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A New Approach for Protein Binding Isotherm Analysis by Means of an Electrochemical Microsensor

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A simple method for analysis of binding isotherm in the protein–ligand interaction was introduced using potentiometric membrane micro-sensor data for the first time. The new potentiometric method is able to analysis the binding isotherm to obtain the complete set of biophysical parameters in protein-ligand binding studies. This method was applied to the interaction study of human serum albumin (HSA) with erbium ion, at 27 °C in Tris buffer at pH=7.4. Two binding sites for erbium ion with negative cooperativity and intrinsic association equilibrium constants of $K_1=2.75\times10^8$ $mol^{-1}L$ and $K_2=8.74\times10^7$ $mol^{-1}L$ were found on HSA.

Keywords: Protein binding isotherm analysis, potentiometric microsensor, erbium ion, human serum albumin

1. INTRODUCTION

Potentiometric sensors are the most rapidly growing class of chemical sensors [1-5]. Since the 1930s, these devices have found the most practical applicability in different fields due to its low cost, simplicity, high selectivity. The response mechanism of potentiometric chemical sensors is based on the measurement of a potential under no current flow, which can in turn be used to determine the analytical concentration of some components of the analyte. Potentiometric electrodes offer advantages of direct, simple, rapid, inexpensive and selective detection of ionic activities in aqueous media. The selectivity of these sensors originates from selective interactions between the membrane material and the target ion [1-5]. To have a complete potentiometric cell, the sensing electrode should

be used in conjunction with a reference electrode, which is also known as the external reference electrode. The indicator or sensing electrode consist of a transducer or internal reference electrode, commonly an Ag wire coated with AgCl, and a selective sensing species, incorporated in the membrane. The measured potential differences of the resulting electrochemical cell (ISE versus outer reference electrode potentials), in the case of an ion selective electrode (ISE), are linearly dependant on the logarithm of the activity of the target ion in the solution [1-5].

Different methods have been reported for data analysis of ligand binding protein [6-8]. However, potentiometric sensors are portable devices with low cost which offer advantages such as fast, simple, selective, accurate and precise response without destruction of analyte.

During recent decade, lanthanide and their complexes have been an interesting field of research in biological and medical studies [9-13]. Lanthanide ions have similar ionic radii to calcium, but a higher charge density. Thus, Ca^{2+} sites on biological molecules have higher affinity to lanthanide ions. Lanthanides can be used as biochemical probes to study calcium transport in biological environments. Therefore, there is a need to monitor lanthanide ion concentration during the experiments selectively [14-17]. There are different instrumental methods for determination of these ions, such as, inductively coupled plasma atomic emission spectrometry (ICP-AES), Neutron Activation Analysis (NAA), ICP-MS [18-22]. Although these methods have accurate and some of them have a low detection limits, they destruct the analyte and are not able to monitor the concentration during the experiments. However, potentiometric ion selective electrodes can solve this problem. Our research group has recently reported ion selective electrodes and microelectrodes for monitoring most of lanthanide ions [23-34].

In this work, we have introduced an equation with a useful linear graphical method in the ligand binding studies, to obtain equilibrium constant by potentiometry data for anti-cooperative systems with one set of identical and independent binding sites. A graphical fitting simple method for determination of thermodynamic parameters has also been introduced, which has been applied in inhibitor binding on the enzymes [35-37]. Determination of the binding isotherm for a set of identical and independent binding site has been reported [38]. Here, a new potentiometric data analysis is introduced to obtain the binding isotherm for a set of independent or interacting binding site, which is applied on the interaction between human serum albumin (HSA) protein and erbium ion (Er^{3+}).

2. EXPERIMENTAL PART

2.1. Materials and Reagents

Erbium nitrate was prepared by dissolving erbium oxides (Er_2O_3) in a nitric acid aqueous solution. Then, the products were recrystallized. Human serum albumin (HSA) was purchased from Sigma–Aldrich and Tris-HCl from Merck Co. Triply distilled water was used during the experiments.

2.2. Methods

The potentiometric titration experiments were performed by an erbium potentiometric membrane micro-sensor. The erbium micro-sensor, which is used as an indicator electrode, was

prepared as described elsewhere [39]. The Er^{3+} microelectrode exhibits a near Nernstian response of 17.5±0.5 mV per decade of erbium concentration, and a very wide linear range 3.0×10^{-10}-1.0×10^{-3} mol L^{-1}. It can work well in the pH range of 3.0-9.0. The lower detection limit (LDL) of the microelectrode was calculated to be 2.0×10^{-10} mol L^{-1} [39].

The erbium microelectrode was applied as an indicator electrode for the titration of 5.0 mL of 1.0×10^{-6} and 2.0×10^{-6} mol L^{-1} of HSA solution with 1.0×10^{-4} mol L^{-1} of Er^{3+}. Adding of erbium solution into the electrochemical cell was repeated 35 times, and each adding included 10 µL reagents. The potentiometric signal was measured by a Corning ion analyzer model 250-pH/mV meter.

2.3. Sample treatment

A 1×10^{-5} mol L^{-1} of HSA solution was prepared in a Tris–HCl buffer [0.05 mol L^{-1} Tris base (2-amino-2-(hydroxymethyl)-1, 3-propanediol), 0.10 mol L^{-1} NaCl] at the pH value of 7.4. The solutions were stored at 0-4 °C. The Er^{3+} standard solutions (1.0×10^{-2} mol L^{-1}) were also prepared. The working solutions (10^{-9} to 10^{-4} mol L^{-1}) were prepared by serial appropriate dilution of the stock solution.

3. RESULTS AND DISCUSSION

The raw data obtained from potentiometric titration of HSA interaction with Er^{3+} ion in two different concentrations of the protein was shown in Figures 1 and 2.

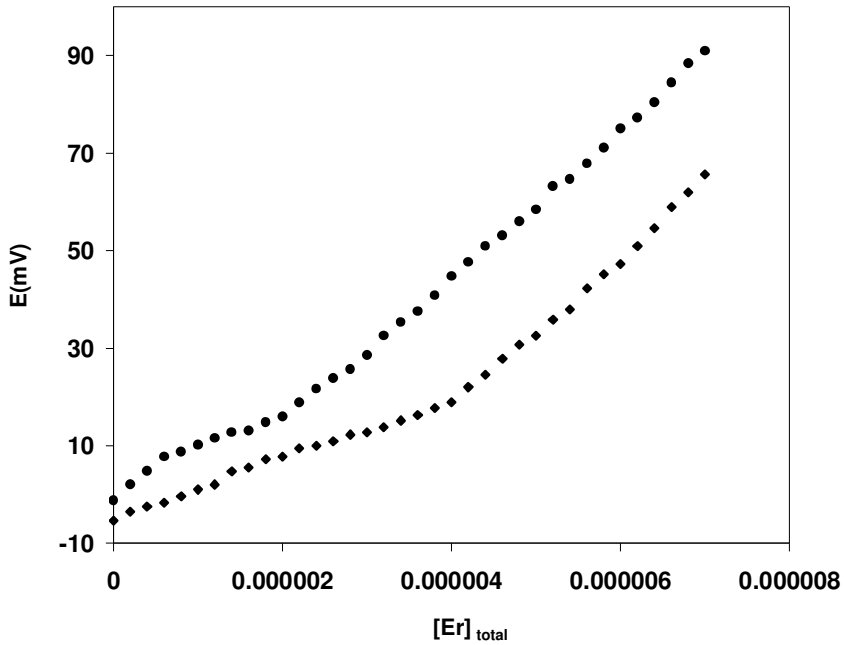

Figure 1. The potential changes of the titration of 5.0 mL of a: 1.0×10^{-6} and b: 2.0×10^{-6} mol L^{-1} of HSA solution with 1.0×10^{-4} mol L^{-1} of Er^{3+}

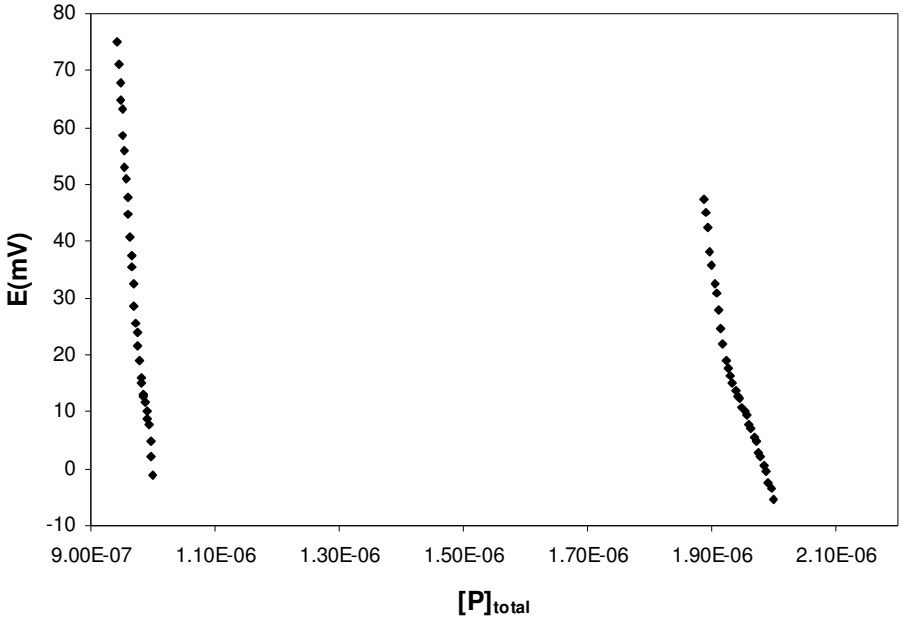

Figure 2. The potential changes of the titration of 10.0 mL of a: 1.0×10^{-6} and b: 2.0×10^{-6} mol L^{-1} of HSA solution with 1.0×10^{-4} mol L^{-1} of Er^{3+} *vs.* total concentration of HSA

In general, there will be "g" sites for binding of erbium ions per protein macromolecule and "ν" is defined as the average moles of bound erbium ions per mole of total HSA. At any constant value of potential (E), the free concentration of erbium ion (L_{free}) and "ν" are also constant at equilibrium on both two curves in Fig. 1. Erbium ions exist in two forms of free and bound. Hence, $L^{free}=L^{total}-L^{bound}$, where L^{total} and L^{bound} are the total and bound concentration of erbium ion, respectively. We do the potentiometric titration experiment in two concentrations of the protein, shown by a and b in Figures 1 and 2. Equality of L^{free} at any constant value of potential on both curves in Fig. 1 results the equation:

$$L_a^{free}=L_b^{free} \quad (1)$$

$$L_a^{total}-L_a^{bound}=L_b^{total}-L_b^{bound} \quad (2)$$

By applying $\nu=L^{bound}/P^{total}$, which P^{total} is the total concentration of the protein, and equality of ν at any constant value of potential on both curves in Fig. 1, it can be deduced Eq. (3) from Eq. (2):

$$L_a^{total}-\nu P_a^{total}=L_b^{total}-\nu P_b^{total} \quad (3)$$

This equation can be rearranged to give Eq. (4):

$$\nu=\frac{L_a^{total}-L_b^{total}}{P_a^{total}-P_b^{total}} \quad (4)$$

Then L^{free} can be calculated by Eq. (5), which obtains from substitution of ν from Eq. (4) into the equation $L^{free} = L_a^{total} - \nu P_a^{total}$ **:**

$$L^{free} = \frac{L_b^{total} P_a^{total} - L_a^{total} P_b^{total}}{P_a^{total} - P_b^{total}} \quad (5)$$

ν and L^{free} can be easily calculated from equations 4 and 5. However, by using the calibration curve of the proposed electrode, the L^{free} can be measured easier.

In this way, one can obtain a binding isotherm (Fig. 3) or the Scatchard plot (Fig. 4) according to the Eq. (6) [40]:

$$\frac{\nu}{[Er^{3+}]^{free}} = gK - \nu K \quad (6)$$

$[Er^{3+}]^{free}$ is the free concentration of erbium ion. It is sometimes the case that binding data does not form a straight line when plotted in a Scatchard plot. In this case, ligand binding at one site affects binding at the other. In fact, the data does not support the independent, equivalent site. The shapes of the Scatchard plots are clearly characteristic of different types of cooperativity [41,42]. A concave downward curve, as shown in Fig. 4, describes a system with negative cooperativity.

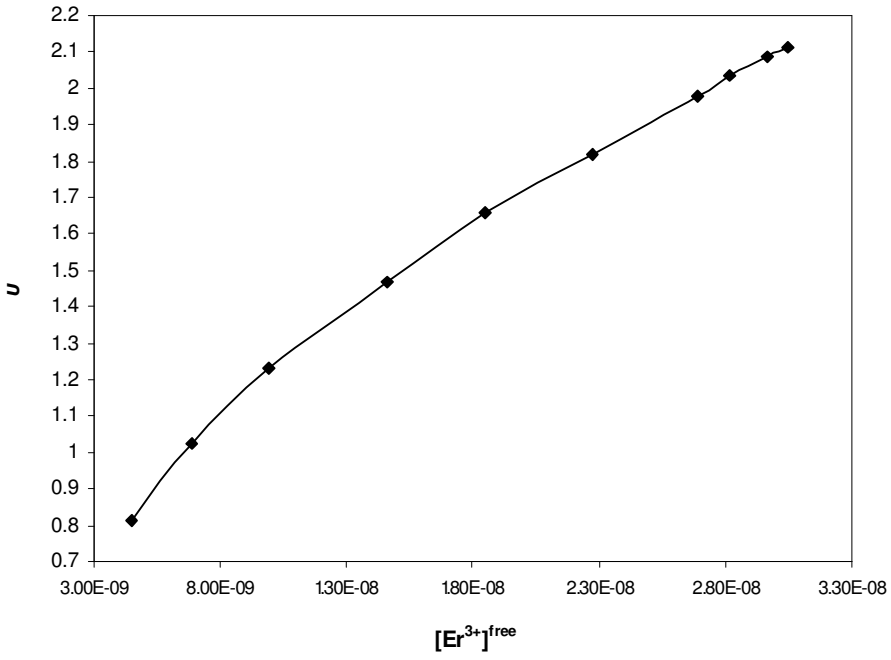

Figure 3. The binding isotherm of binding erbium ion to HSA

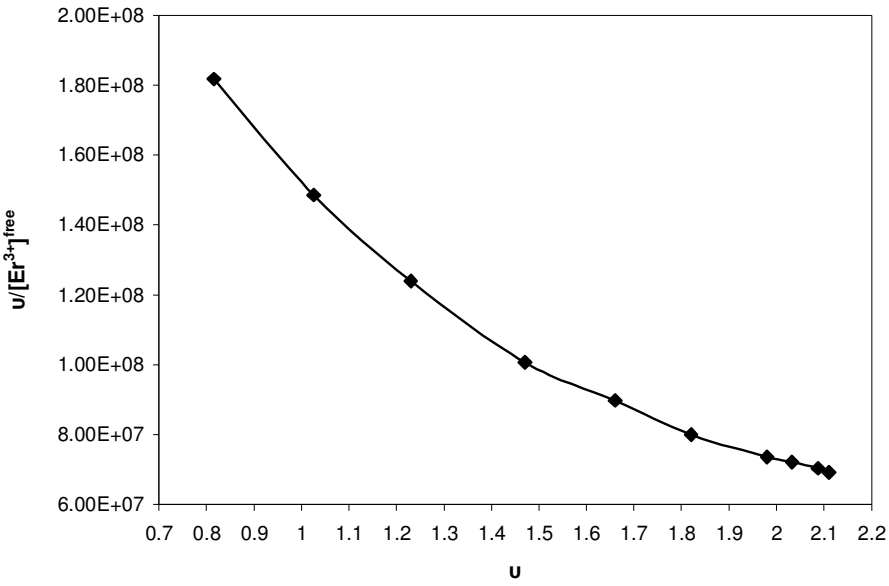

Figure 4. The Scatchard plot of binding erbium ion to HAS

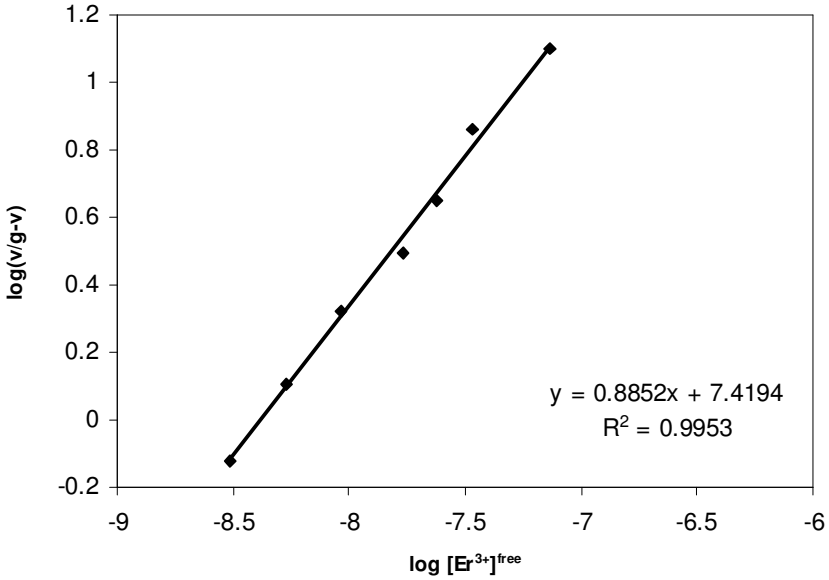

Figure 5. The Hill plot of binding erbium ion to HSA

For obtaining approximated values of binding parameters, it might be possible to fit the binding data to the Hill equation [43]:

$$v = \frac{g(K([Er^{3+}]^{free})^{n_{Hill}}}{1+(K([Er^{3+}]^{free})^{n_{Hill}}} \quad (7)$$

where K and n_{Hill} are the binding constant and Hill coefficient, respectively. The binding data for the binding of erbium ions to HSA can be fitted to the Hill equation using a computer program for nonlinear least-square fitting [44]. A quick and easy alternate to this method is to rearrange the binding function to make it possible to plot a linearized version of the equation. The eye and a straightedge can readily measure the values of n_{Hill} and K and also can detect if the data deviates from the theoretical curve.

$$\log(\frac{\nu}{g-\nu}) = n_{Hill} \log[Er^{3+}]^{free} + n_{Hill} \log K \quad (8)$$

Eq. (7) is in the form of y=m x+b, i.e. a straight line, with slope n_{Hill} and vertical axis intercept n_{Hill} log$K$ if log(υ/g-ν) versus log$[Er^{3+}]^{free}$ is plotted as shown in Fig. 5. Therefore, converting binding data to this form and plotting it will produce the value of the Hill coefficient and the constant K, which is the artificial binding constant for the simultaneous binding of two erbium ions to HSA. The results are: g=2, K=2.4×10^8 mol^{-1}L and n_{Hill}=0.88. The best-fit curve of the experimental binding data was then transformed to a Scatchard plot as shown in Fig. 4. A simple method for calculating intrinsic association equilibrium constants for system with two cooperative sites (K_1 and K_2) has been introduced from the Scatchard plot [45]. It has been shown that, in the limit as ν approaches 0, ν/$[Er^{3+}]^{free}$=2K_1 and when ν=1, or at half-saturation, ν/$[Er^{3+}]^{free}$=$(K_1K_2)^{1/2}$. Thus, K_1 can be obtained from the ordinate intercept of a Scatchard plot and K_2 is derived from the value of ν/$[Er^{3+}]^{free}$ at half-saturation. The results obtained from Fig. 4 are K_1=2.75×10^8 mol^{-1}L and K_2=8.74×10^7 mol^{-1}L. Thus, occupation of the first site has decreased the binding affinity of the second site. Results obtained by this new method are agreement with our previous results by calorimetry [46].

4. CONCLUSIONS

The new potentiometric method described in this paper allows obtaining the binding isotherm for measurement of the complete set of biophysical parameters in protein ligand binding studies. The binding isotherm for ligand–protein interaction can be easily obtained by carrying out titration potentiometric experiment in two different concentrations of a protein.

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