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

Imunoassay 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 quantitative 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 (\(\lambda_{\text{max}} \sim 469\) 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 \([i.e., 0.8-2.0 \, \text{ng/mL} \text{ or } (1.0 \text{ to } 2.5) \times 10^{-9} \, \text{M}]\). 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)-amino methane (Tris) free base, ethylenediaminetetraacetic 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 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 4 M) was serially diluted in assay buffer as ranging in concentration from 1.0×10⁻6 M to 1.0×10⁻13 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. The plate was 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 1.0×10⁻¹³ 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, Spirocolactone 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 immunoassay systems. Several immunoassays have been developed for determining digoxin using enzymes and mutant aequorin as a label\textsuperscript{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 \times 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 \times 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/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 \times 10^{-9} M of aequorin-digoxigenin conjugate, 10:1. Data points are means of triplicate measurements.
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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 \(1.0 \times 10^{-10}\) M and a dynamic range is three orders of magnitude, which was obtained by \(1.0 \times 10^{-10}\) M conjugate and \(0.9\) \(\mu\)g/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 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 \(5.0 \times 10^{-10}\) M aequorin-digoxigenin conjugate, 10 : 1 and 0.9 \(\mu\)g/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 \( 1.0 \times 10^{-10} \) 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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References