Scientific Paper

A SQUARE WAVE VOLTAMMETRIC STUDY OF THE INTERACTION OF PROCAINE WITH DNA

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Abstract

The interaction of procaine with double-stranded DNA was studied in solution by square wave voltammetry to determine the binding constant and the binding site size using the Scatchard binding model. The binding parameters, the binding constant, *K*, and the effective base pair sites involved in binding, *s*, were obtained by the analysis of bound and free procaine concentration corresponding to the limits of slow and fast binding kinetics compared to the experimental timescale. The binding constant and the binding site size for the interaction of procaine with DNA were $K = 8.78\ (\pm 0.2) \times 10^3\ M^{-1}$ and $s = 1.88\ (\pm 0.06)$ base pairs and $K = 1.60\ (\pm 0.3) \times 10^4\ M^{-1}$ and $s = 3.21\ (\pm 0.04)$ base pairs for static and mobile binding equilibria respectively. Ionic strength and viscosity experiments suggest that procaine may bind to DNA with the groove-binding mode.

Key words: DNA, procaine, binding, voltammetry, scatchard model

Introduction

The binding interaction of drugs with DNA is of interest for both therapeutic and scientific reasons.[1-3] Such interactions may be used for conformational recognition to find new structures of DNA and sequence-specific differences along the helix of a DNA molecule.[4-6] In addition to potential therapeutic applications, due to their electrochemical behaviour, many small molecules capable of recognising DNA are anticipated to be useful in analytical applications.[7-10] Several procedures have been used to describe DNA-binding studies including, *e.g.*, UV,[11,12] circular dichroism,[11-13] isothermal calorimetric titration,[11-14] luminescence,[15] electrophoresis,[16] NMR,[17] quartz crystal microgravimetry[18,19] and electroanalytical methods.[20-25] The electroanalytical techniques usually rely on the effect of the macromolecule (e.g., DNA) on the diffusion current of redox active molecule.[8,20,22] Several authors have shown that the binding constants for redox active species can be obtained from straightforward voltammetric experiments in which the DNA is titrated against the redox active molecule. The measurement of

diffusion currents in the presence of excess nucleic acid has been shown that the diffusion coefficient of DNA-bound species was more than one order of magnitude lower than that of the free species.[8,26]

Procaine is one of the oldest and quick-action local anaesthetics. However, it is toxic and care must be paid when used by infiltration.[27] Therefore, its interaction with DNA has drawn attention. In this paper, the interaction of procaine with DNA has been studied by square wave voltammetry to determine the binding constant and the effective base pair sites involved in binding corresponding to the limits of slow and fast binding kinetics compared to the experimental timescale utilising the Scatchard binding model.

Results and discussion

The free procaine exhibits a single oxidation peak at E = 0.91 V. Figure 1 shows the effect of the addition of double-stranded DNA to a solution of 0.15 mM procaine in 100 mM Tris-HCl buffer, pH 7 on the square wave voltammetry of procaine. The current drops on the addition of DNA owing to the binding of procaine.

In the presence of nucleic acids, the current is mainly due to free species, as the diffusion rate of bound species is small.[26] The reason of the decrease in the peak current was that the apparent diffusion coefficient and the apparent concentration of electroactive species decreased.[8,20] The peak current decreased further with increasing concentration of DNA. As can be seen from Figure 2, at high concentrations of procaine, the current-procaine concentration plots in the presence and absence of DNA ran approximately parallel *i.e.* slopes are roughly same.

This shows that the decrease in diffusion current on addition of DNA is due to binding of procaine and not due to an increased solution viscosity causing a reduction in the diffusion coefficient of procaine. Other workers have also reported that the viscosity effect is negligible at DNA concentrations used in this work.[20] Also, to show that the decrease in the peak current is due to diffusion of procaine-DNA adduct and not due to blockage of the electrode surface by an adsorbed layer of DNA that could possibly form at the electrode surface, voltammograms were recorded for 0.1 mM $K_4[Fe(CN)_6]$, which can not interact with DNA in Tris-HCl buffer at pH 7 in the absence and presence of DNA. Voltammetric behaviour of $K_4[Fe(CN)_6]$ was not affected by the addition of a large excess of DNA indicating that the decrease of the peak current of procaine after the

addition of DNA was caused by the binding of procaine to the bulky, slowly diffusing DNA, which results in a considerable decrease in the apparent diffusion coefficient.

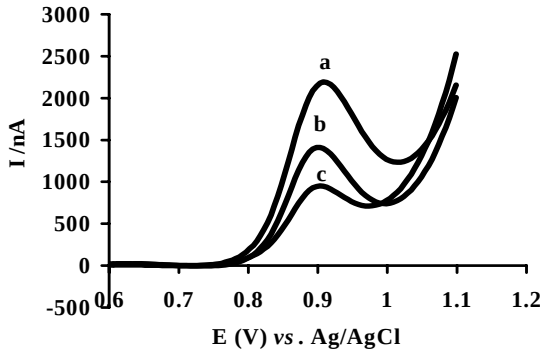

Figure 1. Square wave voltammograms of 0.15 mM procaine in 100 mM Tris-HCl buffer pH 7.0 (a) without DNA, (b) 1.0 mM DNA and (c) 2.0 mM DNA. Equilibrium time: 10 s, Frequency: 10 Hz, Step Potential: 5 mV, Amplitude: 25 mV.

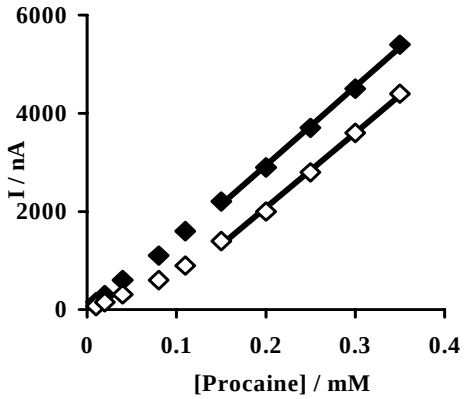

Figure 2. Plots of square wave voltammetry of diffusion current against the total concentration of procaine in the (a) absence and (b) presence of 1.0 mM DNA. The supporting electrolyte was 100 mM Tris-HCl buffer at pH 7. The solid lines are a guide to the eye and indicate the gradient of the plots at high concentrations are roughly equal.

The effect of ionic strength on the interaction of procaine with DNA was also investigated to acquire possible binding modes. Since DNA is a polyanion, electrostatics may be expected to strongly influence the binding of positively charged species with the phosphate groups of DNA. The peak current increased, in other words the magnitude of binding constant decreased as the ionic strength was increased. It is also known that when a charged ligand is added to a polyelectrolyte solution its binding constant, *K*, depends on the total counterion concentration, $[M^+]$, as:[28,29]

$$\frac{d \log K}{d \log[M^+]} = \Delta r = -z\Psi \qquad (1)$$

where z is the charge on the ligand molecule, Ψ is the fraction of counterions and Δr is the number of counterions released upon the binding of the ligand with charge *z*. The theoretical slope of the log*K vs*. log$[M^+]$ relation is -0.88 for a singly charged ligand bound to the B form of DNA in equeous solutions at 25 ºC.[29] The binding constants for the interaction of procaine to DNA up to 100 mM KCl concentration were determined

from the corresponding voltammetric measurements of currents. The plot of log*K* *vs.* log[M^+] was linear with a slope of -0.62. The experimentally determined value indicates that the moles of counterions released to the solution per mol of bound procaine are less than that of the theoretically predicted value. This behaviour supports the interpretation that the charge on procaine is not strongly involved in the binding process.

Viscosity measurements were also carried out to provide clues for a binding model between procaine and DNA. In the presence of various concentrations of procaine, no significant changes were observed in the relative viscosity of DNA. Ligands that bind exclusively in the DNA grooves typically cause less pronounced changes (positive or negative) or no changes in the DNA solution viscosity.[30] This behaviour suggests that procaine may bind to DNA in a groove-binding mode.

To determine the binding constant and binding site size the DNA was titrated with procaine and the diffusion currents plotted against the total concentration of procaine (Figure 2). Titration results were analysed by the combination of the equations that describes the binding equilibrium with that of the voltammetric response. Figure 3 shows a plot of $(i\text{-}i_{dna})/i_{dna}$ *vs.* C_T for procaine in the presence of 1.0 mM DNA from square wave voltammograms and the corresponding least squares fit to equation (4). The binding constant and binding site size obtained at 25 °C were $K = 8.78\ (\pm 0.2) \times 10^3\ M^{-1}$ and $s = 1.88\ (\pm 0.06)$ base pairs for a static binding equilibrium.

The analysis was also carried out for the limiting condition of mobile equilibria by plotting $(i^2\text{-}i^2_{dna})/i^2_{dna}$ *vs.* C_T of procaine (Figure 4). The binding constant and binding site size obtained at 25 °C in this case were $K = 1.60\ (\pm 0.3) \times 10^4\ M^{-1}$ and $s = 3.21\ (\pm 0.04)$ base pairs.

However, the Scatchard model of the binding equilibrium is obtained by assuming each binding species binds independently and that the number of the binding sites is proportional to the concentration of DNA. Data acquired from the interaction of procaine with DNA were analysed in terms of Scatchard plot:[31]

$$\frac{v}{C_f} = sK - vK \qquad (2)$$

where *v* is the number of moles of bound ligand per mol of total base pairs.

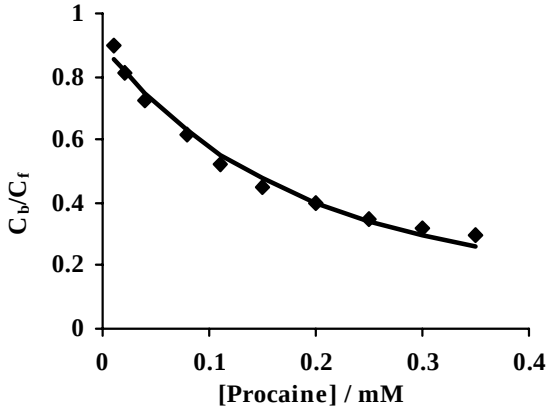

Figure 3. A plot of the ratio of C_b/C_f *vs*. C_T from the square wave voltammetric titration of procaine against 1.0 mM DNA. The ratio of bound to free procaine was determined from the measured currents in the presence and absence of DNA according to equation (9). The solid line shows a least squares fit to the data.

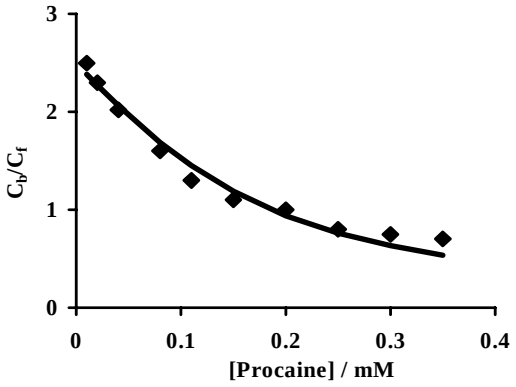

Figure 4. A plot of the ratio of C_b/C_f vs. C_T from the square wave voltammetric titration of procaine against 1.0 mM DNA. The ratio of bound to free procaine was determined from the measured currents in the presence and absence of DNA according to equation (10). The solid line shows a least squares fit to the data.

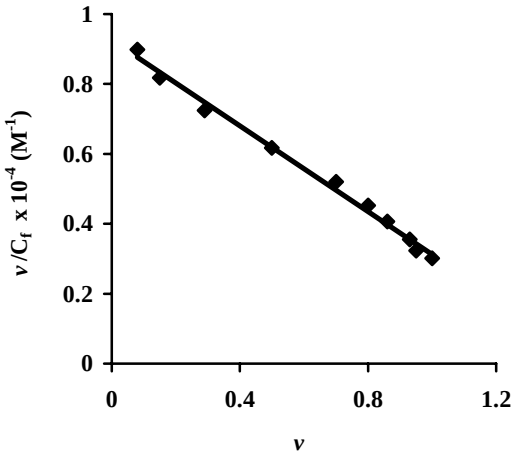

Figure 5. Scatchard plot of the interaction of procaine to DNA. Data were acquired from the corresponding voltammetric measurements of currents.

C_f is the concentration of free ligand, K is the binding constant and s is the binding site size in base pairs. Figure 5 shows a plot of v/C_f vs. v, in which v and C_f values were obtained from the corresponding voltammetric measurements of currents. The Scatchard plot gives a straight line indicating that the binding constant is identical for all binding sites. That is, the binding of one molecule of ligand does not influence the subsequent binding of other ligand molecules. The binding constant, K and the binding site size, s,

obtained from the Scatchard plot are $K = 6.14 \times 10^3$ M^{-1} and $s = 1.5$ which are also in good agreement with the K and s values obtained from the plot of C_b/C_f *vs.* C_T. Furthermore, the Scatchard binding model has previously been found to be adequate and precise to fit the experimental data by several workers.[8,20,21]

Conclusions

Square wave voltammetry has been used to investigate the interaction of procaine with DNA. The binding parameters, the binding constant, K and the effective base pair sites involved in binding, s, are obtained by the analysis of bound and free procaine concentration corresponding to the limits of slow and fast binding kinetics compared to the experimental timescale. The effect of the ionic strength on the binding constant and viscosity experiments suggest that procaine may bind to DNA with the groove-binding mode.

Experimental

Materials and instrumentation

An EcoChemie Autolab 12 potentiostat with the electrochemical software package GPES 4.9 (Utrecht, The Netherlands) was used for voltammetric measurements. A three electrode system was used: a 2 mm sized Pt disc working electrode, an Ag/AgCl reference electrode and a Pt wire counter electrode. For viscosity measurements, the viscometer was maintained at 25 ºC in a thermostated water-bath. Type XIV Herring Testes ds-DNA sodium salt was purchased from Sigma (Taufkirchen, Germany). Solutions of DNA were prepared fresh before each experiment using doubly distilled water containing 100 mM Tris-HCl buffer, pH 7. The concentration of DNA solutions expressed in moles of nucleotide phosphate [NP] was determined by UV/VIS spectrophotometry at 260 nm using a value of 6600 M^{-1} cm^{-1} for the absorption coefficient.[32] The purity (freedom from bound protein) was assessed from the ratio of the absorbances at 260 nm and 280 nm.[33] In general, the commercial DNA preparation was found to be free of protein ($A_{260nm}/A_{280nm} = 1.9$) according to this criterion and no further purification was attempted. Procaine hydrochloride was purchased from Sigma (Taufkirchen, Germany). All other reagents were of analytical grade or equivalent and obtained from Merck (Darmstat, Germany) or Sigma (Taufkirchen, Germany).

Procedure

A known volume of buffered DNA solution was added to the cell and voltammograms were recorded as aliquots of procaine solution were added. The procedure was then repeated using a buffer solution containing no DNA to generate a current-concentration calibration plot. The working electrode was polished with 0.05 μm alumina on a polishing pad and washed with doubly distilled water prior to each experiment. Oxygen-free nitrogen was bubbled through the solution for 10 min before each experiment. All experiments were carried out at 25 ºC.

Data Analysis

The effect of polyelectrolytes on the diffusion of electroactive species has been the subject of several papers.[16,20,26,34,35] In order to quantify the binding of procaine to DNA, two factors need to be considered. First, a binding model must be chosen in order to relate the concentrations of free and DNA-bound procaine in bulk solution to the binding constant and binding site size. Second, voltammetric equations relating the measured current to mass transfer of the mixture of free and DNA-bound procaine and their concentrations must be chosen. This choice depends on whether the exchange of free and bound species is slow or fast on the timescale of the voltammetric experiment. The binding equilibrium in each case is referred to as ‘static’ or ‘mobile’ respectively. The simplest model of the binding of electroactive species to DNA assumes a chemical species-DNA binding equilibrium with a fixed number of binding sites of identical affinity on a given length of DNA. This is equivalent to the well-known Scatchard treatment of binding equilibria.[31] This model has previously been found to be adequate to fit the experimental data by several workers and was therefore also employed in this work. If the concentration of DNA is expressed in terms of nucleotide phosphate concentration, [NP], then the Scatchard model binding constant K is given by:

$$K = \frac{C_{\mathrm{b}}}{C_{\mathrm{f}} \dfrac{[NP]}{2s}} \qquad (3)$$

where C_{f} and C_{b} are the concentrations of free and DNA-bound species respectively and s is the size of the binding site in base pairs or as the average distance between binding sites on the nucleic acid. Using the mass balance equations for the concentration of

nucleotide phosphate and procaine, C_f can be expressed in terms of total concentration of procaine, C_T, total concentration of nucleotide phosphate, $[NP]_0$, K and s:[20]

$$KC_f^2 + (1 - KC_T + \frac{K[NP]_0}{2s})C_f - C_T = 0 \qquad (4)$$

experimentally, C_f and C_b can be obtained from the diffusion currents in the absence of DNA, i, and in the presence of DNA, i_{dna}:

$$i_{dna} = BD_b^x C_b + BD_f^x C_f \qquad (5)$$

$$i = BD_f^x (C_T) \qquad (6)$$

where B is the appropriate collection of constants for the experiment (electrode area, or radius, sweep rate, *etc*.) and $x = 0.5$. D_b and D_f are the diffusion coefficients of the bound and free procaine. It should be noted that equation (5) assumes that the kinetics of the equilibrium are slow on the voltammetric timescale. For mobile equilibria equation (5) should be replaced by:[20]

$$i_{dna} = BC_T (D_b \frac{C_b}{C_T} + D_f \frac{C_f}{C_T})^x \qquad (7)$$

Estimation of K and s from the measured currents (i_{dna} and i) in equations (5), (6) and (7) requires the choice of a regression model. Several possible choices have been suggested.[36,37] In this work, I have computed C_b/C_f and obtained K and s by least squares regression of C_b/C_f on C_T. Using $C_T = C_f + C_b$, I obtain:

$$\frac{D_f^x (i - i_{dna})}{D_f^x i_{dna} - D_b^x i} = \frac{C_b}{C_f} \qquad (8)$$

Since $D_f/D_b \approx 50$, I can make the approximation $D_b << D_f$ and the left-hand side of equation (8) reduces to:

$$\frac{i - i_{dna}}{i_{dna}} = \frac{C_b}{C_f} \qquad (9)$$

Similarly for a mobile equilibrium with $x = 0.5$:

$$\frac{i^2 - i_{dna}^2}{i_{dna}^2} = \frac{C_b}{C_f} \qquad (10)$$

The left-hand sides of equations (9) and (10) can be obtained directly from the experimental diffusion currents. The experimental data were therefore expressed as plots

of $C_b/C_f = (i-i_{dna})/i_{dna}$ (the limit appropriate for a static binding equilibrium) or $C_b/C_f = (i^2-i^2_{dna})/i^2_{dna}$ (the limit of mobile binding equilibrium) against total concentration of procaine at a fixed concentration of DNA and *K* and *s* were obtained by least squares fitting. The correlation between *K* and *s* was reduced by the application of a non-linear fitting model based upon the minimisation of sum of squares with *K* and *s* as the only adjustable parameters. Standard deviations of the fitted parameters were obtained by a bootstrap method which avoids the necessity to make assumptions about the standard deviation and distribution of errors of individual data points other than that they are independent and identically distributed.[38] Briefly, the bootstrap method consists of drawing, with replacement, a random sample of *n* data points from the dataset of *n* datapoints. This process was repeated to generate 250-1000 simulated data sets and fitted values of the parameters *K* and *s* were obtained by the Levenberg-Marquardt method for each data set. This bootstrap sample was used to approximate the sampling distribution of *K* and *s* and to compute standard deviations. Bootstrap resampling was performed using software written in-house in MS Fortran 5.1™ incorporating commercial routines for non-linear least squares fitting by the Levenberg-Marquardt method.[38] Uncertainties in values of *K* and *s* were reported as mean ± standard deviation.

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Povzetek

Interakcije procaina z dvojno vijačnico DNK v vodnih raztopinah so bile študirane z uporabo "square wave" voltametrije in Scatchardovega modela vezanja ter izražene z ustreznimi konstantami vezanja in velikostmi veznega mesta. Parametra vezanja, konstanta vezanja, K, in efektivno število baznih parov sodelujočih pri vezavi posamezne molecule procaina, s, sta bila določena s pomočjo analize koncentracij vezanega in prostega procaina, ki se vzpostavijo pri počasni in hitri kinetiki vezanja. Konstanta vezanja in velikost veznega mesta za interakcijo procaina z DNK sta bili K = 8.78 (± 0.2) x 10^3 M^{-1} in s = 4.88 (± 0.6) baznih parov za statično ravnotežje vezanja ter K = 1.60 (± 0.3) x 10^4 M^{-1} in s = 3.21 (± 0.4) baznih parov za dinamično ravnotežje vezanja. Eksperimenti izvedeni pri različnih ionskih močeh in viskoznostih kažejo, da bi se procain lahko vezal v ožji kanal DNK.

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