Development of Bioluminescence Immunoassay Using Photoprotein, Aequorin and Site-directed Immobilization

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The heterogeneous bioluminescence immunoassay for digoxin was developed using photoprotein, native aequorin as a label and the site-directed immobilization technique based on avidin/biotin interaction. Aequorin is a bioluminescence protein originally isolated from the jellyfish *Aequoria Victoria* and an attractive label in analytical applications because of sensitive detection due to virtually no background bioluminescent signal. Digoxin is a cardioactive drug, and its therapeutic level in serum is at low concentration with very narrow therapeutic index. The aequorin-digoxigenin conjugates were synthesized by the *N*-hydroxysuccinimide ester method and characterized in terms of bioluminescent residual activity. The resulting dose-response curve shows that the detection limit is 1.0×10^{-10} M and a dynamic range is three orders of magnitude, which was obtained by 1.0×10^{-10} M conjugate and 0.9 μg/mL anti-digoxin antibody. Three structurally similar molecules to digoxin were examined for their cross-reactivity. None of these three compounds showed any cross-reactivity with digoxin antibody employed in this study. Standard amounts of digoxin corresponding to the therapeutic range were spiked into the each serum solution. Study of the serum matrix effect indicated that correlation coefficient shows good agreement between luminescence light intensity between in buffer and in serum.

Key Words : Bioluminescence immunoassay, Site-directed immobilization, Native aequorin, Digoxin

Introduction

Immunoassay has emerged as the most powerful tool with simplicity, specificity and sensitivity in the selective detection of various physiological, biological and environmental substances at trace levels.[1-3] The last decade has seen the development and refinement of many new immunoassay reagents and systems.

Bioluminescence is defined as luminescence derived from a biological reaction at ordinary temperature. In contrast to fluorescent and phosphorescent molecules, bioluminescent proteins generate the emission of light on the return to the ground state from an electronically excited species produced by a biological reaction without any associated generation of heat, which means it does not need optical excitation. Thus, It is virtually no background signal that degrades sensitivity in assay. Bioluminescence immunoassay was developed using components of bioluminescence reactions as a label. Thus, the advantages of bioluminescence immunoassay are sensitivity and speed due to no background signal and signal generated in few seconds in case of flash type, respectively, and simple procedures.

Aequorin is a photoprotein originally found in the jellyfish *Aequoria Victoria*. It has been used extensively as a calcium indicator[4] and more recently as a highly sensitive quantitive label in analytical assay systems.[5-7] Aequorin consists of the apoaequorin (22,400 Mw), coelenterazine (luminophore) and molecular oxygen. When Ca^{2+} binds to the aequorin complex, aequorin undergoes a conformational change, and then coelenterazine is oxidized to coelenteramide, with release of CO_2 and light (λmax ~469 nm).[8] The flash-type emission (less than 5 s) of blue light occurs as a single-turnover event.

Bioluminescence immunoassay can be classified as either heterogeneous or homogeneous. Heterogeneous arrangements are outstanding on low detection limit at the expense of time consuming washing steps.[9,10] The first step of heterogeneous assay is immobilization of the proteins on the plastic surfaces. In most case, it has been found that protein binding sites (or enzymatic activity) decrease after the protein gets immobilized randomly. Immobilization through physical adsorption usually gives leakage problem of the immobilized protein from the support. Thus, the well-oriented immobilized techniques should be developed as site-directed immobilization. This gives several advantages such as reproducibility and orientation of the protein over random immobilization.

Digoxin was selected as a model compound for this study, which is the most widely used cardioactive drug for treating congestive heart failure and various disturbances of cardiac rhythm.[11,12] The clinical use of digoxin as a therapeutic agent is very complicated because each patient has different metabolism on digoxin. Also, the therapeutic range of digoxin in serum is very narrow [*i.e.*, 0.8-2.0 ng/mL or $(1.0 \text{ to } 2.5) \times 10^{-9}$ M]. Therefore, it is very important to monitor the accurate digoxin levels in serum.

In this work, we developed heterogeneous bioluminescence immunoassay for digoxin using photoprotein, native aequorin as a label and the site-directed immobilization technique based on avidin/biotin interaction. A derivative of digoxin was chemically conjugated to aequorin and the resulting aequorin-digoxigenin conjugates were characterized

in terms of their luminescence properties. From these, heterogeneous digoxin assay was optimized and their dose-response behaviors were examined. In order to evaluate this assay, serum matrix analysis with spiked digoxin was performed, and structurally similar molecules were also employed to determine the specificity of this assay.

Experimental Section

Reagents. Digoxigenin-3-0-methylcarbonyl-e-aminocaproic acid-*N*-hydroxysuccinimide ester (NHS-digoxigenin) was purchased from Boehringer Mannheim (Mannheim, Germany). Bovine serum albumin (BSA) was purchased from Pierce (Rockford, IL). Aequorin type III, Tris(hydroxymethyl)-aminomethane (Tris) free base, ethylenediamintetraacetic acid (EDTA), coelenterazine, biotinylated monoclonal anti-digoxin antibody, Sodium azide, Tween20 and all other reagents were obtained from Sigma (ST. Louis, MO, USA). All chemicals were of analytical-reagent grade or better and were used as received. All solutions were prepared using deionized (Milli-Q water purification system, Millipore, Bedford, MA) distilled water.

Apparatus. Bioluminescence measurements were made on an MLX-microtiter plate luminometer from Dynex (Chantilly, VA) using a 100 μL fixed volume injector. All luminescence intensities reported are the average of at least triplicates and have been corrected for the contribution of the blank. Neutravidin-coated white polystyrene flat-bottomed microtiter plates were used.

Preparation of conjugates. Aequorin-digoxigenin conjugates were prepared by reacting aequorin with different amounts of NHS-digoxigenin. The required amount of NHS-digoxigenin dissolved in anhydrous DMF was added to 700 μL of coupling buffer containing a given amount of aequorin (3.12×10^{-10} mol). The coupling buffer was 50 mM sodium bicarbonate buffer, pH 8.5 containing 4 mM EDTA. The reaction was run for 24 hr at 4 °C under stirring. The reaction mixture was dialyzed against 30 mM Tris-HCl, pH 7.5 containing 4mM EDTA using a centricon YM-3 (3,000MW cut off, Millipore) to remove the excess NHS-digoxigenin and to change the bicarbonate buffer (pH 8.5) to Tris-HCl buffer (pH 7.5), and then diluted to a final concentration of 1.0×10^{-7} M aequorin (stock solution of conjugate) with dialysis buffer. The resulting aequorin conjugates were characterized by their residual activities.[13-16] All conjugates were kept at 4 °C until the additions of reagents for activity measurements.

Activity study of Aequorin-digoxigenin conjugates. The bioluminescence activity of aequorin-digoxigenin conjugates was determined by measuring the bioluminescence emission at 469 nm. A calibration curve for this aequorin-digoxigenin conjugate was constructed in order to determine the lowest amount of aequorin-digoxigenin conjugates to be used in this study. Serial dilutions of aequorin-digoxigenin solution were made from the stock aequorin-digoxigenin solution (1.0×10^{-7} M) using assay buffer (30 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 0.1% (w/v) BSA, 4 mM EDTA). The calibration curve of aequorin-digoxigenin conjugates was prepared with 100 μL of varying concentrations of aequorin-digoxigenin solution in a 96-well microlite 1 plate. The contents of the wells were mixed and positioned in the MLX luminometer, and the bioluminescence light was measured after injection of 100 μL of bioluminescence-triggering solution, 100 mM $CaCl_2$ in 100 mM Tris-HCl, pH 7.5. A test well containing 100 μL of assay buffer only without aequorin-digoxigenin conjugate was used as a blank. The emission of reacting mixture in each well was read over 3 s time period.

Binder dilution study. Neutravidin-coated plate was pre-washed 4 times with wash buffer (30 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 0.2% (w/v) BSA, 0.05% Tween 20, 4 mM EDTA) before using it. A biotinylated monoclonal anti-digoxin antibody of differing concentrations were prepared in the dilution buffer (10 mM Phosphate buffered saline, pH 7.5 containing 150 mM NaCl, 0.1% BSA) and 100 μL of each dilution was immobilized on a neutravidin-coated plate with constant shaking (~150 rpm) for 2 hr at room temperature. The plate was then washed 4 times with wash buffer. Aequorin-digoxigenin conjugate (100 μL, 1.0×10^{-10} M), in assay buffer was then added to the plate containing site-directed immobilized antibody. The plate was incubated for an additional 2 hr with shaking at room temperature. And then the plate was washed and bioluminescence intensity was measured by injecting 100 μL of the luminescence-triggering buffer.

Dose-response curves. A stock solution of digoxin standard (1.0×10^{-4} M) was serially diluted in assay buffer as ranging in concentration from 1.0×10^{-6} M to 1×10^{-13} M. 100 μL of each digoxin standard incubated with biotinylated monoclonal anti-digoxin antibody (0.9 μg/mL) immobilized on neutravidin-coated plate, for 2 hr at room temperature with shaking. Following this incubation period, the plate was washed 4 times with wash buffer. A volume of 100 μL of conjugate, 10 : 1, of concentration 1.0×10^{-10} M was then added and allowed to incubate with shaking for an additional 2 hr at room temperature. After a washing step, the luminescence intensity was measured as described previously. Dose-response curves were also obtained in a similar manner with differing concentrations of conjugate, 10 : 1 and differing anti-digoxin antibody concentrations

Cross-reactivity study. A cross-reactivity study was accomplished in the same manner as the studies performed for digoxin in serum. The compounds structurally similar to digoxin such as Digitoxigenin, Ouabain, Spironolactone were applied. And various concentrations of them were spiked in the serum and the percent cross-reactivity was calculated from the dose-response curve obtained for digoxin.

Matrix effect study. Standard amounts of digoxin were spiked to 100 μL of serum solution. 100 μL of these samples were incubated with immobilized biotinylated monoclonal anti-digoxin antibody, for 2 hr at room temperature followed by a washing step. A volume of 100 μL of the aequorin-digoxigenin conjugate, 10 : 1 of 1.0×10^{-10} M concentration

was then added to the plate and incubated for another 2 hr. After a washing step, the luminescence intensity was measured as described previously.

Results and Discussion

For the sensitive immunoassay development, the choice of an appropriate signal generator is very essential. As a signal generator, bioluminescent reporter proteins have been used as an excellent label, which have advantages of low background, large dynamic range, high sensitivity, and no need of substrate or enhancer in mmunoassay systems. Several immunoassays have been developed for determining digoxin using enzymes and mutant aequorin as a label,[13-15] however, it is the first time to develop the bioluminescence immunoassay by employing native aequorin for digoxin assay.

In heterogeneous assays, label-analyte conjugate should retain a useful residual activity, and be desired a less inhibition by an excess binder. Therefore, various aequorin-digoxigenin conjugates were prepared by reacting each aequorin with different amounts of NHS-digoxigenin. In general, higher initial NHS-digoxigenin/aequorin ratios used during the conjugation reaction yielded lower residual activity and greater conjugate inhibition by a given excess of binder. Aequorin contains 14 lysine residues in its primary structure. The initial molar ratios of 10 : 1, 25 : 1, 50 : 1 and 100 : 1 of NHS-digoxigenin/aequorin were examined. And all conjugates were characterized. Figure 1 shows activity study for no-conjugate, 10 : 1 and 100 : 1 conjugates, respectively. Here, we decide to use 10 : 1 conjugate due to good bioluminescence activity with digoxigenin conjugated for following studies. Also, 1.0×10^{-10} M concentration of the conjugate, 10 : 1 was employed for the further studied. This relatively low concentration of conjugate is enough to generate the signal above the background and obtain the low detection limit. This chemically conjugated aequorin-digoxigenin conjugate shows a flash-type bioluminescence emission with more than 95% of the total light being emitted within 3 s. Therefore, luminescence light intensity was collected over 3 s period of time in all subsequent experiments.

In order to determine the amount of anti-digoxin antibody to be used in this experiment, a binder dilution study was performed. Here, we used neutravidin-coated plate, which was already coated with avidin on the base plate, for site-directed immobilization. There is a very strong relationship between protein stability and immobilization. In random immobilization, the biomolecules, in particular, proteins and enzymes are lost their activity due to the microenvironmental change exerted by both the matrix support and the immobilization procedure itself. Thus, in order to improve the protein stability and maintain their functionality after immobilized on the surfaces, site-directed immobilization was applied. This allows the active/binding site to be fully accessible by its substrates and gives advantages on reproducibility and oriented immobilization of a protein molecule. Here, we accomplished site-directed immobilization through avidin/biotin interaction. The biotinylated monoclonal anti-digoxin antibody was immobilized on a neutravidin-coated plate. Figure 2 shows binder dilution curve obtained by varying the amount of anti-digoxin antibody in the presence of 1.8×10^{-9} M conjugate, 10 : 1. From the linear portion of the binder dilution curve, 0.9 μg/mL concentration of anti-digoxin antibody was selected.

According to association time study, the optimum incubation time required was determined. To gain maximum signal change, 2 hr incubation was selected both incubation steps on anti-digoxin antibody/digoxin and anti-digoxin antibody/digoxin/digoxigenin-aequorin.

After evaluating the various parameters that influence the assay, several dose-response curves were constructed using aequorin-digoxigenin conjugate, 10 : 1 as a sequential manner

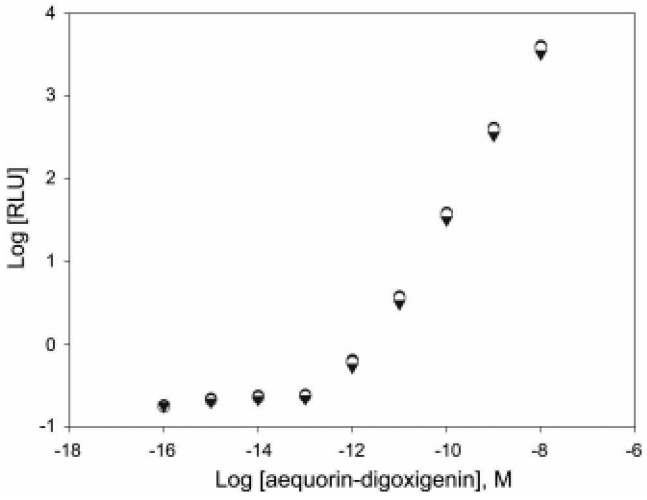

Figure 1. Activity study of aequorin-digoxigenin conjugates: The initial molar ratios of NHS-digoxigenin *vs.* aequorin are no conjugate (●), 10:1 (○) and 100:1 (▼). Data points are means of triplicate measurements.

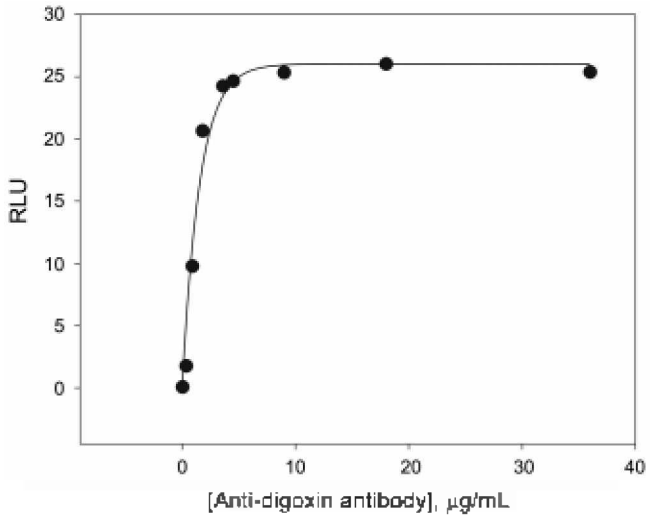

Figure 2. Binder dilution curve obtained by varying amount of anti-digoxin antibody in the presence of 1.8×10^{-9} M of aequorin-digoxigenin conjugate, 10:1. Data points are means of triplicate measurements.

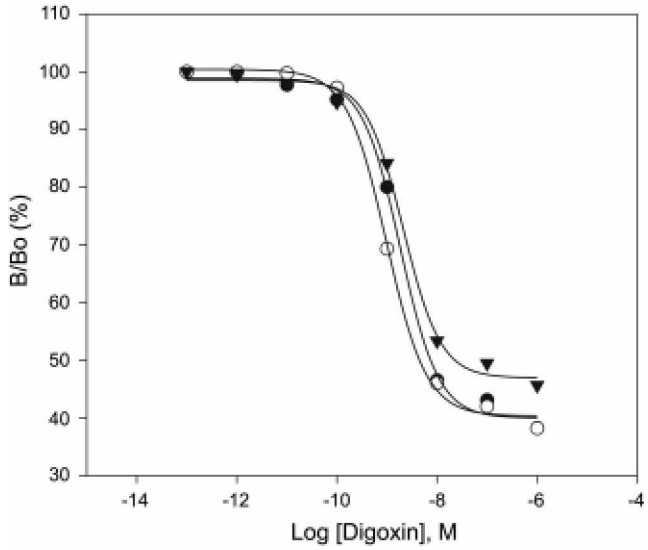

Figure 3. Dose-response curves for digoxin. Aequorin-digoxigenin conjugate, 10:1 was used. The amount of conjugate (M) and anti-digoxin antibody (μg/mL) were : A (●): 1.0×10^{-10}M and 1.8 μg/mL; B (○): 1.0×10^{-10}M and 0.9 μg/mL; C (▼): 5.0×10^{-10} M and 0.9 μg/mL. Data points are means of triplicate measurements.

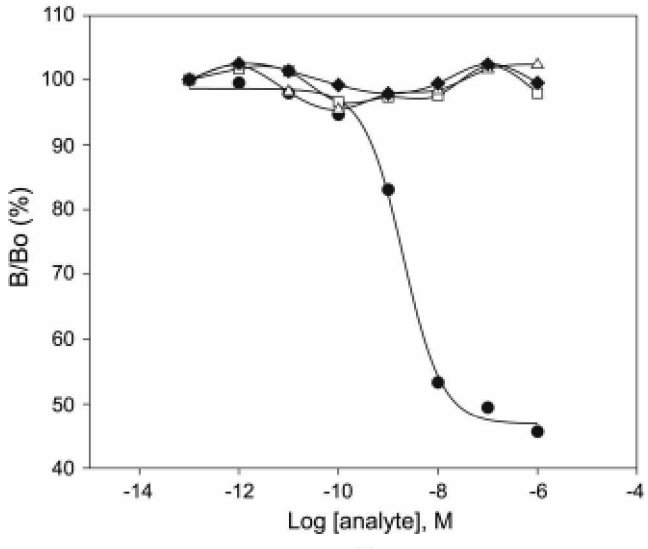

Figure 4. Cross-reactivity study for analyte. 5.0×10^{-10} M aequorin-digoxigenin conjugate, 1:10 and 0.9 μg/mL of anti-digoxin antibody were used. A. (●) Digoxin; B. (○) Digitoxigenin; C. (△) Ouabain; D. (◆) Spinolactone. Data points are means of triplicate measurements.

in competitive assay. In competitive assay a sequential mode takes the advantages of yielding a low detection limit compared to the equilibrium mode. A standard, digoxin solution was first incubated with an anti-digoxin antibody immobilized through avidin/biotin interaction on neutravidin-coated plate for 2 hr followed by washing step. And then aequorin-digoxigenin conjugate, 10 : 1 was added to this plate and incubated for additionally 2 hr followed by washing step again. The Ca^{2+}/Tris luminescence triggering solution was injected into this mixture through fixed volume injector and the resulting luminescence intensity was measured. Dose-response curves were constructed by plotting B/B_0 vs. digoxin concentration in the standards. Figure 3 shows the dose-response curves for heterogeneous digoxin assay by fixed amount of aequorin-digoxigenin, 10 : 1 conjugate and anti-digoxin antibody with varying concentration of digoxin. Among these three conditions examined, the resulting dose-response curve (Figure 3B) shows that the detection limit is 1.0×10^{-10} M and a dynamic range is three orders of magnitude, which was obtained by 1.0×10^{-10} M conjugate and 0.9 μg/mL anti-digoxin antibody. The detection limits were determined by measuring the signal at 3 times the standard deviation of the blank.

In drug assay with narrow therapeutic range such as digoxin assay, the specificity of the assay is very critical in assay development.[17] Specificity in immunoassay describes the ability of an antibody to yield a measurable response only for the target molecule and an important part of the evaluation of any immunoanalytical technique. Because many proteins have closely related structures with highly conserved epitopes. Thus, the presence of similar structure to the target molecule in the sample solution can reveal the serious errors in results if the assay has poor specificity. Three structurally similar molecules[18-20] to digoxin, of

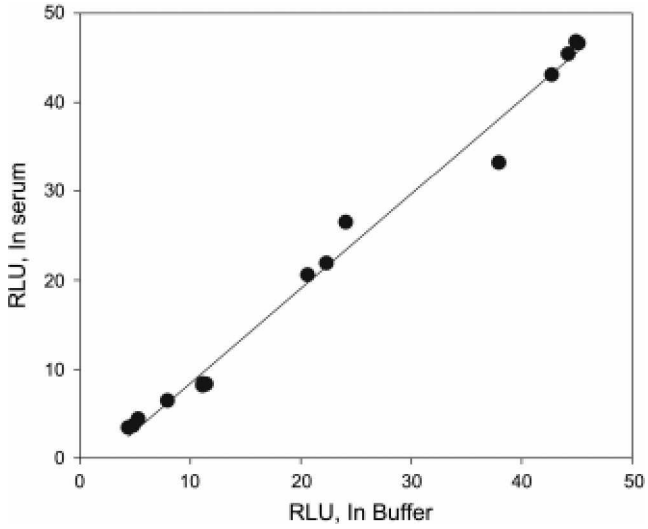

Figure 5. The correlation diagram of luminescence light intensity between in buffer and in serum: RLU (in serum) = 1.06 × RLU (in buffer) −2.19 (R^2=0.988).

digitoxigenin, quabain, and spironolactone were examined for their cross-reactivity in our immunoassay. Various concentrations of these compounds spiked to aliquots of serum in the absence of digoxin. The assay was conducted using 5.0×10^{-10} M aequorin-digoxigenin conjugate, 10 : 1 and 0.9 μg/mL anti-digoxin antibody, and the percent cross-reactivity was determined from the dose-response curve. Figure 4 represented that none of these three compounds showed any cross-reactivity with the digoxin antibody used.

In order to examine the effect of the serum matrix, standard amounts of digoxin corresponding to the therapeutic range were spiked into the each serum solution. Even though this condition isn't exactly same as the real patients samples, it can serve as a good model. Figure 5 shows the correlation

plot of the luminescence light intensity obtained both in buffer and in serum. Least-squares regression of this data gave the following relationship: RLU (in serum) = 1.06 × RLU (in buffer) − 2.19. (R^2 = 0.988). The correlation coefficient. R^2 serves as an index of the degree of agreement[21] and the value 0.988 shows strong agreement between them.

In conclusion, bioluminescence immunoassay using photoprotein, native aequorin as signal generator was examined for the first time in an attempt to develop a digoxin assay system employing anti-digoxin antibody and neutravidin-coated plate with site-directed immobilization based on avidin/biotin interaction. A detection limit for digoxin of 1.0×10^{-10} M was accomplished using this experimental condition. The structurally similar compounds, digitoxigenin, quabain, spironolactone were examined for their cross-reactivity. None of these three compounds showed any cross-reactivity. Additionally, Study of the serum matrix effect indicated that correlation coefficient shows good agreement between luminescence light intensity between in buffer and in serum.

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