Binding Study of the Fluorescence Probe 1-Anilino-8-naphthalenesulfonate to Human Plasma and Human and Bovine Serum Albumin Using Potentiometric Titration

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The binding of 1-anilino-8-naphthalenesulfonate (ANS) to bovine serum albumin (BSA), human serum albumin (HSA), and human plasma has been studied by potentiometric titration utilizing a laboratory constructed ion selective electrode (ISE) of ANS. Three classes of ANS binding sites were found on BSA, HSA, and plasma at 25 and 37°C. Computer analysis of the data resulted in estimates for the association constants, number of binding sites (HSA, BSA), and binding capacity of each class. The association constants for the first class of binding sites at 25°C were found to be 7.53 (±0.59) × 10^5, 2.70 (±0.20) × 10^5, and 2.64 (±0.26) × 10^5 M^{-1} for BSA, HSA, and plasma, respectively. Lower values for the association constants of all binding classes were estimated at the higher temperature (37°C). The binding capacity for ANS decreased in the order BSA, plasma, HSA.

KEY WORDS: protein binding; 1-anilino-8-naphthalenesulfonate (ANS); bovine serum albumin; human serum albumin; plasma binding; ion selective electrodes; potentiometric titration.

INTRODUCTION

Many experimental techniques have been used to investigate the interaction of drugs with proteins. The fluorescence technique utilizing the model ligand 1-anilino-8-naphthalenesulfonate (ANS) as a fluorescence probe (1–6) has been extensively applied. Although fluorescence techniques have proven useful in investigating drug–protein interactions, their application to studies involving biological specimens, i.e., plasma or serum, is limited (1). This lack is associated with the inherent drawback of all spectroscopic techniques requiring extensive dilution of the biological sample to avoid spectral interferences.

In recent years ion selective electrodes (ISEs) have been applied to clinical (7) and pharmaceutical analysis (8,9) and to binding studies (10,11). ISEs are electrochemical transducers responding selectively, directly, and continuously to the free ion activity (concentration) in solution. The advantage of ISEs, when applied to binding studies, is their ability to measure directly the concentration of the free ion of interest in the presence of the protein and the bound form. Moreover, ISE potentiometry is capable of measuring the activity of the free ion directly in the biological specimen, thus overcoming the major drawback of fluorescence techniques. These advantages prompted us to construct an ISE for ANS in order to study its binding to bovine serum albumin (BSA), human serum albumin (HSA), and human plasma at room temperature (25°C) and at 37°C.

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MATERIALS AND METHODS

Reagents

All solutions were prepared in deionized water. 1-Anilino-8-naphthalenesulfonate ammonium salt, bovine serum albumin fraction V, human serum albumin fraction V, tetraheptylammonium bromide, and *p*-nitrocumol were obtained from Fluka (Buchs, Switzerland). Polyvinyl chloride (PVC) of high molecular weight (d = 1.385) was from Janssen Chimica (Beerse, Belgium).

Phosphate Buffer, 0.1 M, *pH 7.4.* This was prepared by dissolving sodium dihydrogen phosphate in water and adjusting the pH with a 18 *M* NaOH solution.

ANS Stock Solution. This solution was 0.100 *M* with respect to ANS in phosphate buffer, 0.1 *M*, pH 7.4. More dilute solutions were prepared from the stock solution by dilution in the same medium.

BSA and HSA Solutions. These solutions contained 22.5 g/L of BSA or HSA in phosphate buffer, 0.1 *M*, pH 7.4. The solutions were stored at 4°C for no more than a week.

Plasma Preparation. Blood was collected from nine healthy volunteers of either sex, aged between 25 and 35 years, who had not received any drug for at least 1 week before the blood collection. The plasma was obtained by centrifugation at 3000 rpm for 10 min. Prepared plasma was used on the same day after dilution 1:1 with phosphate buffer, 0.1 *M*, pH 7.4. The remaining was divided into 5-ml-volume glass tubes and stored at −40°C. The total plasma protein concentration was determined by the Biuret reaction and was found to be 71.5 (±6.2) g/L.

ANS–BSA and ANS–HSA Mixed Solutions. These solutions contained ANS at a concentration of 0.006 *M* and BSA or HSA at a concentration of 22.5 g/L in phosphate buffer, 0.1 *M*, pH 7.4.

Electrode Construction

Electrode Assembly. The electrode was of the PVC membrane type and its assembly was laboratory made. The body of the electrode was a 0.4-cm-diameter Pasteur pipette filled with the internal reference solution, and a silver wire, coated with AgCl, was used as internal reference electrode. The electrode membrane (liquid ion exchanger entrapped in PVC membrane) was attached to the open end of the electrode body through a small PVC tube-cup. The indicator electrode was used with an external Corning Ag/AgCl single-junction reference electrode filled with a 4 *M* KCl solution. After its preparation the ISE was conditioned, in a magnetically stirred 0.0010 *M* ANS solution, for 24 hr before use. When not in use the electrode was stored in a 0.0010 *M* ANS solution or (for overnight or longer periods of storage) the

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PVC tube-cup, having the membrane, was removed and kept dried.

Liquid Ion Exchanger. The liquid ion exchanger for ANS was its ion pair with the tetraheptylammonium cation in *p*-nitrocumol at a concentration of ≈0.1 *M* and was prepared as follows. A 0.010 *M* tetraheptylammonium bromide solution in 5 ml *p*-nitrocumol was shaken three times with a 0.100 *M* ANS solution in order to exchange Br^- with ANS, and the aqueous phase was separated by centrifugation and decanted. The organic phase was then dried with anhydrous sodium sulfate to remove any water traces. A small amount (0.5 ml) of this ion exchanger was used for the preparation of the PVC membrane according to the method introduced by Graggs *et al.* (12).

Internal Reference Solution. This solution was 0.010 *M* with respect to ANS in 0.10 *M* sodium chloride, saturated with silver chloride.

Apparatus

The system used for the measurements consists of an Orion Model 801 digital pH/mV meter, with a readability of ±0.1 mV, connected to a Radiometer strip-chart recorder (REC 21) through a high-sensitivity unit (REA 112) interface. All measurements were carried out in a 25-ml double-walled glass cell, kept at the appropriate temperature with an Edmund Buhler 7400 Tubingen Type 7TH-4 water bath, with constant magnetic stirring of the solutions. For pH measurements, a Metrohm Model E-350B pH-meter with a combination glass electrode was used.

Procedures

Calibration Curve. Five milliliters of phosphate buffer, 0.1 *M*, pH 7.4, was pipetted into the measurement cell, the electrodes were immersed, and after the potential was stabilized, various aliquots of a 0.0060 *M* ANS solution were added, with a 100-μl Hamilton microsyringe (concentration range covered, 1.2×10^{-6}–2×10^{-3} *M*). The emf values were recorded and measured after stabilization (±0.1 mV) following each addition. The potential values *E*, were plotted against $-\log C$ (*pC*) to give the calibration curve using a least-squares fitting program (10). Corrections for the changes in volume after addition were performed by the program.

Binding Experiments (Potentiometric Titration). Solutions of BSA, HSA, or plasma (5.0 ml) were pipetted into the measurement cell, and the electrodes were immersed in it. After the potential was stabilized (±0.1 mV), small amounts of either a 0.0060 *M* ANS solution (in the case of plasma) or the mixed ANS–BSA or ANS–HSA solution were added with a 100-μl Hamilton microsyringe. The emf values were recorded to check stabilization (±0.1 mV) and measured after each addition. Corrections for the dilution of the protein solution after each addition were performed, when needed (in the case of plasma), by the program (10) used for the data analysis (see below).

Data Analysis. The generalized Scatchard model (13) was used to describe the binding phenomenon. According to this model the protein is considered to contain *m* distinct classes of independent and noninteracting binding sites. The *i*th class contains n_i equal binding sites which are characterized by the same binding constant K_i (M^{-1}). Equation (1) relates the molar concentration of the bound ligand *B* and the binding parameters (m, n_i, K_i)

$$B = \sum_{i=1}^{m} \frac{n_i K_i F}{1 + K_i F} P_t \qquad (1)$$

where P_t and *F* are the total protein and the free ligand molar concentration, respectively.

In our binding experiments, for every ANS addition, *F* was calculated from the calibration curve and *B* from the equation $B = T - F$, where *T* is the total ligand molar concentration. The binding parameters were estimated by a computer program (10) performing nonlinear least-squares fitting of the Scatchard model to the experimental data (electrode potential *E* and *T* values are the dependent and independent variables, respectively).

In the case of ANS binding to plasma, where the molar concentration of the protein is not known, a modified (14) Scatchard equation was used:

$$B = \sum_{i=1}^{m} \frac{N_i K_i F}{1 + (K_i F/1000\ \mathrm{MW})} P_t \qquad (2)$$

where *B* and *F* are given as μg/ml, P_t as g/L, and K_i as L/mol, N_i is the binding capacity of the *i*th class of binding sites expressed as mol/g, and MW is the molecular weight of ANS. The computer program (10) utilized for the analysis of data was appropriately modified to accommodate Eq. (2) for the estimation of N_i and K_i.

A paired Student *t* test ($P = 0.05$) was used to evaluate differences between association constants.

RESULTS AND DISCUSSION

Electrode Characteristics

The electrode shows near-Nernstian response in the range of 2.0×10^{-6}–1×10^{-2} *M* ANS concentration. The slope at 25°C is 56–58 mV/decade ($r = 0.9998$) and the detection limit is 1×10^{-6} *M*. The potential was independent of pH in the range 4–8. Graphs of *E* (potential) vs pH were constructed at various ANS concentrations. The pH of the initial solution (containing also 0.100 *M* Na_2SO_4 to adjust the ionic strength) was varied by the addition of very small volumes of the appropriate NaOH or H_2SO_4 solutions. A typical plot is shown in Fig. 1.

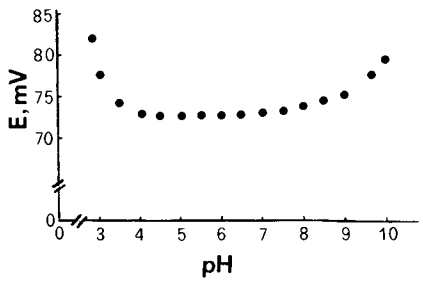

Fig. 1. Effect of pH on the potential *E* of the ANS ion selective electrode. ANS concentration, 0.0010 *M* at 25°C.

The electrode was found to have an operative life of about 1 month after which a new membrane was attached on the PVC tube-cup. The long-term stability of the electrode was checked through the calibration curves obtained on every experimental day. The slope of the electrode remained constant during its operative life, while the potential for the same aqueous standard solutions varied ±2 mV. The behavior of the electrode in the protein solutions used and the 1:1 diluted plasma was adequate for precise binding studies. The response time was short (the continuously recorded potential was stabilized in about 5–10 sec after each ANS addition). The long-term stability for operation in protein solutions is substantiated by the fact that reproducible binding parameters were obtained (Table I).

Binding Studies

The Scatchard plots for the ANS–BSA and ANS–HSA interactions at 25 and 37°C (Figs. 2 and 3) indicate that more than one class of binding sites is involved in the binding. Computer analysis of the data according to Eq. (1) revealed three classes of binding sites in both cases. The estimates for the binding parameters (Table I) indicate that the binding of ANS to both BSA and HSA is consistent with strong binding at the first binding class, at three and one binding sites, respectively.

Controversial conclusions concerning the number of classes of binding sites of ANS to BSA and HSA have been derived from previous studies (2–6,15) in spite of the fact that all studies were based on spectrofluorometry. Some reports (2–6) support that ANS is bound to HSA and BSA at only one class, while an additional, second weaker binding class of low capacity has been also suggested (4,15). In our study, computer analysis of the data revealed three classes of binding sites. It is worthy to mention that this finding was observed in all experiments irrespective of the binder (BSA, HSA, plasma) and temperature (25, 37°C) examined (Table I). It is obvious that the novel methodology utilized allows the study of binding over a wide range of ANS:albumin ratios. Thus, more complete and consistent Scatchard plots, compared to those previously reported (2–6,15), were observed (Fig. 2). The inability of fluorescence techniques to unmask the weakest class of binding sites could be linked with the basic assumption of fluorescence studies, i.e., the fluorescent quantum yield is the same for ANS bound at each site of the binder (15). It seems likely that this assumption is not valid for the weakest binding class since the results of Sudlow *et al.* (15) show that the quantum yield was constant up to a saturation of 3.7 mol of ANS per mol of HSA. In our study, this level of HSA saturation is reached shortly after the completion of ANS binding to the tight and intermediate sites since $n_1 + n_2 = 3.20$ (Table I). The arrow

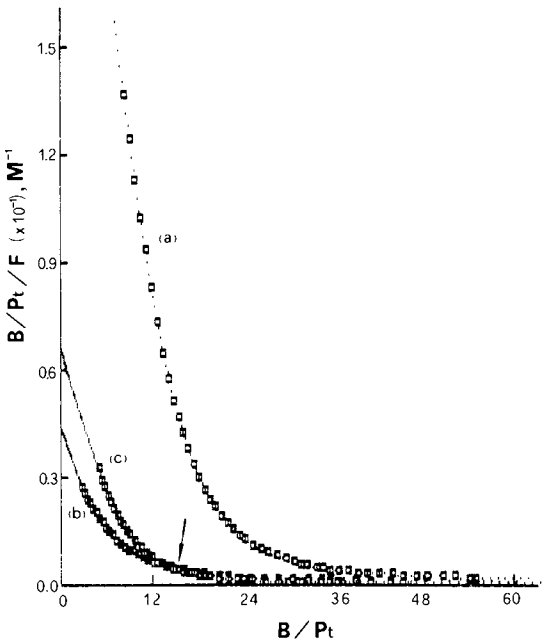

Fig. 2. Scatchard plots for the binding of ANS to (a) BSA, (b) HSA, and (c) human plasma at 25°C. BSA and HSA concentration, 22.5 g/L, in phosphate buffer, pH 7.4, plasma diluted 1:1 with phosphate buffer, pH 7.4. The theoretical Scatchard curves, based on the calculated binding parameters according to Eq. (2), are drawn over the experimental points. See text for explanations concerning the arrow.

Table I. Estimates (±SD)[a] for the Binding Parameters of the ANS Interaction to BSA,[b] HSA,[b] and Plasma[c] at 25 and 37°C

Binding parameter	BSA		HSA		Plasma	
	25°C	37°C	25°C	37°C	25°C	37°C
n_1	2.7 (0.2)	2.72 (0.03)	0.88 (0.05)	1.03 (0.06)	—	—
n_2	2.8 (0.2)	2.67 (0.02)	2.32 (0.03)	2.48 (0.08)	—	—
n_3	14.7 (1.0)	11.06 (0.09)	16.30 (0.50)	18.15 (1.31)	—	—
10^5N_1 (mol/g)	4.0 (0.2)	4.07 (0.03)	1.32 (0.08)	1.53 (0.08)	2.29 (0.17)	2.25 (0.05)
10^5N_2 (mol/g)	4.2 (0.2)	3.98 (0.03)	3.47 (0.05)	3.70 (0.11)	2.40 (0.10)	2.40 (0.13)
10^5N_3 (mol/g)	21.8 (1.4)	16.52 (0.13)	24.28 (0.74)	27.07 (1.95)	11.41 (0.31)	9.15 (0.45)
$10^{-5}K_1$ (M^{-1})	7.53 (0.59)	4.66 (0.07)	2.70 (0.20)	2.02 (0.12)	2.64 (0.26)	1.82 (0.05)
$10^{-5}K_2$ (M^{-1})	0.34 (0.05)	0.27 (0.01)	0.24 (0.02)	0.15 (0.01)	0.23 (0.04)	0.13 (0.02)
$10^{-4}K_3$ (M^{-1})	0.08 (0.01)	0.08 (0.01)	0.04 (0.01)	0.03 (0.01)	0.08 (0.01)	0.06 (0.01)
SD_{re}[d]	0.4	0.1	0.2	0.2	0.3	0.2

[a] Calculated from three titration experiments (runs), each one consisting of approximately 50 measurement points.
[b] BSA and HSA concentration was 2.25% and kept constant during the experiment.
[c] Plasma was diluted 1:1 with phosphate buffer, 0.1 *M*, pH 7.4.
[d] Standard deviation of the sum of squared residuals utilizing either Eq. (1) (BSA, HSA) or Eq. (2) (BSA, HSA, plasma).

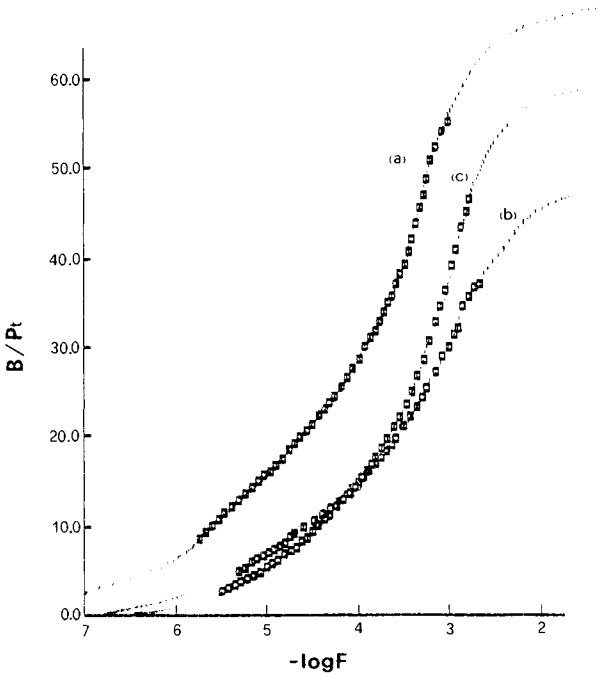

Fig. 3. Plots of the (a) BSA, (b) HSA, and (c) human plasma saturation with ANS at 25°C. Albumin concentration, 22.5 g/L, in phosphate buffer, pH 7.4, plasma diluted 1:1 with phosphate buffer, pH 7.4. The theoretical saturation curves based on the calculated binding parameters according to Eq. (2) are drawn over the experimental points.

in the Scatchard plot in Fig. 2 indicates the exact position of the data point which corresponds to 3.7 mol of ANS bound per mol of HSA.

As far as the estimates of the binding parameters is concerned, the association constant of the high-affinity class of the ANS–BSA interaction at 25°C was found to be 7.53 ($\pm$0.59) $\times$ 10^5 M^{-1}, which is in the range of 0.7 $\times$ 10^6 to 1.6 $\times$ 10^6 M^{-1} reported in the literature (2–6,15). Our results show that there is a considerable difference between the affinity of ANS to BSA and its affinity to HSA; the calculated association constant for the tight site of BSA is almost three times higher than that of HSA (Table I). This finding is not in accordance with similar values reported in a number of other studies (3,4,15) for the association constant of the interaction of ANS with the high-affinity binding sites of BSA and HSA. However, differences in selectivity of individual sites of BSA and HSA are likely to occur, and this possibility has been addressed previously (16). Since the diversity in the estimates for the association constants is not uncommon (3,6), it appeared logical to assume that the estimates derived from fluorescence studies could be biased due to the limitations arising from the incomplete study of the binding phenomenon. Despite these differences, the high-affinity binding class was found to be composed of three binding sites for BSA and one binding site for HSA (Table I), in full agreement with previously reported values (2–6,15).

The binding of ANS to human plasma at 25 and 37°C was also studied in plasma diluted 1:1 with phosphate buffer, 0.1 *M*, pH 7.4, in order to obtain a solution of 22.5 g/L with respect to HSA. Three classes of binding sites were again found, as indicated from the Scatchard plots (Fig. 2) and the computer analysis of data according to Eq. (2) (Table I). The first class was found to be of low capacity but appeared, again, determinant of the binding, with almost 10 times higher affinity for ANS than the other two classes.

In order to evaluate the binding capacity of the binding sites of BSA, HSA, and plasma, all binding data were analyzed according to Eq. (2) and the results are summarized in Table I. The estimates for the association constants indicate that the binding was strongest between ANS and BSA, while HSA and plasma showed lower but almost identical binding affinities for ANS. This result is expected since HSA is the major plasma protein responsible for the binding of acidic drugs in plasma. It is interesting to note, however, the observed higher binding capacity of the first class of plasma binding sites. This is possibly explained by the contribution of the other plasma proteins to ANS binding, and especially of a_1-acid glycoprotein, which has recently been reported (6) to bind ANS strongly. For comparative purposes, Scatchard and saturation plots for the binding of ANS to BSA, HSA, and plasma at 25°C are presented together in Figs. 2 and 3. The Scatchard plots (Fig. 2) for the binding of ANS to all binders are nonlinear; the initial almost-linear segments correspond to the first class of binding sites. The slopes of these linear parts represent the values for K_1, while their intersection with the ordinate corresponds to the total binding capacity, $\Sigma_{i=1}^{3} N_i K_i$. The saturation plots of BSA, HSA, and plasma with ANS at 25°C shown in Fig. 3 demonstrate the overall binding capacity of the binders for ANS. The binding capacity was found to decrease in the order BSA, plasma, HSA.

Experiments performed at 37°C, while all other conditions (pH, albumin, and ANS concentration, ionic strength etc.) were kept constant, showed a significant effect of temperature on the binding phenomenon. Decreased binding at the higher temperature was observed, judging from the significant decrease in the association constants (Table I). This observation is indicative of the exothermic character of binding (16). A typical example of the effect of temperature on the binding is presented in Fig. 4, for the interaction ANS–BSA. Similar plots (not shown) were obtained for the interactions ANS–HSA and ANS–plasma. It is interesting to note that the analysis of data for the binding of ANS to all binders at 37°C revealed three classes of binding sites with almost the same number of binding sites to these found at 25°C (Table I). This finding is an additional piece of evidence that ANS is bound to three classes of binding sites on BSA, HSA, and plasma.

In conclusion, the present study has shown that the application of direct potentiometry for the elucidation of the ANS binding to BSA, HSA, and plasma offers the following significant advantages over the fluorescence techniques. First, the wide ANS concentration utilized in the study covered more than three orders of the molar scale of the free ion concentration. This property, which is common for most ISEs, enabled us to study the binding phenomenon over a wider range of ANS/binder ratios compared with fluorescence studies. As a result of this ratio enlargement, three ANS binding classes were found at 25 and 37°C (Table I). Previous studies based on spectrofluorometry were conflict-

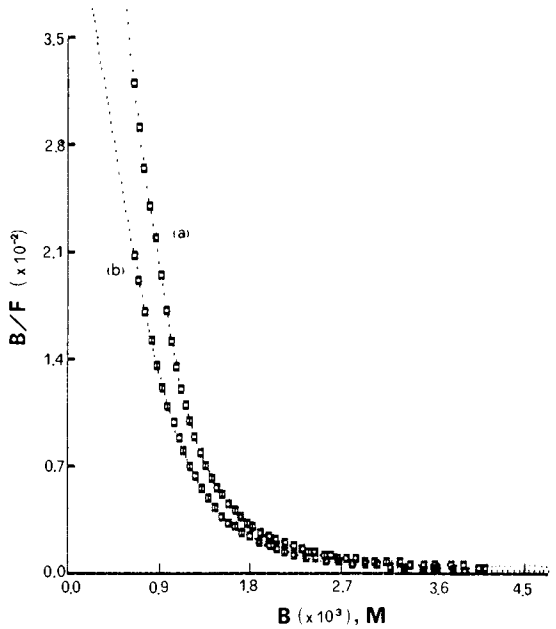

Fig. 4. Scatchard plots for the binding of ANS to bovine serum albumin at (a) 25 and (b) 37°C. Albumin concentration, 22.5 g/L, in phosphate buffer, pH 7.4. The theoretical Scatchard curves, based on the calculated binding parameters according to Eq. (1), are drawn over the experimental points.

ing, suggesting either one (2–6) or two (4,15) binding classes. Second, in our study the binding of ANS to plasma was accomplished with a reasonable dilution (1:1) of the biological specimen. This advantage is unique since the fluorescence techniques cannot be applied to moderately diluted plasma. To the best of our knowledge, only one fluorescence study dealing with ANS–plasma interaction has been published (1); extensively diluted (≤2%) plasma was employed in this study. However, such a dilution can shift the binding equilibria as acknowledged by the authors (1). The same research group in a recent *in vivo* study (17) utilized classical equilibrium dialysis in order to determine the free fractions of ANS in rat plasma. Our results show that the binding of ANS to plasma can be studied employing the constructed ISE of ANS, while the analysis of data according to Eq. (2) provides a means for the comparative assessment of ANS binding to all binders examined.

Finally, it can be anticipated that the use of the ISE potentiometric titration technique will open a new era of research in the field of competitive binding experiments and other areas of protein binding. Moreover, the development of other suitable ISEs for model ligands and drugs will enhance its analytical power and give insight into the binding mechanisms. Preliminary displacement experiments using the ISE of ANS in our laboratory are promising.

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