Original Paper

Determination of Surface-Immobilized Double-Stranded DNA Using a Metallointercalator and Catalytic Voltammetry

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Abstract. DNA films immobilized on an indium tin oxide (ITO) electrode surface were detected and determined employing a high-affinity intercalator, $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$ ($\text{bpy} = 2,2'$ -bipyridine, $\text{dppz} = \text{dipyrido}[3,2\text{-}a:2',3'\text{-}c]\text{phenazine}$), as a redox indicator, and oxalate as a sacrificial electron donor in solution to chemically amplify the voltammetric signal of the indicator. Nucleic acids were immobilized on ITO by layer-by-layer electrostatic adsorption, using avidin as the first layer and nucleic acid as the second layer. In quartz crystal microbalance (QCM) measurements on an avidin-coated gold surface, the amount of adsorption from a $200\ \mu\text{g mL}^{-1}$ nucleic acid solution was found to be $3.2\ \text{ng mm}^{-2}$ for both double-stranded (ds-) and single-stranded (ss-) calf-thymus DNA as well as polycytidylic acid (Poly-C). After binding with $\text{Ru}(\text{bpy})_2(\text{dppz})^{2+}$ (Ru-dppz), voltammetry of the ds-DNA film on ITO was carried out in an indicator-free phosphate buffer. An anodic peak at about 1.15 V was observed, and it was assigned to Ru-dppz oxidation. When measured in an oxalate buffer, however, a catalytic current was observed due to the oxidation of oxalate by electrochemically generated $\text{Ru}(\text{bpy})_2(\text{dppz})^{3+}$, resulting in a 120-fold increase in the signal. Since oxalate itself produces a very low oxidation current on

ITO, catalytic voltammetry produces about a 14-fold improvement in the signal-to-blank ratio over the non-amplified determination. As a result, ds-DNA adsorbed from $20\ \text{ng mL}^{-1}$ solution could be detected, which was estimated by QCM to be $160\ \text{pg mm}^{-2}$ on the surface. The catalytic current of ds-DNA was substantially higher than that of ss-DNA and poly-C, indicative of selective binding of the redox indicator to ds-DNA. The results serve as a basis for the catalytic voltammetric detection of DNA hybridization in future work.

Key words: Electrochemistry; DNA determination; catalytic voltammetry; intercalator.

New analytical methods for nucleic acids have been developed for life science research, environmental monitoring, drugs toxicity screening, and clinical molecular diagnosis. DNA sensors for the detection of mutations in specific genes as diagnostic markers, and for the detection of differences in gene expression levels in healthy and diseased cells have attracted ever increasing attention. Electrochemistry is a widely used analytical method in various types of qualitative and quantitative analysis, including the increasingly popular DNA analysis [1–6]. The main advantages it offers are the relatively high sensitivity, simplicity, low instrument cost and small instrument size. The overview below shows that the sensitivity of some

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of the electrochemical methods are beginning to out-rival the more popular fluorescence methods based on DNA binding dyes such as PicoGreen (detection limit 25 pg mL^{-1}).

Strategies for the electrochemical analysis of DNA can be classified into two groups: label-free and DNA-labeled methods. In most of the label-free strategies, DNA was detected directly, relying on the electroactivity of DNA bases and sugars [7–9]. Recently, Thorp's group developed a novel approach in label-free DNA analysis, employing dissolved ruthenium tris (bipyridine) (Ru-bipy) as a redox mediator for the catalytic oxidation of DNA guanines. An impressive detection limit of 40 pg mm^{-2} was achieved due to the facile electrode kinetics of the mediator and fast second-order reaction between the metal complex and guanine [10–12]. The approach was further developed by Rusling and Forster by immobilizing the mediator on the electrode surface [13, 14].

Detection of DNA has also been accomplished using electroactive labels either covalently or non-covalently attached to DNA, such as $\text{Co}(\text{bpy})_3^{2+}$, methylene blue, $\text{Ru}(\text{NH}_3)_6^{2+}$, and anthraquinone [15–19]. More recently, higher affinity DNA binders were developed and tested as redox indicators. Takenaka's group developed a threading intercalator, ferrocenyl-naphthalene diimide [20], with a binding constant of $1.3 \times 10^5 \text{ M}^{-1}$. Maruyama's group reported a metal complex, $\text{Os}(\text{DA-bpy})_2(\text{dppz})^{2+}$ (DA-bpy = 4,4'-diamino-2,2'-bipyridine, dppz = dipyrido[3,2-a:2',3'-c]phenazine) with a binding constant of $3.7 \times 10^7 \text{ M}^{-1}$ [21]. A detection limit of 1 fmol and 160 pg mL^{-1} was achieved, respectively.

Covalent labeling of DNA permits electrochemical measurements to be carried out in a label-free buffer, thereby minimizing the background current. One popular group of redox molecules for covalent labeling is that of ferrocene derivatives, which have been employed by Umek et al., Nakayama et al. and others in a variety of DNA tests [22, 23]. Most recently, a ferrocene-tagged "hairpin" type DNA probe was developed for the reagentless detection of a specific DNA sequence with a detection limit of 5 fmol [24]. Another group of frequently used covalent labels is that of enzymes, which have produced the most sensitive DNA assays to date (1 fmol L^{-1}) thanks to the power of enzymatic amplification [25–27]. Apart from molecular labels, various nanoparticles have also been shown to be promising materials in DNA detection [28–30].

We employed catalytic voltammetry for the determination of various biological binding assays [31–33]. With this method, a Ru-bipy derivative is attached either covalently or non-covalently to a biological binding pair as a redox label. An artificial electron donor, oxalate, is present in the solution in large excess during the electrochemical measurement. The anodic current of Ru-bipy on the ITO electrode is amplified by the catalytic reaction with oxalate. Because oxalate itself produces a negligible oxidation current on ITO, the signal-to-background ratio is improved substantially compared to non-catalytic detection. The unique features of the metal complex label include high stability, small size, diversity of linking chemistry, and applicability to electrode array-based determination. Using Ru-bipy as a covalent label in a competitive assay for small molecules yielded a determination limit of 1 ng mL^{-1} biotin on an avidin-coated electrode [33]. In another demonstration of the utility of the method, a high-affinity DNA intercalator, Ru-dppz, was employed as a non-covalent redox label in the determination of ds-DNA in solution by catalytic voltammetry [32].

This report describes our work on the determination of surface-immobilized ds-DNA by catalytic voltammetry. The above mentioned Ru-dppz intercalator was employed once again as the redox indicator. As in previous studies, a direct comparison was drawn between catalytic and non-catalytic detection to demonstrate the benefit of catalytic voltammetry. The indicator provided good selectivity towards ds-DNA over ss-DNA and Poly-C. By rough estimation, 160 pg mm^{-2} ds-DNA on the ITO surface was detected. The unique feature of the current system is that the Ru-dppz redox indicator not only produces a catalyzed oxidation signal, but also differentiates between single- and double-stranded DNA.

Experimental

Chemicals

Sodium oxalate and oxalic acid were purchased from Alfa Aesar (Ward Hill, MA, USA, www.alfa.com). Calf thymus ds-DNA (approximately 13 k base pairs), ss-DNA, Poly-C potassium salt, 3-mercaptopropionic acid (MPA) and Ru-bpy were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA, www.sigmaaldrich.com) and used as received. Avidin was purchased from MP Biomedicals (Irvine, CA, USA, www.mpbio.com). Ru-dppz was synthesized according to the published procedure [34, 35]. Solutions were prepared in high-purity water from a Millipore milli-Q (Biocel) water purification system (Billerica, MA, USA, www.millipore.com). Both Ru-dppz and oxalate solutions can be used for over 3 months if kept at 4°C in the dark.

DNA Film Assembly and Probe Binding

ITO conductive glass was obtained from WeiGuang Corporation (Shenzhen, Guangdong Province, P.R. China) and cut into 2.5×0.5 cm size electrodes. The electrodes were cleaned according to a previously described protocol [32]. The cleaned ITO electrodes were first coated with 10 μ L avidin (0.25 mg mL^{-1} , dissolved in 20 mM phosphate buffer, pH 7.3) for 1.5 h, then washed carefully. This surface is denoted as ITO/avidin. The modified electrode was followed by coating with 10 μ L nucleic acids of desired concentration in 10 mM sodium oxalate/oxalic acid, pH 5.5, for 30 min, and is denoted as ITO/avidin/DNA. Finally, Ru-dppz was reacted with the DNA film for 30 min, and unbound metal complex was washed off (ITO/avidin/DNA/Ru-dppz).

Electrochemical Experiment

Cyclic voltammetry was performed on a CHI 660B Electrochemistry Analyzer (Austin, TX, USA) with a Pt counter-electrode and an Ag/AgCl reference electrode. The electrode area in contact with the electrolyte was 0.5 cm^2 .

Quartz Crystal Microbalance

QCM measurements were performed on a CHI 400A instrument (Austin, TX, USA) to monitor the film assembly. Gold-coated resonator electrodes provided by CHI were rinsed with freshly prepared Piranha solution (95°C) for nearly 15 min and then successively with water and ethanol (Caution: Piranha solution should be handled with extreme care, and only small volumes should be prepared at a time). The cleaned gold electrode was coated with MPA by adsorption in 4 mM ethanol solution for 1 h to introduce negative charges onto the surface. Avidin and DNA layers were assembled following the same procedure as described above for ITO electrodes. After assembly of each layer, the surface was rinsed with water and blown dry with nitrogen before QCM measurement. With a basic frequency of 8 MHz and an area of 0.205 cm^2 , the frequency change is related to the film mass change ΔM as follows:

$$\Delta F = -7.05 \times 10^8 \Delta M$$

Results and Discussion

Previously we applied the Ru-dppz/oxalate catalytic system to the voltammetric determination of ds-DNA in solution [32]. In this study, we employed the same system in the determination of surface-immobilized DNA. While there are many ways of immobilizing DNA molecules on solid surfaces, the layer-by-layer electrostatic self-assembly approach is one of the most convenient methods of constructing multi-layer films. Avidin protein has been found to adsorb strongly on ITO surfaces by electrostatic interaction due to its high positive charge [33], and was used successfully in this study for subsequent deposition of the DNA layer.

QCM measurement was performed on a gold crystal to verify the immobilization of the DNA layer and to determine the amount of DNA immobilized. To

Fig. 1. Measured QCM frequency change on an 8 MHz gold crystal (0.205 cm^2) after deposition of (a) MPA; (b) avidin; (c) $200 \mu\text{g mL}^{-1}$ Poly-C; (d) $200 \mu\text{g mL}^{-1}$ ss-DNA; (e) $200 \mu\text{g mL}^{-1}$ ds-DNA; (f) $60 \mu\text{g mL}^{-1}$ ds-DNA; and (g) $20 \mu\text{g mL}^{-1}$ ds-DNA

mimic the situation on the ITO surface, the gold crystal was first modified with MPA to obtain a negatively charged surface, to which an avidin layer was then adsorbed by electrostatic adsorption. We assumed that this surface should be similar to the avidin-coated ITO and would adsorb approximately the same amount of DNA, as found by Lvov et al. [36].

Figure 1 shows the frequency shift (average of 3 replicates) of the gold crystal after deposition of MPA, avidin and nucleic acid, respectively. After deposition of the avidin layer, the QCM frequency decreased by 155 Hz on average. According to Eq. (1), the frequency shift corresponds to 11 ng mm^{-2} of avidin immobilized on the MPA-coated gold-electrode, or $1.6 \times 10^{-11} \text{ mol cm}^{-2}$ surface coverage. The surface coverage of avidin adsorbed on ITO electrodes can be calculated from the voltammogram in our previous study [33], which is estimated to be $2.5 \times 10^{-11} \text{ mol cm}^{-2}$. The rough agreement of avidin coverage between MPA-modified gold and the ITO surface suggests that the former can be used to mimic the latter in order to measure DNA adsorption. After deposition of nucleic acids from a $200 \mu\text{g mL}^{-1}$ solution on the avidin-coated surface, the frequency shift was in the range of -44 to -49 Hz for ss-DNA, ds-DNA and Poly-C, indicating that the three nucleic acids were deposited by the same amount. It can be estimated that 3.2 ng mm^{-2} nucleic acids were immobilized on the avidin-coated gold electrode. These estimates are very close to those measured by Rusling et al. on myoglobin-coated surfaces. The amount of adsorption from $60 \mu\text{g mL}^{-1}$ and $20 \mu\text{g mL}^{-1}$ ds-DNA was estimated by QCM to be 2.1 and 0.55 ng mm^{-2} , respectively. The latter approaches the detection limit of our QCM measurement (due to the change in temperature, humidity, vibration, etc., the smallest measurable frequency change is approximately 10 Hz).

Fig. 2. Voltammograms of (a) ITO/avidin/ds-DNA/Ru-dppz; (b) ITO/avidin/Ru-dppz; and (c) ITO/avidin/ds-DNA. The DNA layer was deposited from a $6 \mu\text{g mL}^{-1}$ solution, and the Ru-dppz concentration was $30 \mu\text{M}$. Voltammetry was measured in 20 mM phosphate buffer, pH 7.3

As mentioned above, ds-DNA was immobilized by an intermediate layer of avidin on the ITO electrode. After reacting with $30 \mu\text{M}$ Ru-dppz for 30 min, the unbound indicator was washed off, and voltammetric measurement was carried out in an indicator-free phosphate buffer. Figure 2a shows an anodic peak at about 1.15 V. Based on the peak position and the absence of such an oxidation peak for the ITO/avidin/ds-DNA surface (Fig. 2c), the response was assigned to Ru-dppz oxidation. From Fig. 1a and c, the signal-to-blank ratio (S/B) is estimated to be roughly 3.5 at 1.15 V for the DNA film deposited from a $6 \mu\text{g mL}^{-1}$ solution. The metal complex is believed to bind to the surface due to specific interaction with DNA, as it produced a much lower signal on a DNA-free (ITO/avidin) surface (Fig. 2b). When the DNA concentration was lowered to $2 \mu\text{g mL}^{-1}$, no difference was observed between a DNA surface and a DNA-free surface.

The benefit of catalytic voltammetry was demonstrated in our previous study by drawing a direct comparison between amplified and non-amplified detection. 60-fold and 100-fold signal enhancement was obtained in oxalate buffer for Ru-dppz intercalated DNA in solution [32] and Ru-bipy labeled biotin [33], respectively. In this report, a similar enhancement effect was also observed for Ru-dppz intercalated into surface-immobilized DNA films. The ITO/avidin/ds-DNA/Ru-dppz surface was prepared following the same procedure as in Fig. 2, and its voltammetry was carried out in a 30 mM sodium oxalate/oxalic acid buffer. A sigmoid-shaped response curve was obtained, as depicted in Fig. 3a, indicative of an electrochemical reaction followed by a catalytic reaction. This is due to the electrochemical oxidation of Ru-dppz followed by the redox reaction between

Fig. 3. Voltammograms of (a) ITO/avidin/ds-DNA/Ru-dppz; (b) ITO/avidin/Ru-dppz; and (c) ITO/avidin/ds-DNA. The DNA layer was deposited from a $6 \mu\text{g mL}^{-1}$ solution, and the Ru-dppz concentration was $30 \mu\text{M}$. Voltammetry was measured in 30 mM sodium oxalate/oxalic acid (pH 5.8)

oxidized Ru-dppz and oxalate. Although guanine bases in DNA can also be electro-catalytically oxidized [11], the contribution is negligible because the number of guanines in the film is limited. The catalytic current at 1.3 V in Fig. 3a is around $60 \mu\text{A}$, which is approximately 120 higher than the non-amplified signal illustrated in Fig. 2a. Because the background current from oxalate oxidation is very low (Fig. 3c), the S/B ratio also increases to 37, representing approximately a 14-fold improvement over the non-amplified measurement. The limiting current is not a perfect plateau, reflecting the kinetic limitation of the metal complex across the non-conducting film.

With the improved S/B ratio, a smaller amount of surface-immobilized DNA could be detected by catalytic voltammetry than with the regular method. In Fig. 4, the concentration of ds-DNA deposition solution was varied from $0.02 \mu\text{g mL}^{-1}$ to $200 \mu\text{g mL}^{-1}$ in 3-fold increments. After DNA film assembly and reacting with $30 \mu\text{M}$ Ru-dppz, the electrode was measured in 30 mM oxalate buffer. As can be seen from the figure, the relationship between the measured catalytic current and the DNA concentration follows the typical adsorption isotherm. The relative standard deviation of the data ranges from 3% to 15%. Also plotted in Fig. 4B is the mass increase of the DNA film obtained in Fig. 1 with respect to the solution concentration. Due to the limitation of the QCM instrument, only films adsorbed from $20 \mu\text{g mL}^{-1}$ solution or higher could be detected reliably. Bearing in mind the limited number of QCM data, the trend of the mass change nonetheless looks very similar to that of the current. If we assume that both voltammetric response and mass change follow the same adsorption isotherm, then we can extrapolate the lowest amount of surface-bound DNA that can be detected by catalytic

Fig. 4. (A) Voltammograms of ITO/avidin/ds-DNA/Ru-dppz electrodes prepared from the following DNA solutions: 0, 0.02, 0.06, 0.2, 0.6, 2, 6, 20, 60 and 200 $\mu\text{g mL}^{-1}$. The Ru-dppz concentration was 30 μM . Voltammetry was measured in 30 mM sodium oxalate/oxalic acid, pH 5.8. The arrow sign indicates increasing DNA concentration. (B) Plots of the catalytic current at 1.3 V (open square, left vertical axis) and QCM mass change (filled triangle, right vertical axis) as a function of DNA concentration

Fig. 5. Voltammograms of ITO/avidin/DNA/Ru-dppz electrodes measured in 30 mM oxalate/oxalic acid buffer, pH 5.8. DNA solutions are 200 $\mu\text{g mL}^{-1}$ of (a) ds-DNA, (b) ss-DNA, (c) Poly-C, and (d) no DNA. The Ru-dppz concentration is 10 μM

voltammetry. The catalytic current for DNA film obtained from 200 $\mu\text{g mL}^{-1}$ solution is 200 μA , and its mass is 3.2 ng mm^{-2} . From Fig. 5A, the current for the lowest DNA concentration is 10 μA , or 20 times less than the 3.2 ng mm^{-2} film. So by extrapolation, the lowest amount of DNA on the surface detectable by catalytic voltammetry is 1/20th of 3.2 ng mm^{-2} , or 160 pg mm^{-2} . For comparison, Armstead and Thorp reported a detection sensitivity of 44 amol mm^{-2} , or 40 pg mm^{-2} by electrocatalysis with dissolved Ru-bpy [11].

There are two reasons why Ru-dppz was selected as the electrochemical indicator for DNA detection. Firstly, its oxidation potential is about 1.25 V vs. Ag/AgCl, and it is able to oxidize oxalate with high efficiency. Secondly, similar ruthenium and osmium complexes with dppz ligands have been shown in fluorescence and electrochemistry studies to intercalate into ds-DNA with relatively high affinity ($K = 10^6$ – 10^7 M^{-1}) and selectivity [37–40]. Figure 5 illustrates

the binding selectivity of Ru-dppz toward ds-DNA films assembled on an electrode surface. DNA films were prepared from a 200 $\mu\text{g mL}^{-1}$ solution of either ds-DNA, ss-DNA or Poly-C. According to the QCM measurement described in Fig. 1, the amount of nucleic acids immobilized on the electrode is the same for all the three samples. After incubating with Ru-dppz, catalytic voltammetry was measured in the oxalate buffer. As shown in Fig. 5, the electrochemical signals of the three films are drastically different. For the Poly-C film, the response is essentially the same as with a DNA-free electrode, suggesting no binding of Ru-dppz with Poly-C. The ss-DNA film produced some oxidation current, indicative of limited binding by the metal complex. This is likely due to the intercalation of the metal chelate into some of the partially hybridized sequences. The largest catalytic current was obtained with the ds-DNA film, which is 7.6 and 20 times higher, respectively, than that of ss-DNA and poly-C, showing a strong preference for binding with ds-DNA.

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