Labeling Digoxin Antibody with Colloidal Gold and Ferrocene for Its Use in a Membrane Immunostrip and Immunosensor

Myung Ja Choi,* So Young Kim,* Jeongeun Choi,* and Insook Rhee Paeng†

*Bioanalysis & Biotransformation Research Center, Korea Institute of Science and Technology, P.O. Box 131 Cheongryang, Seoul 130–650, Korea; and †Department of Chemistry, Seoul Women’s University, Seoul 139–774, Korea

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Current trends in the development of on-site diagnostic tests using immunodetection principles are proceeding in two directions: toward the immunochromatography test strip and toward the immunosensor. To develop both applications for the detection of digoxin, we prepared multilabeled antibody conjugates using colloidal gold and ferrocene. Prior to applying the digoxin antibody in immunochromatography, we constructed a dose–response curve with the digoxin antibody by enzyme immunoassay under optimized conditions. From the results, we found it was possible to detect as little as $10^{-2} \mu g/ml (1.28 \times 10^{-11} M)$ digoxin, which is within the therapeutic and toxic ranges of digoxin.

First, this antibody was labeled with ferrocene as a sensor signal generator. As the ferrocene moiety increased, electroactivity also increased, but immunoreactivity between digoxin and its antibody was constant as ascertained from the molar ratio of its conjugate which was up to 1:35. And the immunoreactivity of the conjugate with molar ratio 1:125 dropped by 50%. Thus, after determining the optimum molar ratio (1:35) of antibody to ferrocene, this ferrocene-conjugated antibody was labeled with colloidal gold for the visual recognition of color in the strip. This immunostrip could detect a digoxin level of $1 \mu g/ml (1.28 \times 10^{-10} M)$ within 2 min.

Key Words: digoxin; enzyme immunoassay; immunochromatography; immunosensor.

INTRODUCTION

Digoxin is a cardiac glycoside that is widely used in the treatment of congestive heart failure and various disturbances of cardiac rhythm. The use of digoxin in patients with congestive heart failure is based on its ability to increase myocardial contractions, thereby increasing the blood output of a failing heart. The clinical use of digoxin as a therapeutic agent is complicated by the fact that individual patients vary considerably both in the dosage required to produce a beneficial therapeutic response and in their sensitivity to the toxic effects of digoxin (1). Accordingly, it is necessary to individually monitor digoxin levels in serum.

The ability to perform such diagnostic tests at a location remote from the laboratory would be highly desirable for speed and economy. Immunochromatographic detection techniques are capable of greater discrimination between chemical structures, so they are often used as the analytical tool (2). One kind of immunochromatographic technique, the on-site test strip, is not dependent on instrumentation, but it has been used widely for many qualitative and semiquantitative assays, for example, pregnancy tests for home use. This is because the test strip labeling substances, such as highly colored colloidal gold particles, generate a change in color density, in accordance with the analyte (3).

1 To whom correspondence should be addressed.
The use of polyclonal antidigoxin antibody–ferrocene conjugate makes it possible to transduce antigen–antibody recognition directly into a sensor signal. And it would facilitate a convenient and relatively inexpensive approach to obtain an analytical signal (4, 5). Recently, these detection methods in immunoassay have been gaining interest, because they have many advantages such as safety, simplicity, easy handling, economy, and high sensitivity (4, 6–8). As immunosensor technology has been attracting interest, personal monitoring technology progresses into two directions: toward the development of an on-site test strip that uses visual identification and a test strip that uses a screen-printed sensor device.

In the present investigation, we confirmed the redox properties of various antibody–ferrocene conjugates by cyclic voltammetry. These conjugates were applied to a heterogeneous enzyme immunoassay to select an antibody–ferrocene conjugate that showed a good reactivity both in immunoreactivity and electroactivity.

In this paper, we describe how we prepared multilabeled antibodies using labels of colloidal gold and ferrocene and how we developed digoxin enzyme immunoassay and immunochromatography detection procedures.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Digoxin, bovin serum albumin (BSA) used to prepare the buffer solution, sodium periodate, sodium borohydride, ethylene glycol, o-phenylenediamine (OPD), ferrocene-acetic acid, tributylamine, and isobutyl chloroformate were purchased from Sigma Chemical Company (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat immunoglobulin G (IgG) fraction to rabbit IgG (whole molecule) and rabbit antidigoxin serum were purchased from Cappel and Biostride Inc., respectively. Digoxin antibody was purified using a CNBr–Sepharose affinity column. Proteins [BSA, keyhole limpet hemocyanin (KLH) and ovalbumin (OVA)] used to prepare digoxin–protein conjugates were purchased from Pierce (Rockford, IL) and colloidal gold was from British BioCell International (Cardiff, England). All chemicals used were of analytical grade, and the solutions were made in deionized water using the Milli-Q water purification system (Millipore, Bedford, MA).

A microwell module (Maxisorp) was purchased from Nunc (Denmark) and a kinetic microplate reader (Molecular Devices) was used to measure the optical density of the ELISA results.

**Preparation of Digoxin–Protein Conjugates**

Ten milligrams of digoxin (0.0128 mmol) was suspended in 1.3 ml of 95% ethanol. To the digoxin suspension, 0.7 ml of 0.05 M NaIO₄ was added dropwise over 2 min. After stirring for 30 min, 0.26 ml of 1 M ethylene glycol was added to oxidized digoxin solution. The mixture was stirred continuously for 5 min. Then 1.3 ml of BSA solution (15 mg/ml in 0.1 M sodium bicarbonate, pH 8.2) was added dropwise into the above mixture, maintaining the pH in the range 9.3–9.5 with 1 M Na₂CO₃ (2 drops). Stirring was continued for 1 h at room temperature. Seven-tenths milliliter of 0.4 M NaBH₄ solution was added and stirred for 30 min at 4°C. The reaction mixture was dialyzed against 0.1 M carbonate buffer, pH 9.3 (I).
Dose–Response Curves for Digoxin

Prior to plotting the dose–response curve, we determined the antibody concentration yielding a response of OD 2.0 at 490 nm (9). First, microtiter plates were coated with 100 µl of digoxin–BSA in 50 mM carbonate buffer, pH 9.6, for 30 min at room temperature. The wells were blocked with 150 µl of 3% BSA in PBS for 30 min and incubated with 100 µl of serially diluted digoxin-specific antibodies (rabbit antidigoxin) for 30 min. Goat anti-rabbit IgG–HRP (7.25 µg/ml) was added to each well and incubated for 30 min. The plate was washed three times with 250 µl of PBS/0.05% Tween-20 after every incubation step. The color reaction was stopped with 50 µl of 2 N H₂SO₄ and the optical density was read at 490 nm.

Based on the digoxin antibody titer level, a dose–response curve for digoxin was constructed. Using the above protocol, 50 µl of free digoxin and 50 µl of digoxin antibody were added to the plate well which was adsorbed digoxin–BSA, instead of serially diluted antibody, and the other assay protocols were the same. The standard curve was constructed by plotting the relative response (%) to the response at zero concentration of digoxin.

Preparation of Ferrocenylated Antibody

The activation of 5 mg of ferroceneacetic acid dissolved in 100 µl of dry dimethylformamide was started by adding 20 µl of tributylamine, followed 15 min later by 50 µl of isobutyl chloroformate. After 15 min of reaction at −10°C and 30 min at room temperature, the activated ferrocene derivative was added to the antibody in 50 mM phosphate buffer, pH 7.4. The coupling was completed after incubation for 2 h at room temperature. Insoluble particles were removed by centrifugation. The reaction mixture was dialyzed against phosphate buffer, pH 7.4 (12, 13).

Cyclic Voltammetry

For cyclic voltammetry, a conventional three-electrode electrochemical cell was used. The electrochemical cell was constructed of glass (100 µl of working volume) and fitted with a water jacket to control the temperature of the cell contents. Cyclic voltammograms (CVs) of antibody–ferrocene conjugates of various molar ratios were obtained using a potentiostate (CV-50W, BAS, West Lafayette, IN) interfaced to a personal computer with software supplied by the manufacturer. A carbon paste microelectrode, a platinum counter electrode, and a saturated calomel reference electrode (SCE) were used in the electrochemical cell. Measurements were carried out at 25°C under anaerobic conditions and the scanning rate for the CV of the cell was 100 mV s⁻¹.

Preparation of Ferrocenylated Antibody–Gold Conjugate

To have strong adsorption between the gold and antibody–ferrocene conjugate, a preliminary titration was performed. One milliliter of gold colloid adjusted to pH 9.0 was pipetted into a series of tubes. The antibody–ferrocene conjugate adjusted to pH 9.2 was added to each colloidal gold solution in a series from 0 to 150 µl. Each tube was made up to 1.15 ml with 2 mM borax and shaken for 5 min. One hundred microliters of 10% NaCl was added and agitated for 1 min. The tube containing the minimum amount of antibody–ferrocene conjugate required to stabilize the colloidal gold was the one in which
the color of the colloidal gold did not change from red to blue. This determined the amount of antibody–ferrocene conjugate that was added to 1 ml of gold sol and agitated for 1 h. Ten percent BSA was added to the multilabeled solution up to 1% BSA solution. This multilabeled solution using colloidal gold and ferrocene was agitated for 30 min and centrifuged to remove the supernatant.

**Preparation of the Immunochromatography Strip**

The sample pad was treated with 50 mM borate buffer, pH 7.4, containing 1% BSA, 0.5% Tween-20, and 0.05% sodium azide, and dried at 60°C. Digoxin–BSA (0.038 mg/ml) and goat anti-rabbit antibody (1.123 mg/ml) were applied to the nitrocellulose membrane to the test and control lines, respectively, and dried at 35°C. An absorption pad was used without treatment. The multilabeled digoxin antibody with ferrocene and colloidal gold was applied to an untreated glass-fiber membrane and completely dried at 35°C. The nitrocellulose membrane, absorption pad, glass fiber membrane, and pretreated sample pad were assembled as the strip and attached to a sticker. A digoxin sample (400 μl) was pipetted into the reaction holder to evaluate the results.

**RESULTS AND DISCUSSION**

Prior to constructing the dose–response curve for digoxin, we employed the titration curves of digoxin antibody and its titer level was 0.03 μg/ml with 1.0 μg/ml of absorbed digoxin–BSA on the plate by ELISA. It is customary to perform a digoxin immunogen dilution study to determine the concentration of digoxin immunogen needed to maximize sensitivity in the immunoassay (10, 11). Under optimized conditions where each reaction was allowed to reach equilibrium during incubation, we differentiated digoxin immunogen amounts with a fixed amount of digoxin antibody. In the dilution study, we found that 1.0 μg/ml digoxin–OVA gave the best sensitivity for detecting low concentrations of digoxin. Based on the digoxin immunogen dilution study, we varied the amount of enzyme conjugate from 1.8 to 14.5 μg/ml at 1.0 μg/ml digoxin–OVA coated on the wells. However, the dose–response curves obtained from the goat anti-rabbit IgG–HRP conjugate dilution study made no difference in the sensitivity of the assay system. Thus, the dose–response curve with the best detection capabilities was established with 3.63 μg/ml goat anti-rabbit IgG–HRP and 1.0 μg/ml digoxin–OVA immunogen coating concentration. As shown in Fig. 1, the detection limit in this assay was determined to be 10⁻² μg/ml (1.28 × 10⁻¹¹ M) with an ED₅₀ of 1.1 × 10⁻³ μg/ml (1.4 × 10⁻⁹ M) where the CV was 2.6% at 10⁻³ μg/ml (1 × 10⁻⁹ M) free digoxin concentration. The response range was determined from 10⁻⁵ μg/ml (1.28 × 10⁻¹¹ M) to 10⁻¹ μg/ml (1.28 × 10⁻⁷ M) in this assay, and the curve showed sensitivity over the therapeutic and toxic concentration ranges of digoxin. The lowest detectable dose was below the clinically relevant concentration; thus, we would detect the digoxin level of undiluted serum samples.

After confirming that digoxin could be detected based on the recognition between antigen and antibody, the redox-labeled antibody was prepared by the mixed anhydride activation method using a ferrocene derivative (12, 13). Ferrocenylation of the antibody required an excess of activated ferroceneacetic acid because the mixed anhydrides were quickly hydrolyzed in aqueous antibody solution (12). Figure 2 shows the cyclic voltagograms of 100-μl samples of antibody–ferrocene conjugates, and Table 1 summarizes
the characteristics of various antibody–ferrocene conjugates. Digoxin antibody concentration was determined with a Bradford assay, and ferrocene concentration was determined with a quantitative assay using a standard curve for ferrocene. As can be seen in Fig. 2, the current itself, at the ferrocene oxidation potential, seemed to increase gradually with conjugates of molar ratios between 3.8:1 and 19.3:1 ferrocene:antibody and, then, began to decrease gradually with conjugates of higher ferrocene:antibody molar ratios (35.4:1 and 125.8:1). From the cyclic voltamograms in Fig. 2, however, when we adjusted the current of the electroactive ferrocene that was conjugated to 2 μg of the digoxin antibody in each conjugate, the current of the electroactive ferrocene was increased as the molar ratio of ferrocene increased (Table 2). The percent OD values resulting from antigen–antibody recognition by EIA for conjugates with molar ratios of 1:3.8, 1:4.6, 1:19.3, and 1:35.4 were almost the same (97% OD) as that of control antibody which was not conjugated with ferrocene. But the OD value of the digoxin antibody–ferrocene conjugate with molar ratio 1:125 decreased by 50%. (The OD value of immunoreactivity was determined using microtiter plate wells coated with 1 μg/ml digoxin–KLH with addition of 0.18 μg/ml antibody–ferrocene conjugate and 1/2000 diluted goat anti-rabbit IgG–HRP. Each step was incubated for 30 min and followed the procedure of dose–response curves for digoxin.) Table 2 summarized the electroactivity and immunoreactivity in these conjugates.

These results suggest that the coupling of activated ferrocene with digoxin antibody stereochemically restricts its binding to the digoxin–KLH coating the microtiter well. As
As can be seen in Table 2, the electrochemical signal was directly proportional to the amount of conjugated ferrocene, whereas the immunoreactivity of the antigen–antibody was inversely proportional to the conjugate whose ferrocene molar ratio was higher than 35.

Based on the above results, we synthesized an antibody–ferrocene conjugate with a molar ratio of 1:35 and applied this to the screen-printed carbon electrode (SPCE) on the nitrocellulose membrane strip. The SPCE was prepared with printed carbon electrode in the working area, followed by Ag/AgCl reference electrode and Ag counter electrode. The results indicate that the conjugate in solution with a current of 170 nA at the ferrocene oxidation potential responded on the SPCE, but we could not confirm by the immunoresponsiveness.

### TABLE 1
Characterization of Various Antibody–Ferrocene Conjugates

<table>
<thead>
<tr>
<th>No. of Ab–Fc conjugates</th>
<th>Raw materials</th>
<th>Ab–Fc conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ab (mmol)</td>
<td>Activated Fc (mmol)</td>
</tr>
<tr>
<td>a</td>
<td>2.3</td>
<td>4.3</td>
</tr>
<tr>
<td>b</td>
<td>2.3</td>
<td>8.7</td>
</tr>
<tr>
<td>c</td>
<td>2.3</td>
<td>17.4</td>
</tr>
<tr>
<td>d</td>
<td>2.3</td>
<td>34.7</td>
</tr>
<tr>
<td>e</td>
<td>2.3</td>
<td>69.5</td>
</tr>
</tbody>
</table>

*Ab, antibody; Fc, ferrocene.
activity whether the antigen–antibody binding reaction had occurred or not. To recognize the immunoreactivity between digoxin and its antibody–ferrocene conjugate on the strip, we attached colloidal gold to this conjugate. Colloidal gold has been popular as a label for antibody in membrane strips using the immunochromatography principle for the home test, because its pink color is formed as a result of an immunoreaction between antigen and the colloidal gold-attached antibody and is easy to recognize visually without instrument. Because conjugation of antibody to gold particles depends on ionic and hydrophobic attraction, it was necessary to perform a preliminary titration to form strong absorption between the colloidal gold and antibody–ferrocene conjugate. Addition of 17.7 mg, the minimum amount, of antibody–ferrocene conjugate to 1 ml of colloidal gold stabilized the synthesized multilabeled conjugate. We applied the multilabeled conjugate to glass-fiber membrane and determined the appropriate concentration that showed the response of OD 1.5 at 540 nm, which would allow good visual identification on the immunochromatography strip.

We also applied various concentrations of digoxin–BSA on the test line of the immunochromatography strip to optimize and improve the detectability of digoxin in sample solution. As the amounts of digoxin–BSA immunogen placed on the nitrocellulose membrane increased, the sensitivity of the immunochromatography for the digoxin solution decreased. Furthermore, both the control and test lines did not appear when using the lower concentration of digoxin–BSA. Thus, it was necessary to optimize the amount of digoxin–BSA dispensed on the nitrocellulose membrane, because it affected the detection capability of the immunochromatography. From the results, 0.038 mg/ml digoxin–BSA was selected, and under this condition, digoxin solution was applied to the immunochromatography strip assembled. On the strip, digoxin in sample solution competed with the digoxin–BSA coating on the nitrocellulose membrane, and the multilabeled antibody could detect a digoxin level of 1 \mu g/ml (1.28 \times 10^{-8} \text{ M}) within 2 min (Fig. 3).

**CONCLUSION**

The dose–response curve for digoxin using digoxin–OVA-coated wells, digoxin antibody, and goat anti-rabbit IgG–HRP conjugate under optimized conditions could be used to detect 10^{-3} \mu g/ml (1.28 \times 10^{-11} \text{ M}) digoxin and to differentiate between the therapeutic

<table>
<thead>
<tr>
<th>No. of Ab–Fc conjugates</th>
<th>Molar ratio (Ab:Fc)</th>
<th>Electroactivity (nA/2 \mu g Ab in Ab–Fc)</th>
<th>Immunoreactivity (% OD at 490 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1:0</td>
<td>n/d (^a)</td>
<td>100.0</td>
</tr>
<tr>
<td>a</td>
<td>1:3.8</td>
<td>1.1</td>
<td>97.5</td>
</tr>
<tr>
<td>b</td>
<td>1:4.6</td>
<td>1.3</td>
<td>97.0</td>
</tr>
<tr>
<td>c</td>
<td>1:19.3</td>
<td>7.0</td>
<td>97.1</td>
</tr>
<tr>
<td>d</td>
<td>1:35.4</td>
<td>17.5</td>
<td>97.6</td>
</tr>
<tr>
<td>e</td>
<td>1:125.8</td>
<td>42.5</td>
<td>50.0</td>
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</table>

\(^a\) Not determined.
and toxic ranges of digoxin in serum. To develop a sensor signal, antibody–ferrocene conjugates with various ferrocene:antibody ratios were synthesized using the mixed anhydride activation method. As the ferrocene moiety increased, antigen–antibody recognition decreased in the application of immunochromatography strips and immunosensor systems. The antibody–ferrocene conjugate was labeled additionally with colloidal gold to prepare colloidal gold–antibody–ferrocene conjugate which could monitor the immuno-reactivity of digoxin to its antibody on the nitrocellulose membrane of the immunochromatography detection strip. This immunodetection strip can detect digoxin at levels of approximately 1 μg/ml (1.28 × 10⁻⁶ M) by visual recognition of color without instruments, using a multilabeled antibody conjugate with colloidal gold and ferrocene.

REFERENCES