Electrochemical Displacement Method for the Investigation of the Binding Interaction of Polycyclic Organic Compounds with DNA

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The binding interaction of many organic carcinogens such as polycyclic aromatic hydrocarbons with DNA is the key step in their genotoxic effect. In this work, an electrochemical displacement method was developed to study such interaction. In the method, a DNA film is deposited on an indium tin oxide electrode surface by layer-by-layer assembly, and a redox-active DNA intercalator Ru(bpy)₂-(dppz)(BF₄)₂ (bpy = 2,2'-bipyridine, dppz = dipyrrodo [3,2-a:2',3'-c] phenazine) is employed as an electrochemical indicator. If an organic compound competes with the indicator for the same binding site on DNA in the film, it would displace the ruthenium complex from DNA, resulting in a reduction in the measured electrochemical signal. From the titration curve, the binding constant of the organic compound with DNA can be calculated. With the use of oxalate as an electron donor to chemically amplify the oxidation current of the indicator, chemicals can be tested at low micromolar concentrations. Five well-known DNA binding polycyclic organic compounds, thiazole orange, 4,6-diamidine-2-phenylindole, H33258, ethidium bromide, and quinacrine, were investigated by the displacement method. The binding constants obtained in our experiments fall in the range of (4.3×10^5) to (1.2×10^7) M⁻¹, which are generally consistent with those reported in the literature by some established methods. The electrochemical method provides a general tool that complements the commonly used spectroscopic methods for the study of DNA/small molecule interactions.

The interaction between small molecules and DNA is important due to its implication in the regulation of gene expression by activators and repressors in vivo.¹ Binding interaction with DNA is also the first step in the DNA-damaging action and genotoxic processes of many polycyclic organic compounds including polycyclic aromatic hydrocarbons (PAHs). In the commonly accepted chemical carcinogenesis, a PAH is converted to a reactive diol-epoxide by three metabolizing enzymes in a stepwise process. The metabolite then intercalates rapidly between the base pairs

of a DNA helix, followed by covalent adduct formation to the deoxyguanosines or deoxyadenosines in DNA.^{2,3} The indication that intercalation is an essential step in DNA adduct formation points to the importance of understanding the mechanism of binding interactions with DNA.

Intercalation, groove binding, and electrostatic interaction are the three major binding modes of small molecules to DNA.⁴ Common techniques employed in the investigation include footprinting, affinity cleavage, NMR, X-ray crystallography, UV-vis spectrophotometry, fluorescence, circular dichroism, and hydrodynamic measurements.^{5,6} Most relevant to the present work is the fluorescence displacement assay developed by Boger's group for the rapid, high throughput assessment of DNA binding affinity, sequence selectivity, and binding stoichiometry of drug compounds.^{7,8} Electrochemistry offers great advantages because it provides rapid, simple, and low-cost detection. Bard's group pioneered the electrochemical study of the binding interaction between metal chelates and DNA in solution.⁹ With the use of metal polypyridine complexes as redox probes, binding and redox chemistry with DNA was elucidated in detail by Thorp's group.¹⁰ More recently, the recognition of various electrostatic and intercalative binders with DNA films immobilized on electrode surfaces was investigated.¹¹ Extensive work has also been done on the electrochemical investigation of DNA/drug¹² and DNA/pollutant interactions¹³ by monitoring the electrochemical signal of either the compound

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itself or the guanine base in DNA. Since the redox potential of the compounds varies from one to another, the detection protocol is rather case-specific. In addition, their binding mode with DNA was not revealed directly by electrochemical measurement.

In our previous work, a DNA film voltammetry technique was developed and employed in the study of the binding and redox reaction of a metallo-intercalator $\text{Ru}(\text{bpy})_2(\text{dppz})(\text{BF}_4)_2$ (bpy = 2,2'-bipyridine, dppz = dipyrro [3,2-a:2',3'-c] phenazine) with DNA.¹⁴ In this report, the technique has been further developed into an electrochemical displacement method to study the binding interaction of redox-inactive molecules with DNA. The association constant of five polycyclic organic DNA binders was measured by the new method and was found to be comparable to the value obtained by other established methods.

EXPERIMENTAL SECTION

Calf thymus double-stranded DNA (CT-DNA, approximately 13K base pairs) and Hoechst 33258 (H33258) were purchased from Merck (San Diego, CA). Thiazole orange (TO), ethidium bromide (EB), poly(diallyldimethylammonium chloride) (PDDA), and quinacrine dihydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). $\text{Ru}(\text{bpy})_2(\text{dppz})(\text{BF}_4)_2$ (Ru-dppz) was synthesized according to the published procedure.¹⁵ 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI) was purchased from Roche (Basel, Switzerland). Solutions were prepared in high-purity water from a Millipore Milli-Q (Biocel) water purification system (Billerica, MA). The concentration of nucleic acids was determined by measuring the absorbance at 260 nm.

Tin-doped indium oxide (ITO) electrodes were prepared and cleaned as described before.¹⁶ The DNA film on the ITO was assembled by layer-by-layer electrostatic adsorption. Cleaned ITO electrodes were first reacted with 10 μL of PDDA (2.0 mg/mL in 20 mM phosphate buffer, pH 7.3) on an area of 0.5 cm $\times$ 0.5 cm for 1.5 h and then washed carefully. The PDDA modified electrode was then incubated with 10 μL of 0.2 mg/mL nucleic acid in 20 mM phosphate (pH 7.3) on the same area for 30 min. This is denoted as the ITO/PDDA/DNA electrode. Finally, Ru-dppz alone or Ru-dppz with an organic compound was reacted with the DNA film for 30 min, and unbound chemicals were washed off with water before electrochemical measurement.

Cyclic voltammetry was performed in 30 mM sodium oxalate/oxalic acid (pH 5.5) on a CHI 830B electrochemistry analyzer (CH Instruments, Austin, TX) with a Pt counter electrode and an Ag/AgCl reference electrode (also from CH Instruments). The electrode area in contact with the electrolyte was 0.25 cm².

UV-visible absorption spectra were collected on a UNICO model UV-2800 spectrophotometer (UNICO Instruments, Shanghai, China). To prepare the sample, a 10 μL solution of 30 μM Ru-dppz alone or 30 μM Ru-dppz mixed with 100 μM DAPI was reacted with a DNA film electrode. After the reaction, a total of 15 μL of solution from three such electrodes were pooled together.

Scheme 1. Illustration of the DNA Film Structure and Principle of the Electrochemical Displacement Method

After dilution with 45 μL of phosphate buffer, its absorbance was measured in a quartz cell with a 1 cm path length.

RESULTS AND DISCUSSION

Scheme 1 illustrates the DNA film structure and the principle of the electrochemical displacement method. DNA films were prepared on ITO electrodes by layer-by-layer assembly.¹⁷ A polycation, PDDA, was first adsorbed on the negatively charged ITO surface, followed by adsorption of calf-thymus DNA. A DNA intercalator, Ru-dppz , is allowed to bind to the DNA film to serve as an electrochemical indicator. To investigate a potential DNA binding compound, it is mixed with Ru-dppz in solution and reacted with the DNA film. If the compound competes with the indicator for the same binding site on DNA, it would displace the ruthenium complex from DNA, resulting in a reduction in the measured electrochemical signal. From the titration curve, the binding constant of the compound with DNA can be calculated.

Many polycyclic organic compounds including PAHs have relatively low solubility in aqueous solutions. To be able to measure these compounds, a previously reported signal amplification mechanism¹⁸ is employed in the electrochemical displacement method. An artificial electron donor, oxalate, is present in the solution in large excess during the electrochemical measurement. Anodic current of Ru-dppz is amplified due to the redox reaction between the oxidized ruthenium complex and oxalate and subsequent regeneration of the reduced metal complex. Because oxalate itself produces negligible oxidation current on ITO, the

Figure 1. Plot of the oxidation current (at 1.25 V) of Ru-dppz bound to the ITO/PDDA/DNA electrode as a function of Ru-dppz concentration in solution for the binding reaction. Scan rate: 30 mV/s. Reference: 3 M Ag/AgCl. Each data point is the average of three electrodes.

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Figure 2. Voltammograms of the ITO/PDDA/DNA electrode in 30 mM oxalate (pH 5.5) after reacting with a mixed solution of 30 μM Ru-dppz and (a) DAPI (0, 1, 3, 10, 30, 100, and 300 μM from 1 to 7) and (b) quinacrine (0, 30, 100, and 300 μM from 1 to 4). Scan rate: 30 mV/s. Reference: 3 M Ag/AgCl.

signal-to-background ratio is increased by about 14-fold over the nonamplified measurement.¹⁷ As shown below, the improved sensitivity allows the use of a low concentration of Ru-dppz and consequently low concentrations of testing compounds in the electrochemical displacement measurement.

To select a suitable indicator concentration for the electrochemical displacement measurement, the binding interaction of Ru-dppz with the ITO/PDDA/DNA film was first investigated. This was done by reacting the metal complex of different concentrations with the DNA film, then measuring its catalyzed electrochemical response in an oxalate-containing electrolyte free of Ru-dppz. As can be seen from Figure 1, the relationship between the measured current and the Ru-dppz concentration follows the typical adsorption isotherm. With increasing Ru-dppz concentration, the current initially increased rapidly and then became a plateau at 30 μM or higher. The voltammogram (shown in Figure 2, curve 1) is not sigmoid in shape, suggesting slow kinetics of the electrode reaction impeded by the nonconducting polymer film. With the use of Scatchard analysis,¹⁹ the binding constant of Ru-dppz with DNA was estimated as $2.1 \times 10^6 \text{ M}^{-1}$, a value which agrees reasonably well with those reported in the literature.²⁰

On the basis of the above results, 30 μM Ru-dppz was used in the displacement measurement. Five polycyclic organic compounds, DAPI, H33258, TO, EB, and quinacrine (see Scheme 2 for their structure), were tested to validate the new methods. These compounds are known to bind to DNA by either groove

binding (DAPI and H33258) or intercalation (TO, EB, and quinacrine).^{21–29} DNA modified electrodes were incubated in a solution containing Ru-dppz (30 μM) and one of the five compounds of various concentrations. After the reaction and rinsing, the electrochemical current was measured in oxalate. As expected, the addition of the DNA-binding polycyclic organic compounds leads to obvious reduction in the measured current, indicating the competition of the compound with Ru-dppz for the DNA binding site. The current kept decreasing as the concentration of the compound was increased. However, the degree of reduction is different for each compound. For the two minor-groove binders, addition of even 1 μM concentration of the compound into the solution resulted in an approximately 20% loss in signal. The current dropped to almost the background level when the compound concentration exceeded 30 μM (Figure 2a). For EB and quinacrine, on the other hand, electrochemical response did not change appreciably until the concentration reached 30 μM . At the highest concentration tested (300 μM), the signal was still significantly above the background (Figure 2b). The compound might bind to DNA at the same site as the indicator or it might not. As long as it binds to DNA, it would interfere with the indicator and displace it.

In order to validate the displacement mechanism described above, a UV-visible absorption measurement was carried out. Figure 3 shows the absorption spectrum of Ru-dppz (curve 1) and DAPI (curve 2). For Ru-dppz, there is a broad absorption peak centered around 450 nm, where DAPI does not absorb appreciably. Therefore, this peak was selected to quantify the amount of Ru-dppz bound to the DNA film. After a Ru-dppz solution was reacted with the DNA film electrode, its absorbance decreased from 0.1325 to 0.0945 (curve 3), indicating binding of Ru-dppz to the electrode. From the absorbance change, the amount of Ru-dppz bound to DNA was calculated to be $3.3 \times 10^{-10} \text{ mol}$, or $4.4 \times 10^{-10} \text{ mol/cm}^2$, which is reasonable for a Ru-dppz saturated surface. When DAPI was added to the Ru-dppz solution and then reacted with the DNA film electrode, the absorbance at 450 nm changed back to its original value (curve 4), indicating no binding of Ru-dppz with the surface. The absorbance results support the displacement mechanism we proposed above to interpret the electrochemistry data.

A plot of the oxidation current measured at 1.25 V (where the signal/background ratio is the highest) against the compound concentration displays a typical competition curve, as illustrated in Figure 4. From the plot, IC_{50} values are obtained, which stands for the concentration of the organic compound required to produce 50% inhibition of indicator binding with

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Scheme 2. Structure of the Five Polycyclic Organic Compounds Tested in the Study

DNA, i.e., 50% loss of its signal. According to the relationship of the binding constant with IC_{50} in the competitive reaction,³⁰ the binding constants (K_b) of the five polycyclic organic compounds with DNA in the film are calculated (Table 1). The numbers fall in the range of (4.3×10^5) to $(1.2 \times 10^7) M^{-1}$, which are reasonable for DNA intercalators and groove-binders. In particular, the binding constants of the two groove-binders are 5–30 times higher than the three intercalators, which is generally the case. The values are comparable with those reported in the literature obtained by the established methods

Table 1. Binding Constants of Five Polycyclic Organic Compounds with DNA^a

	$IC_{50}(\mu M)$	$K_b(10^6 M^{-1})$	$K_{ref}(10^6 M^{-1})$
DAPI	5.2 ± 0.4	12.3 ± 0.6	$29^{21}, 19\text{--}550^{22}$
H33258	6.3 ± 0.5	10 ± 0.6	$1.3\text{--}520^{22}, 11\text{--}97^{23}$
TO	23.4 ± 1.7	2.7 ± 0.1	$0.316^{24}, 1.0\text{--}1.3^{25}$
EB	155 ± 33	0.41 ± 0.06	$0.56^{26}, 0.4,^{27} 0.18\text{--}1.28^{28}$
quinacrine	158 ± 28	0.43 ± 0.05	$0.159\text{--}0.735^{29}$

^a K_b is the binding constant obtained from the electrochemical displacement measurement, and K_{ref} is the reported value in the literature with the superscripts indicating the corresponding references.

Figure 3. UV-vis absorption spectrum of the (1) $7.5 \mu M$ Ru-dppz solution, (2) $25 \mu M$ DAPI solution, (3) $7.5 \mu M$ Ru-dppz solution after reacting with DNA film, and (4) solution of $7.5 \mu M$ Ru-dppz and $25 \mu M$ DAPI after reacting with the DNA film. All solutions were prepared in 20 mM phosphate, pH 7.3.

Figure 4. Plot of the oxidation current (at 1.25 V) of the ITO/PDDA/DNA electrode in 30 mM oxalate (pH 5.5) as a function of the concentration of polycyclic organic compound in the mixed solution with $30 \mu M$ Ru-dppz. Scan rate: 30 mV/s. Reference: 3 M Ag/AgCl. Each data point is the average of three electrodes.

such as fluorescence, mass spectrometry, and equilibrium dialysis.^{21–29} The agreement proves that the electrochemical displacement method is a valid approach in establishing the binding constant of small molecules with DNA.

CONCLUSIONS

In conclusion, an electrochemical displacement method has been developed for the investigation of the binding interaction of small molecules with DNA. The method employs a surface-immobilized DNA film and an electroactive DNA binder as the signal-generating indicator. The film fabrication requires as low as a few tens of nanograms of DNA on a conventional electrode, instead of milligrams used in the solution measurement. With the use of oxalate to chemically catalyze the oxidation current of the indicator, chemicals can be examined at low micromolar concentrations. All the five polycyclic organic compounds which are known to bind to DNA displaced the indicator from the DNA film and reduced its electrochemical signal. The binding constants obtained from the displacement curve agree reasonably well with the reported values. If the binding mode of the indicator with DNA is known and specific, the binding mode of the compound can in principle be deduced from the displacement measurement. The electrochemical method is particularly useful for some fluorescent organic compounds which cannot be studied by the established fluorescence displacement assay due to their interference with the indicator. The method is very general and can be employed in the study of a broad range of small molecules. Although the employed DNA electrostatic adsorption approach restricts the method to low ionic strength solutions, the problem can in principle be

circumvented by covalent immobilization. One shortcoming is the deviation of the measurement from true equilibrium and, consequently, the limitation to evaluating chemicals with relatively high binding affinity for DNA. It is hoped that, with further development, the electrochemical displacement can be utilized to investigate the interactions of environmental organic carcinogens such as PAHs with DNA and to elucidate the relationship between DNA binding interaction and their carcinogenesis.

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