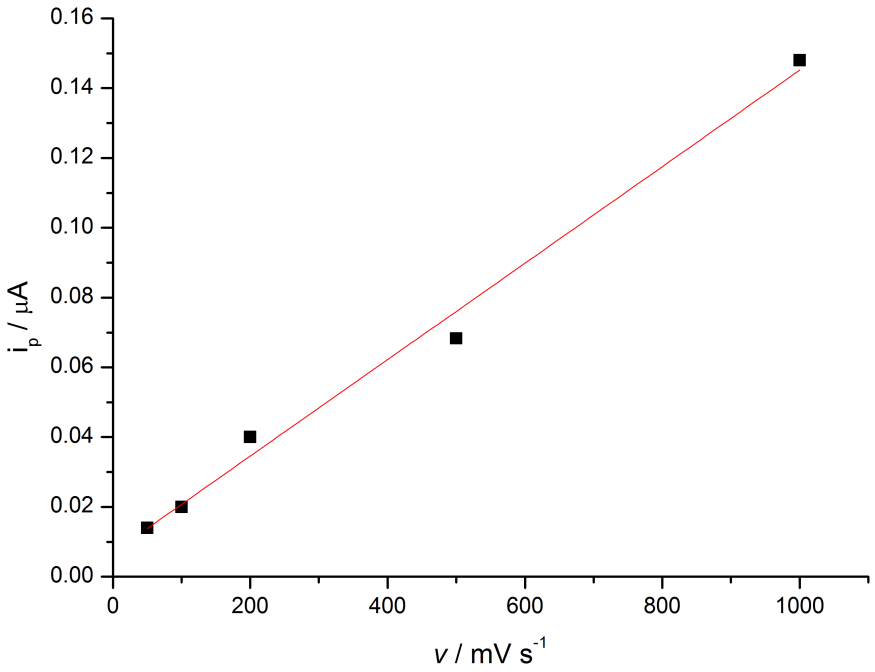

Fig. 8 Plot of the peak current, i_p versus scan rate, v, for the methylene blue labelled DNA modified evaporated gold electrodes.

From Eq. 1 and Figure 8, where the peak current is determined from the reduction peaks, the surface coverage of the electrode can be calculated to be 2.82×10^9 molecules cm^{-2}. Compared to the ferrocene electrodes this is slightly lower; however, this may be due inherently to the fact DNA is larger and so less molecules can pack in the same surface area, but furthermore not every DNA stand may hybridise, thus reducing the effective number of redox groups on the electrode.

Experimental

Gold substrate preparation

Glass and quartz slides were cut into pieces of approx. 1 x 2.5 cm size. The slides were cleaned by immersion in piranha solution for 10 minutes. Piranha solution is a mixture of 3:1 v/v of concentrated sulphuric acid and 30% hydrogen peroxide. The solution was maintained at 60 °C during the 10 minutes cleaning. The slides were then removed and rinsed with deionised water, before being dried under a stream of nitrogen. If drying could not be done immediately, the slides were stored in deionised water. Once dry, the slides were exposed to oxygen plasma at 100% power for 2 minutes. Finally the slides were mounted in the evaporation system, for deposition of chromium (2.5 nm thickness) and gold (55 nm thickness). After removal from the evaporator, the gold electrodes were used immediately for further experiments.* No cleaning procedure was undertaken on the deposited gold.

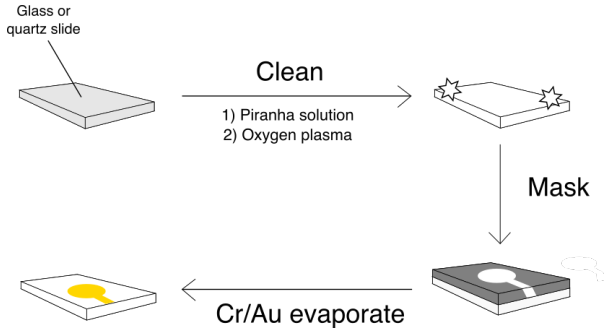

Fig. 9 Schematic of the fabrication of evaporated gold electrodes.

6-(ferrocenyl)hexanethiol modification

A commercially available gold electrode (2 mm diameter) was cleaned abrasively using alumina, and then electrochemically by cycling between –0.05 and +1.1 V repeatedly in 1 M sulphuric acid.

Evaporated gold electrodes were fabricated by creating a contact between the gold and a wire using silver conducting paint, before being sealed with epoxy. It is important to not let the epoxy come into contact with the ethanol solution used subsequently, as this will cause the electrode contact to become detached.

* Gold electrodes, particularly those that were sputtered, left exposed to air for more than 24 hours would be seen peeling off the glass or quartz slides.

The gold electrodes were immersed into an ethanol solution containing the redox active thiol, 6-(ferrocenyl)hexanethiol (1×10^{-4} M) and a diluent molecule, 1-pentanethiol (9×10^{-4} M). The electrodes were exposed to this solution for 24 hours. After this, they were rinsed with and then stored in absolute ethanol. Electrochemical measurements were obtained by running in a 0.1 M $HClO_4$ electrolyte solution.[7]

DNA modification

DNA was synthesized and provided to use by Robert Johnson, University of Utah. The surface (probe) strand is modified at the 3' end (Figure 10a), whilst the target strand is modified at the 5' end (Figure 10b) to ensure the MB redox label is as close to the surface as possible.

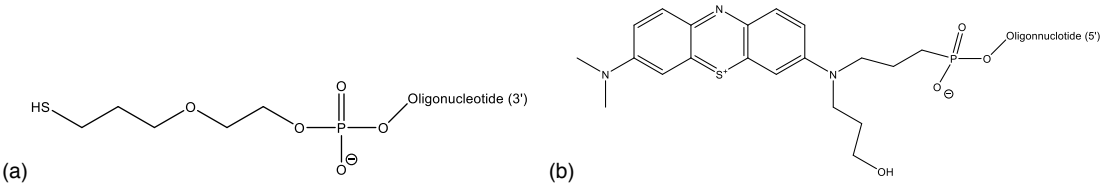

Fig. 10 Structures of the modified ends of the (a) probe and (b) target DNA strands.

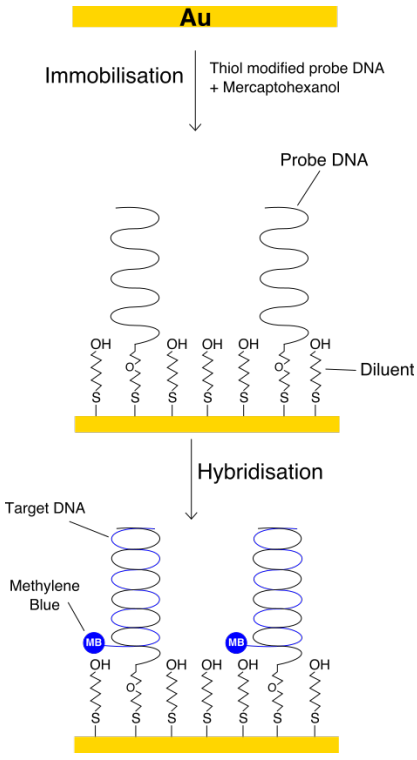

Fig. 11 Schematic of the preparation of double stranded DNA gold electrodes.

Evaporated gold electrodes were immersed into a solution containing the probe DNA and a diluent molecule mercaptohexanol in phosphate buffer (10 mM phosphate, 1 M KCl, pH 7.3). The ratios of DNA to mercaptohexanol (MCH) concentrations were varied as follows:

Solution	DNA / µM	MCH / µM
1	5	0
2	5	5
3	5	25
4	5	50

The electrodes were exposed to these solutions for 24 hours. They were rinsed with buffer, and then immersed in a solution of target DNA (5 µM). The solutions were heated to 80 °C and then slowly cooled to room temperature over a period of 2 hours. After this, the electrodes were rinsed with and then stored in buffer. Electrochemical measurements were obtained by running in buffer.

[7] JF Smalley, HO Finklea, CED Chidsey, MR Linford, SE Creager, JP Ferraris, K Chalfant, T Zawodzinsk, SW Feldberg, MD Newton, *J. Am. Chem. Soc.*, 2003, **105**, 2004-2013.