

Cysteine-Free Mutant of Aequorin as a Photolabel in Immunoassay Development

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The bioluminescent protein aequorin is a sensitive label that has been employed in a number of analytical applications. A mutant of aequorin with enhanced stability produced recombinantly in our laboratory has been employed as a label in the development of an immunoassay for digoxin. Digoxin is a cardiac glycoside used in the treatment of congestive heart failure. This drug has a very narrow therapeutic range of 0.8–2.0 ng/mL (1.0–2.5 nmol/L), thus requiring therapeutic drug monitoring. In this study, a derivative of digoxigenin was chemically conjugated to the mutant aequorin, and the resulting protein–digoxigenin derivative conjugates were characterized in terms of their luminescence properties. A solid-phase immunoassay for digoxin was then developed. The detection limit of the assay for digoxin was 1×10^{-12} M. To demonstrate the use of this mutant aequorin as a label in biological sample analysis without any need for pretreatment of the samples, the assay was tested in serum spiked with digoxin. Interference from digoxin analogues was also evaluated to determine the specificity of the assay.

INTRODUCTION

Aequorin, a calcium-binding photoprotein, was originally isolated from the jellyfish *Aequorea victoria* in Friday Harbor, Washington (1). The holoprotein consists of apoaequorin (189 amino acid residues), a chromophore (coelenterazine), and molecular oxygen (2). The photoprotein is activated by calcium, which binds to the three EF-hand structures present within the protein molecule (3). These EF-hand structures are characteristic of the calcium-binding protein family. Upon addition of calcium, aequorin undergoes a conformational change leading to oxidation of bound coelenterazine to coelenteramide with the subsequent release of CO₂ and a flash of light ($\lambda_{\text{max}} = 469$ nm) (4).

Aequorin has been employed in a variety of analytical and biological applications. It has been used in the measurement of calcium in various intracellular compartments (5). Highly sensitive binding assays and immunoassays have been developed for peptidic and nonpeptidic analytes using aequorin as a label (6, 7). A proteolytic bond cleavage assay has also been developed using an aequorin fusion protein (8). Since aequorin can be detected down to attomole levels, it has been employed in the determination of biotin in single cells and in picoliter-volume vials (9–11). In addition, aequorin-labeled probes have been used as an alternative to radiolabels in nucleic acid hybridization assays (12, 13).

Several mutational studies have been performed on aequorin to elucidate the effects of various amino acids on the activity of the protein (14–16). Among them, cysteine-free mutants have shown to increase the protein's luminescence activity. The luminescence activity of the mutant was found to be higher (124%) when

compared to that of native protein (100%) (17, 18). Although the specific cause for the higher activity is not yet clear, it has been suggested that the cysteines present in the native protein are not essential in the generation of the luminescence activity (19). We have postulated that presence of disulfide bonds in the native protein may introduce conformational constraints in the protein structure leading to a reduction in the bioluminescence activity (19). In addition to an increased activity, the cysteine-free mutant does not need the addition of a reducing agent to prevent dimerization of the protein, thus increasing the stability of the protein.

The goal of this study was to investigate the performance of this cysteine-free mutant of aequorin constructed previously in our laboratory as a label in immunoassay development. Bioluminescence is a relatively rare phenomenon in nature; thus, when aequorin is used as a label in biological sample analysis it offers the advantage of producing a low background signal (3). Therefore, we decided to investigate the detection ability of this mutant of aequorin in the analysis of biological samples and without performing any pretreatment steps.

Digoxin was selected as a model analyte for the study. Digoxin is a cardiac glycoside used in the treatment of congestive heart failure and supraventricular arrhythmias (20, 21). It has a narrow therapeutic window of 0.8–2.0 ng/mL with a potential of fatal toxicity. Hence, it frequently needs therapeutic drug monitoring (TDM) (22).

For the purpose of this study, a derivative of digoxigenin was employed for its chemical conjugation to the lysine residues of the mutant aequorin. Several mutant aequorin–digoxigenin derivative conjugates with various mole ratios of digoxin to aequorin were prepared and were characterized in terms of their luminescence properties and binding ability to an anti-digoxin antibody. Dose–response curves were generated for digoxin in a sequential binding format. The optimized assay was used

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to perform serum analysis with spiked digoxin without any pretreatment of the samples. To determine the specificity of the immunoassay, the assay was also employed to determine the effect of structurally similar compounds to digoxin.

MATERIALS AND METHODS

Reagents. Tris(hydroxymethyl)aminomethane (Tris) free base, ethylenediaminetetraacetic acid (EDTA) sodium salt, glucose, digoxin, monoclonal biotinylated anti-digoxin antibody, digitoxin, bufalin, oleandrin, and human sera were purchased from Sigma (St. Louis, MO). Digoxigenin-3-*O*-methycarbonyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester (derivative of digoxigenin) was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Reactibind 96-well neutravidin-coated white polystyrene microtiter plates and biotin-free bovine serum albumin (BSA) were from Pierce (Rockford, IL). Microlite 2 polystyrene microtiter plates were purchased from Dynex (Chantilly, VA). The Bradford protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Coelenterazine was obtained from Biosynth International (Naperville, IL). Luria Bertani (LB) broth was from Gibco-BRL (Gaithersburg, MD). All solutions were prepared using deionized (Milli-Q Water Purification System, Millipore, Bedford, MA) distilled water. All chemicals were reagent grade or better and were used as received.

Apparatus. Ion-exchange chromatography was performed using a BioCAD-SPRINT perfusion chromatography system by Perseptive Biosystems (Cambridge, MA). Fractions containing the mutant aequorin protein were lyophilized using a VirTis Bench Top 3 freeze-dryer (Gardiner, NY). The purity of the aequorin protein was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a Phast System by Pharmacia Biotech (Uppsala, Sweden). Absorbance measurements were performed using a HP 8453 UV–Vis spectrophotometer from Hewlett-Packard (Waldbronn, Germany). Bioluminescence measurements were taken on a MLX microtiter plate luminometer from Dynex (Chantilly, VA) using a 100 μ L-fixed volume injector. All luminescence intensities reported are the average of a minimum of three replicates and have been corrected for the contribution of the blank.

Expression and Isolation of Mutant Aequorin. *Bacillus subtilis* (*B. subtilis*) cells containing plasmid pSD110 consisting of the gene for mutant apoequorin were grown in 250 mL of LB broth containing kanamycin (30 μ g/mL) for 16 to 18 h. The plasmid pSD110 consists of sequence for signal peptide fused to the gene for mutant apoequorin. Thus, the expressed protein was secreted out in the culture media. The culture was centrifuged at $13800 \times g$ for 30 min at 4 °C, and the supernatant was collected. The supernatant containing the mutant protein was filtered through 0.2 μ m membrane filter to remove any cell debris. The pH of the protein solution was then adjusted to 4.2 by adding glacial acetic acid in order to precipitate the protein. The precipitated protein was collected by centrifugation at $13800 \times g$ for 30 min at 4 °C.

Purification of the Mutant Protein. The precipitate containing the mutant protein was resuspended in 30 mM Tris-HCl, pH 7.0 containing 2 mM EDTA, and it was filtered using 0.2 μ m filter. The filtered protein was purified by perfusion anion exchange chromatography using a column consisting of a quarternized polyethyl-eneimine (HQ) as a stationary phase. A salt gradient

from 0.0 to 0.5 M NaCl was employed to elute the protein. Fractions containing the photoprotein were pooled together and lyophilized after adding glucose at a final concentration of 30 mM for protein stability. The lyophilized protein was resuspended in distilled deionized water and immediately dialyzed against 50 mM sodium bicarbonate buffer of pH 8.3 containing 4 mM EDTA to remove excess salt from the protein solution. The purity of the mutant protein was verified by SDS–PAGE using 12.5% polyacrylamide gels, which were developed by silver staining. The protein concentration was determined using the Bradford protein assay, with BSA as the standard.

Preparation of Mutant Apoequorin–Digoxigenin Derivative Conjugates. To prepare apoequorin–digoxigenin derivative conjugates, various amounts of derivative of digoxigenin were reacted with a fixed amount of mutant apoequorin. Five different mole ratios of digoxigenin derivative to mutant apoequorin, namely, 25/1, 50/1, 100/1, 250/1, and 500/1 were prepared. The digoxigenin derivative in anhydrous dimethyl sulfoxide was added to a solution of mutant apoequorin in 50 mM sodium bicarbonate, pH 8.3, containing 4 mM EDTA. The reaction was allowed to proceed for 4 h at 4 °C while stirring. After that, a 5-fold molar excess of coelenterazine was added to the reaction mixture, and the solution was incubated for 15 h at 4 °C. After the conjugation reaction was completed, an excess of unconjugated analyte and an excess of coelenterazine were removed by passing the mixture through a Sephadex G-25 size-exclusion column. The concentration of each conjugate was determined using a standard Bradford assay.

Bioluminescence Emission Study. A volume of 100 μ L of various conjugates at a concentration of 1.0×10^{-7} M were added to a Microlite 2 polystyrene microtiter plate, and the bioluminescence was measured by injecting 100 μ L of luminescence triggering buffer (100 mM Tris-HCl, 100 mM CaCl₂, pH 7.5). The bioluminescence signal was collected at 0.1-s interval over a 3-s time period.

Calibration Plot of Mutant Aequorin–Digoxigenin Derivative Conjugates. A stock solution of conjugates (8.0×10^{-7} M) were serially diluted in assay buffer (30 mM Tris-HCl, 4 mM EDTA, 150 mM NaCl, pH 7.5, containing 0.1% (w/v) biotin-free BSA) and the bioluminescence intensity of 100 μ L of each dilution was measured after injecting 100 μ L of luminescence-triggering buffer.

Binder–Dilution Study. A biotinylated anti-digoxin antibody of differing concentrations were prepared in the dilution buffer (100 mM sodium phosphate, pH 7.4, containing 150 mM NaCl and 0.1% (w/v) biotin-free BSA) and 100 μ L of each dilution was immobilized on a neutravidin coated plate with constant shaking for 2 h at room temperature. The plate was then washed three times with wash buffer (30 mM Tris-HCl, 4 mM EDTA, 150 mM NaCl, pH 7.5 containing 0.1% (w/v) biotin-free BSA). Digoxigenin derivative–aequorin conjugate, 50/1 (100 μ L, 1×10^{-9} M), in assay buffer was then added to the plate containing immobilized antibody. The plate was incubated for an additional 2 h with shaking at room temperature. The plate was then washed three times with the wash buffer, and the bioluminescence intensity was measured by injecting 100 μ L of the luminescence-triggering buffer.

Association–Time Study. For this study, a biotinylated anti-digoxin antibody (100 μ L, 2.0 μ g/mL) was immobilized on the neutravidin-coated wells of the microtiter plate, as described previously. Next, a volume of

100 μL of the conjugate, 50/1, of concentration 1×10^{-9} M was added to the plate, and the plate was incubated for various time periods with shaking. The plate was then washed three times with the wash buffer, and the luminescence intensity was measured in the presence of 100 μL of luminescence-triggering buffer.

Dose-Response Curves for Digoxin. A stock solution of digoxin of a concentration 1×10^{-3} M was serially diluted in dilution buffer. For the assay, 100 μL of digoxin ranging in concentration from 1×10^{-5} M to 1×10^{-13} M were incubated with immobilized biotinylated anti-digoxin antibody (2 $\mu\text{g/mL}$) for 2 h at room temperature with shaking. Following this incubation period, the plate was washed three times with wash buffer. A volume of 100 μL of conjugate, 50/1, of concentration 1×10^{-9} M was then added and allowed to incubate with shaking for an additional 2 h at room temperature. After a washing step, the luminescence intensity was measured as described previously. Dose-response curves were also generated in a similar manner for the 25/1 digoxigenin derivative-aequorin conjugate with differing anti-digoxin antibody concentrations. For this, a volume of a 100 μL of a protein conjugate of 5×10^{-11} M concentration was employed, along with 0.5 and 2.0 $\mu\text{g/mL}$ of anti-digoxin antibody, respectively.

Determination of Degree of Conjugation. The degree of conjugation for each conjugate was determined by a simultaneous spectrophotometric assay for two component system by following a previously reported protocol (23). For that, absorbance measurement was done at two different wavelengths ($\lambda_1 = 265$ nm and $\lambda_2 = 278$ nm). Following equations were used to calculate the concentration of the digoxin

$$A_{\lambda 1} = a_{x\lambda 1}bc_x + a_{y\lambda 1}bc_y$$

$$A_{\lambda 2} = a_{x\lambda 2}bc_x + a_{y\lambda 2}bc_y$$

where, $A_{\lambda 1}$ = absorbance at λ_1 , b = path length = 1, x = component 1, y = component 2, $a_{x\lambda 1}$ = molar absorptivity of compound 1 at λ_1 , c_x = concentration of compound 1.

Recovery Study of Digoxin in Serum. A volume of 100 μL of serum samples were spiked with standard amounts of digoxin. Next, 100 μL of these samples was incubated with immobilized biotinylated anti-digoxin antibody, for 2 h at room temperature followed by a washing step. A volume of 100 μL of the conjugate (25/1) of 5×10^{-10} M concentration was then added to the plate and incubated for 2 h. After a washing step, the luminescence intensity was measured as described previously. Percent recoveries of digoxin in serum samples were determined from the dose-response curve.

Cross-Reactivity Study. A cross-reactivity study was performed in the same way as the studies conducted for digoxin in serum. Various concentrations of compounds structurally similar to digoxin such as bufalin, oleandrin, and digitoxin were spiked in the serum, and the percent cross-reactivity was determined from the dose-response curve generated for digoxin.

RESULTS AND DISCUSSION

Luminescent proteins have become popular reporters due to their ease of handling and inherent sensitivity. Aequorin is one such luminescent protein that has been used as a highly sensitive label in analytical assay systems. Native aequorin contains three cysteine residues in its primary structure, which necessitates the presence of disulfide reducing agents at all times to prevent dimerization of the protein leading to loss in its activity

Table 1. Characteristics of Aequorin-Digoxin Conjugates.

initial ratio (aequorin:digoxigenin derivative)	degree of conjugation	detection limit (M)	half-life (s)
cysteine-free aequorin	N/A	7.0×10^{-12}	1.22
25/1	1.1	8.0×10^{-12}	1.30
50/1	2.0	5.0×10^{-11}	1.17
100/1	4.0	2.0×10^{-10}	1.27
250/1	4.0	4.0×10^{-10}	1.22
500/1	4.1	1.0×10^{-9}	1.22

(14). Furthermore, the presence of reducing agents can also have a negative effect on the binding capacity of antibodies as they consist of disulfide bonds linking their heavy chains with light chains that together form the binding site for antigens. We have demonstrated that a cysteine-free mutant of aequorin constructed using genetic engineering tools can overcome these problems associated with the native protein. Moreover, this cysteine-free mutant protein has been found to be more active than the wild-type protein (17).

In this study, the performance of this mutant aequorin as a label in the development of an immunoassay for the determination of digoxin in biological samples was investigated. As mentioned earlier, patients taking digoxin need to be monitored frequently because of the narrow therapeutic range of this drug. Thus, assays for digoxin that are highly sensitive, rapid, and selective are required. Several immunoassays have been developed for analyzing digoxin and are commercially available. For example, Vitros digoxin immunoassay by Johnson & Johnson Clinical Diagnostics is based on competition between the digoxin and digoxin-peroxidase conjugate for binding to anti-digoxin antibody (22). AxSYM Digoxin II is an automated immunoassay for digoxin developed by Abbott Laboratories which uses an alkaline phosphatase conjugate of digoxin in a heterogeneous format (22).

The mutant aequorin can be produced using recombinant DNA technique in a highly reproducible manner and in unlimited amounts, thus reducing the reagent cost (1). For that, the cysteine-free mutant aequorin was expressed in *B. subtilis* using plasmid pSD110 constructed in our laboratory (6). The expressed protein was collected from the culture media by acid precipitation. The crude protein was purified using perfusion anion-exchange chromatography, and the concentration of the protein was determined by Bradford assay. The yield of the protein was found to be 8.38 mg of protein per liter of culture.

Several aequorin-digoxigenin derivative conjugates were prepared by reacting an excess of an *N*-hydroxysuccinimide ester derivative of digoxigenin at various mole ratios with the lysine residues of mutant aequorin as described in the Experimental Section. All the conjugates were characterized in terms of their luminescence properties, detection limits, and half-lives, as shown in Table 1. The detection limit for each conjugate was determined using calibration curves generated by employing varying amounts of conjugates. As it can be seen in Table 1, the detection limit increased with the increasing molar ratios of digoxigenin derivative to aequorin. This can be explained by the fact that an increased molar ratio of digoxigenin derivative to aequorin in the conjugation reaction results in the conjugates with a higher number of digoxigenin derivative bound to the protein. Some of these digoxigenin derivative can bind to the active site of the protein affecting the protein activity, and consequently providing a poor detection limit. However, con-

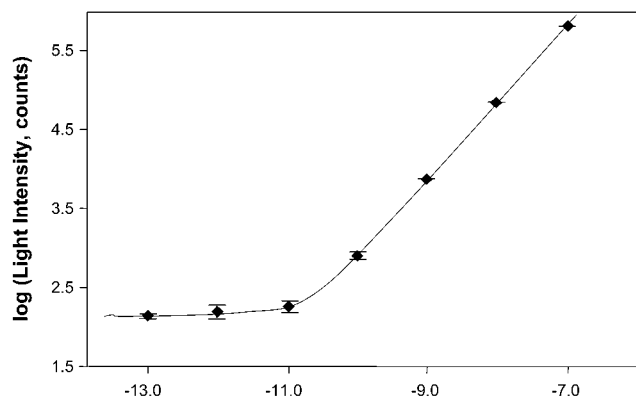


Figure 1. Calibration curve study of the 50/1 aequorin–digoxigenin derivative conjugate. Data are the average plus \pm one standard deviation ($n = 3$).

jugates containing approximately one digoxigenin derivative per aequorin molecule were as active as unmodified aequorin. The bioluminescence half-lives of all the conjugates were found to be comparable to the half-life of nonconjugated mutant aequorin. Performance of the conjugates was also evaluated in terms of change in the luminescence activity of aequorin–digoxigenin derivative conjugate in a solution phase in the presence of anti-digoxin antibody. None of the conjugates exhibited any homogeneous change in bioluminescence signal, indicating that the binding event between aequorin-labeled analyte and antibody does not have any effect on the activity of the protein. On the basis of the results obtained above, we decided to use an digoxigenin derivative–aequorin conjugate, 50/1, for further studies. The conjugate 50/1 showed good bioluminescence activity with a low detection limit. The conjugate 25/1 provided a better detection limit than the conjugate 50/1; however, the probability of attachment of digoxigenin derivative to aequorin in a conjugation reaction is higher for the molar ratio of 50/1 of aequorin to digoxin.

A concentration of 1×10^{-9} M of the conjugate 50/1 was selected from the calibration study for further studies as this is a relatively low concentration of protein capable of generating a signal that is well above the background. A typical calibration curve for 50/1 conjugate is presented in Figure 1.

The next step in the study was to determine the amount of antibody to be used in subsequent experiments. For that purpose, a binder–dilution curve was generated (see Figure 2). The biotinylated anti-digoxin antibody was immobilized on a neutravidin-coated plate by taking advantage of the strong binding affinity of neutravidin for biotin. A concentration of antibody of 2 $\mu\text{g/mL}$ was selected from the linear portion of the binder–dilution curve for further studies. This concentration of antibody was chosen in order to achieve a low detection limit while keeping a high signal.

To reduce the total assay time, the optimum time required for binding of the aequorin–digoxigenin derivative conjugate to the antibody was determined by generating an association curve. The association study shown in Figure 3 was performed in the same manner as the binder–dilution study, except that the incubation time for binding the mutant aequorin–digoxigenin derivative conjugate to the antibody was varied. An association time of 2 h was selected since there is no substantial increase in binding after 2 h of incubation.

After evaluating various parameters, such as optimum amount of conjugate, antibody, and time, a dose–

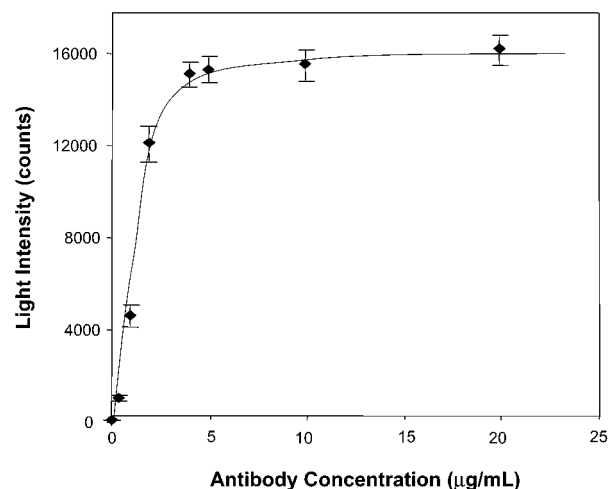


Figure 2. Binder–dilution study conducted using a 100 μL of 1×10^{-9} M 50/1 conjugate with 100 μL of the varying anti-digoxin antibody concentrations. Data are the average plus \pm one standard deviation ($n = 3$).

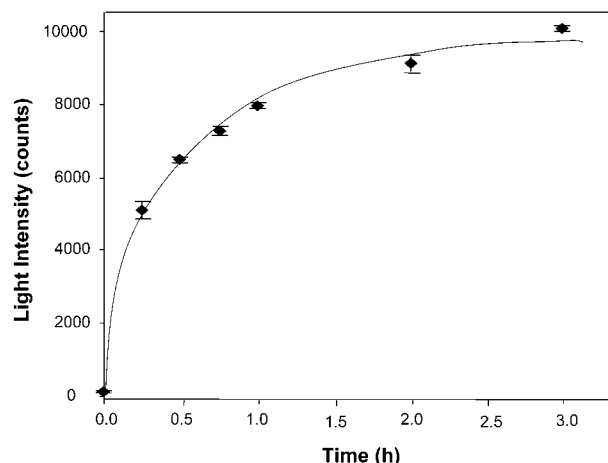


Figure 3. Association time study performed using 100 μL of 1×10^{-9} M 50/1 conjugate, and 100 μL of 2 $\mu\text{g/mL}$ anti-digoxin antibody varying the incubation time period. Data are the average plus \pm one standard deviation ($n = 3$).

response curve was generated for digoxin using the conjugate 50/1 in a sequential binding mode. The sequential binding mode has the advantage of producing a lower detection limit compared to the competitive binding mode. When the dose–response curve was generated using the competitive binding mode, a detection limit of 1×10^{-9} M was obtained (data not shown). To generate a dose–response curve in sequential binding format, various concentrations of free digoxin were incubated with the immobilized antibody followed by a washing step. Next, the conjugate was added and the luminescence intensity was measured. The dose–response curve obtained is shown in Figure 4. The study indicated that as expected the luminescence intensity was lower at higher concentrations of free digoxin and that the intensity of the signal increased with decreasing amounts of the digoxin. From this curve, a working range of 4 orders of magnitude was obtained and the detection limit was determined to be 3×10^{-10} M ($S/N = 3$).

The degree of conjugation was determined for all the conjugates prepared using a spectrophotometric method reported previously (Table 1) (23). The results showed that the conjugate 25/1 has 1.1 digoxigenin derivatives per aequorin molecule. Conjugate 50/1 resulted in 2.0 digoxigenin derivatives per aequorin. The other conju-

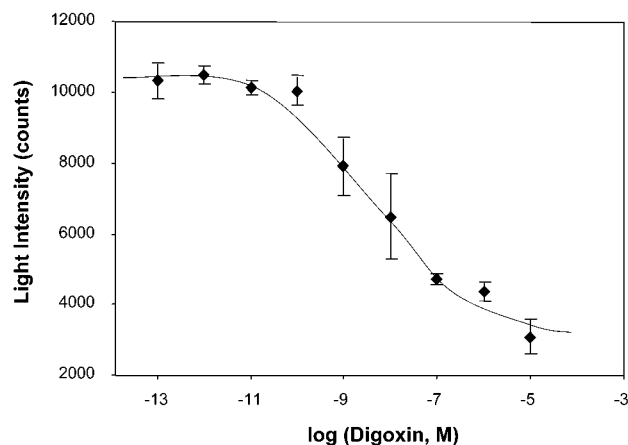


Figure 4. Dose-response curve generated in a sequential binding mode using 100 μL of 1×10^{-9} M 50/1 conjugate and 100 μL of 2 $\mu\text{g/mL}$ anti-digoxin antibody. A stock solution of digoxin was serially diluted and used in the concentration range from 1×10^{-5} to 1×10^{-13} M. Data are the average plus \pm one standard deviation ($n = 3$).

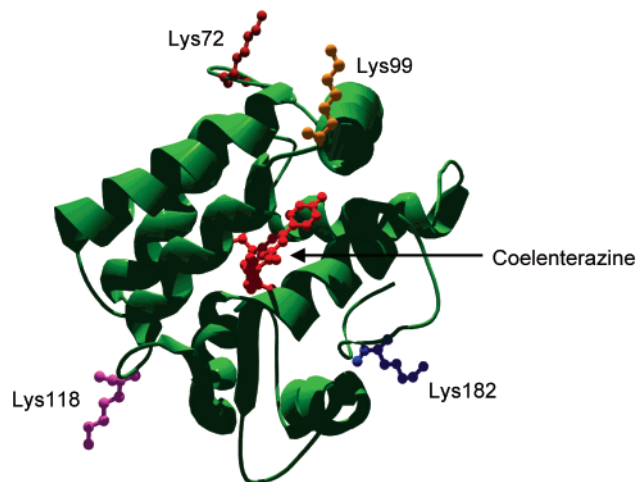


Figure 5. The three-dimensional X-ray crystal structure of aequorin showing the lysine residues 72, 99, 118, and 182. The figure was prepared using Swiss-PdbViewer (24) and rendered using POVray (32).

gates, 100/1, 250/1, and 500/1 had a degree of conjugation of 4.0, 4.0, and 4.1 digoxigenin derivatives per aequorin, respectively. Given these results, it appears that a maximum of four lysine molecules are being conjugated with the digoxigenin derivative employed. An examination of the X-ray crystal structure of aequorin using Swiss-PdbViewer (24) revealed that out of the fourteen lysines in the protein, Lys118 and Lys99 are the most accessible to the solvent with $\geq 62\%$ and 52% accessible surface, respectively (Figure 5). The residues Lys72, Lys182, and Lys88 are also exposed to the solvent but to a lesser degree. The residue Lys72 has $\geq 43\%$ accessible surface and residues Lys182 and Lys88 have $\geq 41\%$ accessible surface. Among other lysines, Lys17 and Lys56 have 35% and 31% accessible surface, respectively. The remaining lysine residues, Lys59, 30, 39, 87, 96, 130, and 134 have less than 25% accessible surfaces. We also examined the crystal structure using Swiss-PdbViewer for the possibility of lysine residues forming ionic bonds with nearby aspartate or glutamate residues. We found that Lys30, 56, and 88 are within 3 \AA from Glu76, Asp60, and Asp92, respectively. These lysine residues may form ionic bonds and thus may not be available for conjugation. Therefore, on the basis of our conjugation results

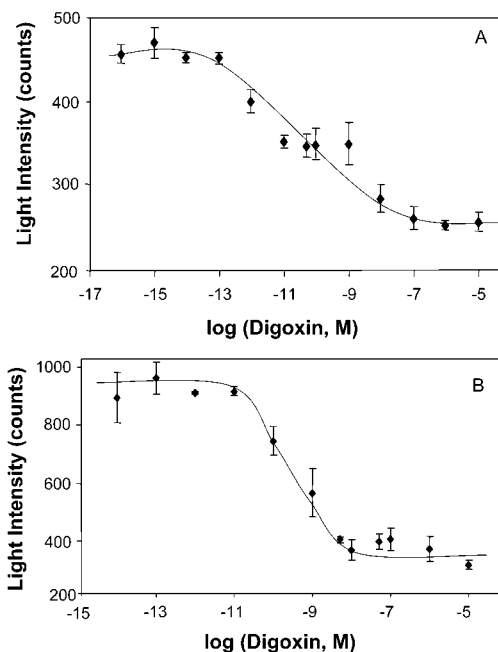


Figure 6. Dose-response curves for digoxin generated using 100 μL of 5×10^{-11} M 25/1 aequorin-digoxigenin derivative conjugate with 100 μL of (A) 0.5 $\mu\text{g/mL}$ and (B) 2 $\mu\text{g/mL}$ anti-digoxin antibody. Data are the average plus \pm one standard deviation ($n = 3$).

as well as on the X-ray crystal structure analysis, we postulate that Lys118 and Lys99 are the ones that are most likely conjugated with digoxigenin derivative followed by Lys72 and Lys182.

Since better detection limits will be achieved from the system with only one analyte conjugated per label, digoxigenin derivative-aequorin conjugate 25/1 would provide better detection limit than the conjugate 50/1 (25). Thus, dose-response curves were also generated using conjugate 25/1. Two different concentrations of anti-digoxin antibody, 0.5 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$, were employed in the study (Figure 6). When the concentration of anti-digoxin antibody was increased from 0.5 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$, the curve became steeper, and as expected the detection limit for digoxin worsened. This is because as the amount of binder is increased, more free-analyte is required to compete with the labeled analyte. A detection limit of 1.0×10^{-12} M was obtained for digoxin using 0.5 $\mu\text{g/mL}$ of antibody (Figure 6A). The dose-response curve generated using 2 $\mu\text{g/mL}$ of anti-digoxin antibody has a dynamic range of 3 orders of magnitude and a detection limit for digoxin of 1.0×10^{-11} M (Figure 6B). The detection limits were calculated by measuring the signal at three times the standard deviation of the blank. The sensitivity of the assay when calculated in terms of standard deviation at zero dose (σ) (26) showed that a lower value of σ was obtained using 2 $\mu\text{g/mL}$ antibody as compared to 0.5 $\mu\text{g/mL}$ of the antibody. Thus, the system is more sensitive when 2 $\mu\text{g/mL}$ of antibody is employed in the study. In general, a better detection limit for digoxin was obtained employing the conjugate 25/1 than when the conjugate 50/1 was used.

The assay developed was employed in the analysis of digoxin spiked in serum samples to determine the effect of the serum matrix on the detection of aequorin. Although this study is not equivalent to real patients sample analysis, it can serve as a good model. It has been found that myocardial digoxin concentration reaches steady state with serum digoxin concentration after several hours of the oral dose (20). Thus, digoxin is

Table 2. Percent Recovery of Digoxin in Serum.

spiked concentrations (ng/mL)	percent recovery ^a
0.5	116.7 ± 0.4
0.8	101.5 ± 2.4
1.5	108.2 ± 0.5
2.2	105.1 ± 1.8

^a Percent recovery is calculated as follows: (concentration obtained from immunoassay)/(spiked concentration) × 100%.

routinely monitored in the serum of the patients. The advantage of using aequorin as a label in serum analysis is that the samples do not need any pretreatment due to the absence of background signal. Before performing the serum sample analysis, we investigated the effect of serum on aequorin activity by incubating the aequorin–digoxigenin derivative conjugates in both buffer and in serum. No effect of the serum matrix was observed on the activity of conjugates (data not shown). To mimic digoxin in the real sample, standard amounts of digoxin corresponding to the therapeutic range were spiked into the serum aliquots and employed in the study. Serum sample analyses were performed using the conjugate 25/1. The percent recoveries of digoxin in serum samples were determined from the dose–response curve generated for digoxin standards (Table 2). The results indicated that the matrix in the serum has very little effect on the assay.

In the development of an immunoassay, the specificity of the assay is very important, especially in assays for drugs with narrow therapeutic range. The presence of compounds similar in structure to the target analyte in the sample can provide with erroneous results if the assay is not selective. Since the therapeutic range of digoxin is narrow, its accurate determination in the presence of interferents is essential (27). There are several other drugs used in this cardiac treatment that have the structures similar to digoxin. For example, digitoxin, a structurally related cardiac glycoside to digoxin, has been found to decrease the apparent digoxin concentration in the sample (28, 29). Oleandrin, another cardiac glycoside, present in the oleander plant and used in low doses to treat heart diseases competes with digoxin for the binding to the antibody (28, 30). Bufalin is a cardioactive compound found in the venom of the Chinese toad, and it is also known to interfere in the assessment of digoxin concentration (28, 31). These three compounds were tested for their cross-reactivity in our immunoassay. Aliquots of serum were spiked with various concentrations of these compounds in the absence of digoxin. Serum samples were spiked with the therapeutic amount of digitoxin and bufalin. Oleandrin was used in higher concentration than the other two as the micromolar concentration of oleandrin in serum has been found to provide apparent digoxin concentration in nanomolar range. The assay was conducted using aequorin–digoxin conjugate 1:25, and the percent cross-reactivities were determined from the dose–response curve generated for digoxin. None of the three compounds showed any significant cross-reactivity with the digoxin antibody (Table 3).

Stability studies were also performed for the conjugates in terms of retention of the luminescence activity of aequorin over time. All the conjugates were found to be stable for at least four months when stored in liquid form in Tris buffer (30 mM Tris-HCl pH 7.5 containing 2 mM EDTA) at 4 °C.

Table 3. Cross-Reactivity Study.

compounds added in serum	spiked concentrations (ng/mL)	percent cross-reactivity ^a
digitoxin	10.0	3.2 ± 1.3
	50.1	0.7 ± 0.1
	100.2	0.2 ± 0.1
bufalin	10.0	1.1 ± 1.4
	50.3	0.2 ± 0.1
	100.1	0.1 ± 0.1
oleandrin	9976.9	2.5 × 10 ⁻³ ± 1.9 × 10 ⁻³
	49884.6	6.4 × 10 ⁻⁴ ± 1.0 × 10 ⁻⁴
	99769.1	3.3 × 10 ⁻⁴ ± 2.0 × 10 ⁻⁴

^a Percent cross-reactivity is calculated as follows: (Concentration obtained from immunoassay)/(Spiked concentration) × 100%.

In conclusion, a highly sensitive assay for digoxin was developed using a cysteine-free mutant of aequorin. We have demonstrated that a cysteine-free mutant of aequorin offers an excellent alternative to native aequorin to use as a label in the development of immunoassays where cysteines in the native protein are undesirable. This mutant of aequorin was found to be as sensitive as the native aequorin. Its performance in the development of immunoassay for digoxin has shown that this mutant can be used as a label in any immunoassays that require sensitive detection and therapeutic drug monitoring. The conjugates prepared retained their bioluminescence activity and had a long shelf life. This study also showed that the mutant aequorin can be effectively used in the assays requiring real sample analysis, and more importantly, no pretreatment of the samples is required, indicating minimal matrix effect. A detection limit for digoxin of 1 × 10⁻¹² M (femtomoles of drug in the sample) was achieved using this cysteine-free mutant of aequorin. Furthermore, no significant cross-reactivity was observed from structurally similar compounds to digoxin.

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