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Potentiometric Homogeneous Enzyme-Linked Binding Assays for Riboflavin and Riboflavin Binding Protein

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Adenosine deaminase (ADA) has been utilized as the label in devising a potentiometric homogeneous assay for riboflavin and riboflavin binding protein (RBP). The proposed homogeneous assay method employs an ADA-biotin conjugate as the signal generator and an avidin-riboflavin conjugate as the signal modulator in the solution phase. The catalytic activity of the ADA-biotin conjugate is inhibited in the presence of an excess amount of the avidin-riboflavin conjugate, and the observed inhibition is reversed in an amount proportional to the concentration of RBP added. When the analyte riboflavin is added to this mixture of ADA-biotin, avidin-riboflavin and RBP, the activity of the enzyme conjugate is re-inhibited in an amount proportional to the concentration of riboflavin. Since the enzyme label used in this system is ADA, an ammonia-producing enzyme, a potentiometric rather than photometric detection scheme is used to monitor the enzymatic activity in the assay.

Introduction

Enzyme-linked binding assay methods have become important analytical methods for the selective detection of various physiological, biological and environmental substances at trace levels.¹⁻⁶ Such methods may be classified as either heterogeneous or homogeneous. The homogeneous types

such as the enzyme-multiplied immunoassay technique (EMIT),^{5,6} rely on the ability of analyte molecules to reverse the inhibition of enzyme-analyte conjugates induced by analyte-specific binders (*e.g.*, antibodies, binding proteins, etc.). Homogeneous assays do not require time-consuming separation of the free and bound enzyme label, and, thus, are simple, fast and easily automated.

Very recently, we^{7,8} have developed a generic type homogeneous method by employing synthetic conjugates in conjunction with riboflavin and riboflavin binding protein (RBP) as the model analyte and analyte-specific binder system. The proposed method utilizes an enzyme-biotin conjugate as a signal generator and an avidin-analyte conjugate as a signal modulator in the solution phase. The binding reaction between the avidin-analyte and enzyme-biotin conjugates inactivates the enzyme conjugate. In the presence of analyte-specific binder, the enzymatic activity of the conjugate is regained since the binding of the binder to the avidin-analyte conjugate prevents the enzyme inactivation by sterically hindering the binding between the avidin-analyte and enzyme-biotin conjugates. When free analyte is added to the assay mixture, activity is re-inhibited in an amount proportional to the analyte concentration.

Unlike the EMIT type homogeneous assays, the proposed avidin-biotin mediated homogeneous assays can utilize single-substrate enzymes such as alkaline phosphatase and adenosine deaminase (ADA) as generic labels, as demonstrated in our previous work.^{7,8} In the conventional EMIT homogeneous methods, two-substrate enzymes (*e.g.*, glucose-6-phosphate dehydrogenase and malate dehydrogenase) are typically used as the enzyme labels, and the enzymatic activity is monitored by photometric detection of NADH at 340 nm.¹⁻⁶ An inherent limitation of assays based on photometric detection is the background absorbance due to the color and turbidity of physiological samples.⁹ This problem may be circumvented by employing a potentiometric detection scheme. The electrochemical detection method is desired particularly for homogeneous type assays, where separation of the turbid or colored sample phase is not possible prior to measuring the activity of labeling enzymes. Thus, such a detection system could permit the least dilution of samples, resulting in assays with better detection limits.

Recently, an EMIT type homogeneous assay for biotin, employing ADA as the labeling enzyme, was reported by Kjellstrom and Bachas.⁹ ADA is a single-substrate, deaminating (*i.e.*, ammonia releasing) enzyme, and its activity can be monitored potentiometrically with an ammonia gas sensor system. The biotin-labeled ADA was reported to be inhibited up to 97% in the presence of an excess amount of avidin.⁹ It is interesting to note that the same group¹⁰ used an ADA-folate conjugate in devising a heterogeneous (rather than homogeneous) type assay employing a potentiometric ammonia gas sensor. Indeed, ADA-folate conjugates were inhibited by less than 10% in the presence of folate binding protein.⁹ Another attempt to inhibit ADA, highly conjugated with adenosine 3',5'-monophosphate (cyclic AMP), by an anticyclic AMP antibody were also unsuccessful.^{9,11} It appears, however, that most enzymes unable to produce inhibited conjugates by other binding systems could produce biotin conjugates that can be highly inhibited by avidin. A model based on the effect of the depth of the binding site was proposed to explain the observed homogeneous inhibition of the ADA-biotin conjugate by avidin.^{9,12}

In our previous work,⁷ we demonstrated the feasibility of using ADA as an enzyme label in the biotin-avidin mediated homogeneous assay for biomolecules other than biotin by using a photometric detection scheme. The aim of the present work is to further examine the characteristics of the

biotin-avidin mediated method and to devise a homogeneous assay for riboflavin and its binder protein (*i.e.*, RBP) that employs a potentiometric detection system to monitor the activity of ADA conjugates.

Experimental

Reagents. Poly(vinyl chloride), bis(2-ethylhexyl) adipate nonactin and N,N-dimethylformamide (DMF) were purchased from Fluka (Buchs, Switzerland). Adenosine deaminase (ADA) from calf intestinal mucosa, adenosine, 2-iminobiotin, N-hydroxysuccinimidobiotin (NHS-biotin), bovine serum albumin (BSA), avidin from egg white, riboflavin binding protein (RBP) from chicken egg white and riboflavin, as well as all other biochemicals, were obtained from Sigma (St. Louis, MO, U.S.A.). The working assay buffer for ADA-biotin conjugates was 0.05 M Tris-HCl (pH 7.4) containing 0.1% BSA (w/v). Dilutions of conjugates, binders, standards, and sample solutions were made using this assay buffer. Deionized water was used to prepare all buffers.

Preparation of conjugates. The ADA-biotin and avidin-riboflavin conjugates were synthesized using the N-hydroxysuccinimide ester method, as described previously.⁷ Briefly, the required amount of NHS-biotin dissolved in DMF (5-50 mg/mL) was added to 500 μ L of coupling buffer containing a given amount of ADA (12.5 units) dissolved in 0.1 M sodium bicarbonate, pH 8.2. The reaction was run for 24 h at 4 $^{\circ}$ C under stirring. The reaction mixture was then dialyzed against 0.05 M Tris-HCl, pH 7.4, and diluted to a final volume of 2.0 mL with the dialysis buffer.

The avidin-riboflavin conjugates were prepared by first synthesizing 3-carboxymethylriboflavin and its NHS-activated ester.^{7,8,13} Prior to the coupling reaction, 3.43 mg of avidin was dissolved in 3 mL of coupling buffer, 0.1 M bis-Tris propane, pH 6.5. In order to protect the active site of avidin,^{7,8} 1 mg of 2-iminobiotin dissolved in 100 μ L of DMF was added to the avidin solution. The required amount of NHS-activated ester in DMF (21.3 mg/mL) was added to 500 μ L of the avidin/iminobiotin solution. The coupling reaction was run for 24 h at 4 $^{\circ}$ C under stirring. The resulting avidin-RF conjugates were dialyzed against 50 mM ammonium acetate/0.5 M NaCl buffer, pH 4.0, and diluted to a final volume of 2.0 mL with 0.05 M Tris-HCl buffer, pH 7.4.

The resulting ADA conjugates were characterized by their residual activities and percent inhibitions induced by an excess amount of avidin, and the avidin conjugates by their ability to inhibit the ADA-biotin activity in the presence or absence of RBP as described previously.^{7,8} All conjugates were kept at 4 $^{\circ}$ C until the additions of reagents for activity measurements.

Flow injection potentiometry. The activity and inhibition of the ADA conjugates were determined by measuring the amount of ammonium ions, liberated from the enzyme-catalyzed reaction, by utilizing an ammonium-selective electrode in a large-volume wall-jet detection arrangement as described elsewhere.^{14,15} A schematic diagram of the complete flow injection system is shown in Figure 1. An ammonium-selective membrane prepared by the method of Simon and coworkers¹⁶ consisted of 1.0 wt% nonactin, 66 wt% bis(2-ethylhexyl) adipate and 33 wt% poly(vinyl chloride). A piece of this membrane was mounted in a Philips electrode body

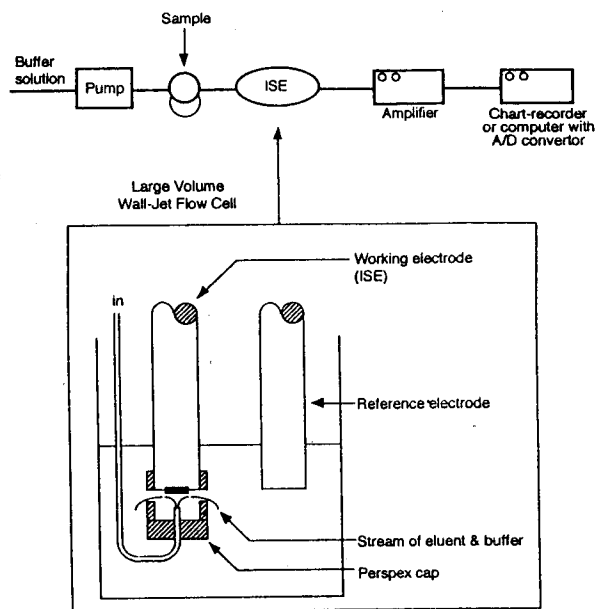


Figure 1. Schematic of the membrane electrode-based flow injection system with a large volume wall-jet flow cell.

(IS-561, Glasbläserei Müller, Zürich, Switzerland) with 0.1 M KCl as the internal filling solution. The electrode was fitted with a special flow cap for use as a flow-through detector in a large volume wall-jet cell. An Orion Model 90-02 double junction Ag/AgCl electrode along with the working ammonium ion-selective electrode were placed in a large beaker of reagent buffer (0.05 M Tris-HCl, pH 7.4). Teflon tubing (0.012 in. i.d.) connected the flow cap to the injection valve. Solution delivery was accomplished with a Ismatech Model 7331-00 peristaltic pump (Zürich, Switzerland). For sample introduction, a Rheodyne Model 5020 four-way rotary valve (Cotati, CA, U.S.A.) equipped with a sample loop was employed. In this work, 270 μL of mixture solutions was injected into a buffer stream (1.7 mL/min) of 0.05 M Tris-HCl, pH 7.4. Electrodes were connected through high impedance amplifiers to an IBM AT-type computer equipped with an analog-to-digital converter, or through a Fisher Accumet Model 620 pH-mV meter (Romulus, MI, U.S.A.) to a Shimadzu Model U-135 recorder (Kyoto, Japan).

To measure the activity of ADA conjugates, 400 μL of 16 mM adenosine was added to a gelatin-coated tube containing 200 μL of an ADA-biotin (1:20 dilution) and 2300 μL of assay buffer. The mixture was then allowed to react for 15 min before terminating the enzymatic reaction by adding 100 μL of 0.01 M AgNO_3 . After mixing and agitation (1-2 s), 270 μL of the reaction mixture was introduced to the flow injection system described above.

Dose-response curves. In order to determine the optimum concentration of avidin-riboflavin to be used in the assays, 200 μL of solutions containing different amounts of avidin-riboflavin was incubated with 200 μL of ADA-biotin (1:20 dilution) and 2300 μL of assay buffer for 10 min. The substrate was then added and the enzymatic activity was measured potentiometrically after adding AgNO_3 as above. A dilution curve was prepared by plotting observed potentials (ΔmV) vs. the amount of avidin-riboflavin used. For the

dose-response curve for RBP, 200 μL of solutions containing different amounts of RBP was incubated with 200 μL of avidin-riboflavin (0.11 mg/mL) and 1900 μL of assay buffer for 15 min. After adding 200 μL of the enzyme-biotin conjugate (1:20 dilution), the mixture was incubated for an additional 10-min period. The resulting enzymatic activity was measured as outlined above. In the case of the riboflavin dose-response curve, 200 μL portions of the riboflavin standards were added to an assay tube containing 200 μL of an RBP solution (125 $\mu\text{g}/\text{mL}$) and 1700 μL of assay buffer. After incubation for 10 min, 200 μL of avidin-riboflavin (0.11 mg/mL) was added, and the mixture was further incubated for a 5-min period. A volume of 200 μL of the ADA conjugate (1:20 dilution) was then added, and the mixture was incubated for 10 min prior to the addition of the substrate for an activity measurement.

Results and Discussion

Although a biotin-labeled ADA was used previously in an EMIT type homogeneous assay for biotin,⁹ this is the first time that ADA has been used as the label in a homogeneous type assay employing a potentiometric detection scheme for the determination of biomolecules (*i.e.*, riboflavin and RBP) other than biotin. In this study, an ADA-biotin conjugate was prepared by employing an initial biotin/ADA molar ratio of 750, and an avidin-riboflavin by an initial riboflavin/avidin ratio of 60 during the conjugation reactions. In our previous report,⁷ it was shown that this ADA-biotin conjugate possessed a residual activity of 66% and was shown to be inhibited up to 90% by an excess of avidin, and that the avidin-riboflavin conjugate exhibited a high inhibitory effect of 90% and reversed the observed inhibition up to 92% with an excess of RBP.

Since ADA catalyzes hydrolysis of adenosine to inosine and ammonia, the ADA activity can be monitored by detecting the amount of ammonia produced in an aqueous solution. In the present study, a flow injection system equipped with an ammonium ion-selective membrane electrode, as depicted in Figure 1, was used for activity measurements in the assay. Enzymatic reaction was terminated by adding AgNO_3 to an assay mixture prior to introducing the mixture to the flow injection system, as described in the Experimental section. Figure 2 illustrates the effect of the incubation period between additions of the substrate and AgNO_3 on the resulting emf signal of the flow injection ammonium ion detector. Since the analytical signal of the proposed homogeneous assay is dependent on the extent of inhibition of the ADA activity, an incubation period longer than that needed for reaching an equilibrium of the enzymatic reaction will degrade the final detection capability of the assay. On the other hand, a reduced incubation period will decrease the analytical signal of the assay. Therefore, based on the results shown in Figure 2, a 15-min incubation period that led to >95% of the equilibrium was selected to gain a nearly maximum change in signal in the subsequent experiments.

A binder dilution curve can be used to determine the reagent concentrations required in an assay tube to obtain a desired signal change (enzyme activity and/or percentage inhibition). For the present method, two types of binder dilution curves are needed: different amounts of avidin-riboflavin

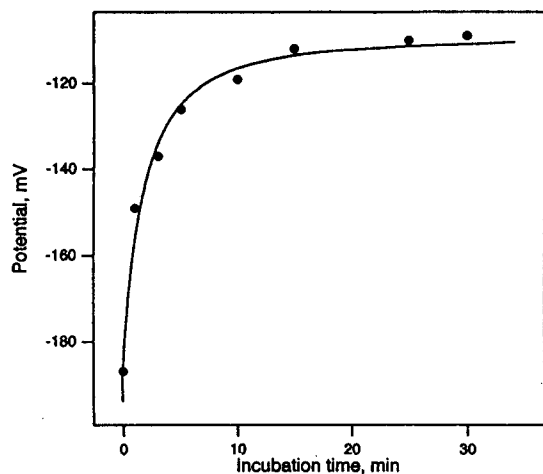


Figure 2. Effect of varying the incubation period for the ADA-catalyzed reaction on the resulting emf signal of the flow injection ammonium ion detector. ADA-biotin (0.25 units) was employed with 1.1 mM of adenosine, and the reaction was terminated by adding 0.33 mM of AgNO_3 in an assay tube. Values shown are average of triplicate measurements.

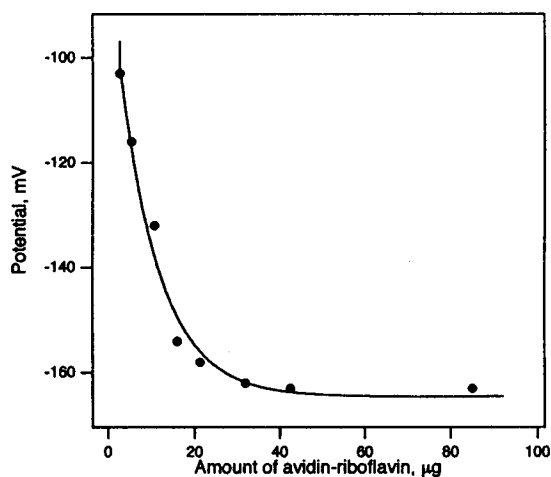


Figure 3. Effect of varying the levels of avidin-riboflavin on the activity of ADA-biotin (0.25 units). Values shown are average of triplicate measurements.

incubated with a fixed amount of ADA-biotin (Figure 3), and different amounts of RBP with fixed amounts of ADA-biotin and avidin-riboflavin (Figure 5). As shown in Figure 3, as the amount of avidin-riboflavin increases in the assay tube, the extent of inhibition induced by binding of avidin-riboflavin to ADA-biotin increases. This results in a decreased amount of ammonium ions (corresponding to a more negative potential in Figure 3) produced from adenosine by the ADA-biotin conjugate. This type of binder dilution curve has been utilized to determine the optimum concentration of binder to be used in homogeneous competitive binding assays. For example, Kabakoff and Greenwood¹⁷ suggested that the amount of binder that corresponds to 85% of the maximum homogeneous inhibition will result in a dose-response curve with the best detection capabilities. Thus, 21.25 μg of the avidin-riboflavin conjugate with 0.25 units (refer to the origi-

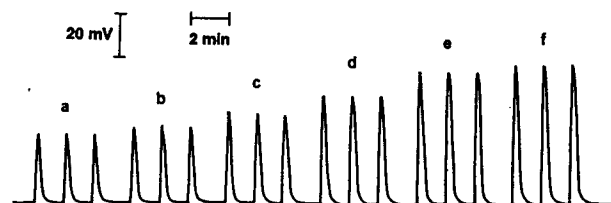


Figure 4. Potentiometric flow injection response peaks for ammonium ions generated in assay tubes containing different amounts of RBP with 0.25 units of ADA-biotin and 21.25 μg of avidin-riboflavin: a) 8 μg ; b) 12.5 μg ; c) 18.75 μg ; d) 25 μg ; e) 50 μg ; and f) 100 μg of RBP.

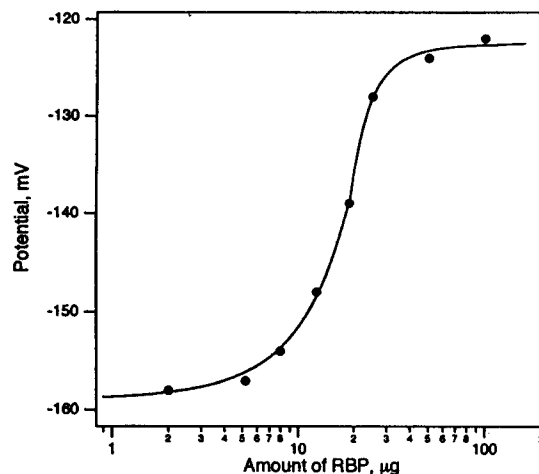


Figure 5. Effect of varying the levels of RBP on the inhibition of ADA-biotin (0.25 units) induced by avidin-riboflavin (21.25 μg) (i.e., a dose-response curve for RBP).

nal specific activity) of the ADA-biotin conjugate was selected for the subsequent competitive binding experiments.

Figure 4 shows flow injection response signals to ammonium ions generated in assay tubes containing different amounts of RBP with fixed amounts of ADA-biotin and avidin-riboflavin as specified above. The response of the flow injection detection system was highly reproducible and the slope of the linear portion of the calibration plots were in the range of 54-56 mV/decade change in ammonium concentration. As can be seen in Figure 4, as the amount of RBP increases, the amount of ammonium ions increases in the assay tube. This is because the binding of RBP to avidin-riboflavin conjugate sterically hinders the binding between avidin-riboflavin and ADA-biotin conjugates, preventing the enzyme inactivation. As a result, the activity of the ADA conjugate is regained in an amount proportional to the concentration of RBP added in the assay mixture. Based on these data, a dilution curve for RBP was constructed by plotting emf response of the ammonium-selective electrode vs logarithm of the RBP concentration added to the assay mixture (Figure 5). Such a curve can be used not only for determining the optimum RBP concentration to be used in the final riboflavin assay, but also for assaying RBP itself in samples. As shown, the curve is extremely steep over a very narrow concentration range (8-50 μg), indicating that the as-

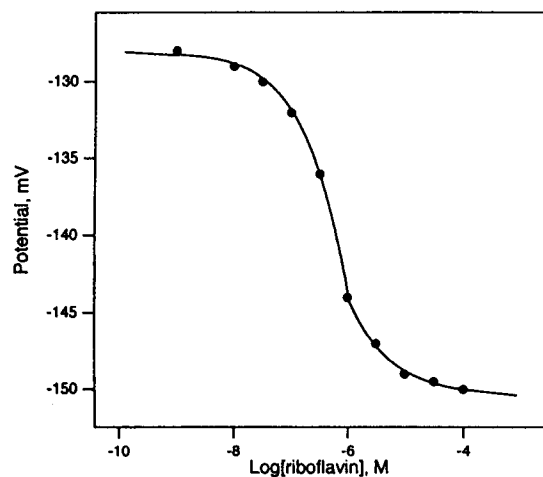


Figure 6. Dose-Response curves obtained for riboflavin with 0.25 units of ADA-biotin, 21.25 μg of avidin-riboflavin and 25 μg of RBP. The x-axis refers to concentrations of riboflavin in the standards.

say can be used for the sensitive determination of RBP. The amount of RBP (25 μg) that yields approximately 85% of the maximum reversal was chosen for use in the subsequent experiments.

Under the above conditions, a competitive dose-response curve for riboflavin was constructed, as shown in Figure 6. The pH of the assay buffer was 7.4 which had been selected as the compromising pH by considering the optimum pHs of the enzymatic and binding reactions involved⁷ and the flow injection detector system employed: pH 7.5 for ADA; pH 6-9 for RBP/riboflavin; extremes of pH for avidin/biotin, and <pH 7.3 for the ammonium-selective electrode. As can be seen in Figure 6, the ammonium concentration in the assay tube decreases as the amount of riboflavin increases: the added riboflavin competes with the avidin-riboflavin conjugate for the limited number of binding sites of RBP. Thus, the inhibition of the ADA-biotin conjugate induced by the free avidin-riboflavin conjugate is restored to an extent proportional to the riboflavin concentration in the assay mixture.

The detection capabilities obtained with the present potentiometric ADA-based riboflavin assay (Figure 6) are not very different from those with the previous photometric ADA-based assay⁷: the dose-response curve is steep over a very narrow concentration range (0.4-2.5 μg of riboflavin). However, it should be realized that the wall-jet style electrode-based flow injection system employed in this preliminary study has several inherent limitations, in terms of lowering the detection limits. As can be seen from flow injection response signals in Figure 4, only a fraction of the response to the injected ions is observed because such a flow injection system operates in a nonequilibrium mode. Furthermore, a relatively large sample injection volume is required to help maintain a constant level of effluent in the wall-jet detector cell for an optimal performance of the electrode detector. Thus, an electrode-based assay with better detection capabilities may be possible when a stop-flow measurement system

is adapted for maximizing the response signal and minimizing the sample injection volume.

In summary, the feasibility of using a single-substrate ADA as a generic label in homogeneous assays has been demonstrated by employing the biotin-avidin mediated assay protocol with riboflavin and RBP as the model analyte system. The use of such enzyme conjugates allows the easy adaptation of electrochemical detection means to enzyme-linked binding assay arrangements. The electrochemical detection method should be advantageous for use in turbid and colored samples. The ability of the avidin-analyte conjugate to inhibit the ADA-biotin activity may prove valuable in the design of electrochemical homogeneous methods for other types of biomolecules. Work in this direction is progress.

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