Gold nanoparticle-based novel enhancement method for the development of highly sensitive immunochromatographic test strips

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Abstract

The immunochromatographic assay that is in widespread use for pregnancy diagnosis is a method for easy visual judging of the antigen–antibody reaction using gold nanoparticle. The rapid observation of results directly by the naked eye ensures the convenience of performing bioassays on-field. Therefore, gold nanoparticle-based immunochromatographic assays have provided attractive means for developing biosensors without the handling of toxic reagents, while allowing an easy and rapid procedure. However, the detection limit of this method is higher than the conventional method, enzyme-linked immunosorbent assay (ELISA). In this report, we developed a highly sensitive immunochromatographic assay for the detection of human chorionic gonadotropin hormone (hCG) as the model case. In this research, we are reporting the application of a new ‘sensitizer’ that contains gold nanoparticle conjugated primary antibody and the antigen. The sensitizer was added to the membrane after finishing the application of the normal method. The antigen of the sensitizer was captured by the secondary antibodies at the test line on the strip. As a result, the accumulation of the gold nanoparticle increased at the test line, and the sensitivity was higher. The sensitivity of our method could be enhanced by the sensitizer to almost the same level of ELISA assay. The test line intensity of hCG at 25 pg/ml treated with sensitizer was almost equal to the density that we observed at 1.0 ng/ml with the normal method. We also tested the performance of the sensitizer by using the surface plasmon resonance (SPR) technology of BIACORE™. The sensitizer using immunochromatographic assay is a promising candidate for decentralized diagnosis in clinically important fields such as the sensitive detection of cancer markers.

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1. Introduction

An immunochromatographic test strip is one of the promising tools for the development of easy-handling biosensors. The rapid observation of results directly by the naked eye ensures the convenience of performing bioassays on-field. Therefore, gold nanoparticle-based immunochromatographic assays have provided attractive means for developing biosensors without the handling of toxic reagents, while allowing an easy and rapid procedure [1–4]. Test strips for bioanalysis based on gold nanoparticle as the visual indicator have already been commercialized for pregnancy diagnosis. The gold nanoparticle are more stable and easy to use than the conventional systems utilizing fluorescence or enzymatic labels. Moreover, nanoscale surfaces of gold nanoparticle are appropriate for accelerating the antibody–antigen recognitions, which enhances the immunoassay signals [5]. The basic operation procedure of an immunochromatographic test strip is described as follows. In the presence of an antigen, a sandwich-type assay is formed between the secondary antibody–immobilized gold nanoparticle immunocomplex and the primary antibody immobilized on the membrane. After the antigen–antibody reaction, the red color caused by the accumulation of gold nanoparticle at that location, appears on the membrane [6]. Through these detection processes, the results are obtained in approximately 10 min after the sample introduction. However, the conventional test strips based on immunochromatography have a disadvantage. The sensitivity of immunochromatographic assay is significantly lower than ELISA.

There are some reports to enhance the immunochromatographic assay. The enhancing methods involves gold/silver...
staining, enzyme labeling in connection with fluorescence or electrochemistry [7–11]. However, these methods are not rapid and easy to perform. The gold/silver staining and enzyme labeling methods require an additional process to stop the staining reaction. Moreover, the fluorescence and electrochemical detection systems require sophisticated analytical devices.

In this study, we are reporting a novel ‘sensitizer’, which is a gold nanoparticle-based enhancement method for obtaining a better sensitivity in immunochromatographic test strips. The sensitizer does not require a procedure to stop the staining, and an additional device for the measurement of the analytical signal. The sensitizer consists of only gold nanoparticle and the primary antibody. These reagents are already the parts of the normal sandwich-type immunochromatographic test strip, thus there is no need for extra reagents to enhance the sensitivity. We present the performances of ‘sensitizer’ to detect the human chorionic gonadotropin hormone (hCG) as the model case. We also examined the binding of gold nanoparticle-conjugated secondary antibodies and ‘sensitizer’ by using surface plasmon resonance (SPR).

2. Materials and methods

2.1. Materials

Monoclonal anti-human α-subunit of follicle-stimulating hormone (MabHzS) and monoclonal anti-human chorionic gonadotropin (MabhCG) were purchased from Medix Biochemica (Kauniainen, Finland). Polyclonal anti-mouse IgG was purchased from DakoCytomation (Glostrup, Denmark). The recombinant human chorionic gonadotropin (hCG) as a measurement subject was purchased from Rohto Pharmaceutical Co., Ltd (Tokyo, Japan). Gold nanoparticle for labeling the antibodies were purchased from Tanaka Kikinzoku Kogyo (Tokyo, Japan). High-flow nitrocellulose membrane (FH180AK020) and absorption pad were kindly donated by Nihon Millipore K.K. (Tokyo, Japan). For fabrication of the immunochromatographic assay system, disodium hydrogen-phosphate (Na2HPO4), sodium dihydrogenphosphate dihydrate (NaH2PO4·2H2O), sucrose, polyethylene glycol (PEG), and potassium dihydrogenphosphate (KH2PO4) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA) for blocking the antibody immobilized nitrocellulose membrane was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Sodium azide (NaN3) purchased from Nakarai Tesque (Kyoto, Japan) for preserving the proteins in blocking and diluting solutions.

2.2. Apparatus

The dispensing system (Biojet Quanti 300) for the immobilization of the primary antibody on the nitrocellulose membrane was purchased from BioDot, Inc. (CA, USA). After the immunochromatographic assay, the images were scanned by EPSON EU-34 from Seiko Epson (Nagano, Japan) in order to detect the color intensity of the test lines. The scanned images were converted into gray scale readings by Adobe™ Photoshop™5.5. The intensities of each signal were quantified with Image SMX vol. 1.74.

2.3. Preparation of immunochromatographic test strip

MabhCG solution at 1 mg/ml was prepared by diluting with 50 mM phosphate buffered saline (PBS, pH 7.4). For the immobilization at the test line, the MabhCG solution of 650 μL was mixed with 20% sucrose solution diluting with 50 mM potassium dihydrogenphosphate buffer (pH 7.5) of 50 μL, 50 μL of 2-propanol and 50 μL of 50 mM potassium dihydrogenphosphate buffer. For the control line, polyclonal anti-mouse IgG of 40 μL was mixed with 60 μL 2-propanol and 1100 μL 50 mM potassium dihydrogenphosphate buffer. The MabhCG solution and polyclonal anti-mouse IgG solution were applied to the nitrocellulose membrane as test and control lines by using dispensing system. After drying for 1 h at room temperature, the membrane was blocked against the non-specific protein adsorption by immersing in 50 mM boric acid buffer containing 0.5% casein (pH 8.5), and incubating for 30 min at room temperature. Then, the blocked membrane was washed by immersion in 5.0 mM phosphate buffer (pH 7.5) containing 0.01% sodium dodecyl sulfate for 30 min at room temperature. After drying the membrane for overnight, the membrane was prepared on a backing sheet, and absorbent pad were pasted. The sheet was cut with an adequate size for the test strip (Fig. 1).

2.4. Preparation of gold nanoparticle-conjugated antibodies

MabhCG solution at 50 μg/ml was prepared by diluting with 5 mM KH2PO4 solution (pH 7.5) in ultra pure water (18.3 MΩ·cm) to a final volume of 250 μL. The diluted MabhCG solution at 200 μL was added in 1.8 ml of gold nanoparticle solution (Φ = 40 nm, 0.0065 wt%) and mixed immediately. The mixture

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Fig. 1. Schematic representation of the immunochromatographic test strip. A, a top view; B, cross-section; C, image for the experimental set-up.
was kept for 10 min at room temperature for the immobilization of antibody onto the gold nanoparticle surfaces. After the immobilization, 100 µL of 1% (w/v) PEG, which was dissolved in 50 mM KH₂PO₄ solution (pH 7.5) and 200 µL of 10% (w/v) BSA, which was also dissolved in 50 mM KH₂PO₄ solution (pH 9.0), were added for blocking the non-coated surfaces. After the immobilization and blocking procedures, gold nanoparticle-conjugated MabhCG was separated by a centrifugal operation (8000 g for 15 min at 4 °C). The gold nanoparticle-conjugated MabhCG was pulse-sonicated for a few sec, and was added to 2 ml of preserving solution (1% (w/v) BSA, 0.05% (w/v) PEG 20000, 0.1% (w/v) NaN₃ and 150 mM NaCl in 20 mM Tris–HCl buffer, pH 8.2).

After mixing, gold nanoparticle-conjugated MabhCG were collected by the same process as described above. After pulse-sonication, the gold nanoparticle-conjugated MabhCG solution was diluted with the preserving solution to OD₅₂₀ = 6.

2.5. Preparation of the sensitizer

Sensitizer consisted of gold nanoparticle-conjugated Mabh-Hzs (primary antibody) and hCG (antigen). The gold nanoparticle-conjugated Mabh-Hzs was prepared by the same procedure of the gold nanoparticle-conjugated MabhCG as described above. The diameter of gold nanoparticle-conjugated Mabh-Hzs was changed for the estimation of the effect of size on the sensitizer. The diameter of gold nanoparticle ranged between 5, 40, 60, 80 and 100 nm. The sensitizer was mixed with the antigen solution. The ratio of sensitizer (OD₅₂₀ = 6) to the solution of hCG was 1:10.

2.6. Confirmation of the binding event between the secondary antibodies and the sensitizer by SPR

The binding to secondary antibodies and sensitizer was confirmed by using the surface plasmon resonance technology (SPR) of Biacore 2000 and Sensor Chip Au (Biacore international AB., Tokyo, Japan). The first, 200 µL of Mabh-Hzs solution which were the same procedure of immobilized test line, were dropped on the sensor chip for over night at 4 °C. The Mabh-Hzs immobilized sensor chip was washed by PBS buffer. The sensor chip was docked into the Biacore instrument, then, hCG and gold nanoparticle-conjugated MabhCG mixed solution was introduced to sensor chip with a volume of 10 µL at a flow rate of 1 µL/min. After the signal

![Fig. 2. The schematic illustration for the sensitizer enhancing the test line.](image)

![Fig. 3. Result of immunochromatographic test strip treated with sensitizer containing different sizes of gold nanoparticle.](image)
of Biacore was stable, running buffer (HEPES buffer) was introduced to remove the non-specifically bound gold nanoparticle. The sensitizer was introduced to confirm the binding of the secondary antibodies and the antigen of the sensitizer with a volume of 10 μL at a flow rate of 10 μL/min.

3. Results and discussion

3.1. Evaluation of the sensitizer

Fig. 2 shows the principal of our newly developed method for enhancing the sensitivity of immunochromatographic test strip. The control results involved the normal sandwich-type method in comparison with the sensitizer. First, we prepared several concentrations of antigen solutions (0, 100, 50, 25 pg/ml) and the gold nanoparticle-conjugated MabhCG were mixed into the solution. The fundamental ratio of the gold nanoparticle-conjugated MabhCG to solution of hCG was 1:10 (40 μL/stripped). The mixed solution was absorbed to the test strip by capillary force. The intensity of the red color, which was produced by the accumulation of the gold nanoparticle-conjugated MabhCG on the test line of the strip, was measured by an image analyzer. Briefly, the sensitizer was prepared by the interaction of the gold nanoparticle-conjugated MabhHzS and the hCG. The 40 μL of sensitizer was added to the test strips to increase the intensity of the test line after a normal sandwich-type immunochromatographic assay. After drying the membrane, we also measured test line by an image analyzer. We also checked the effect of gold nanoparticle with different sizes on the performance of the sensitizer.

3.2. Comparison of the normal sandwich-type method and the sensitizer

Several concentrations of hCG solutions were prepared, and the gold nanoparticle-conjugated MabhHzS was mixed with the solution. The mixed solution was absorbed to the test strip by capillary force. The detection of hCG at 100 pg/ml was possible by the naked eyes following the normal sandwich-type method (Fig. 3). We could also detect a weak signal at 50 pg/ml of hCG when analyzed image analyzer, however, the red color at test line was difficult to recognize by the naked eyes (Fig. 4). The 40 μL of sensitizer was added to the test strips to increase the intensity of the test line after a normal sandwich-type method. The intensity of test line could not be enhanced by using the sensitizer of Φ5 nm gold nanoparticle-conjugated. The intensity was decrease after using sensitizer. We concluded that Φ5 nm diameter of gold nanoparticle was too small to produce the red color for the immunochromatographic assay. We made Φ5 nm gold nanoparticle-conjugated MabhCG, and tested the immunochromatographic assay by the normal sandwich-type method. The test line measured by Φ5 nm gold nanoparticle-conjugated MabhCG did not present a red color at 1 ng/ml of hCG. We could observe the red color by using gold nanoparticle over Φ20 nm conjugated with MabhCG (data no shown). When we used the sensitizer with the gold nanoparticle size over Φ60 nm, we could detect the low concentration of hCG antigen (0.025 ng/ml). The density of red color at 25 pg/ml by using Φ80 nm gold nanoparticle-conjugated sensitizer was almost similar the result of 1 ng/ml hCG detected by the normal sandwich-type method.

3.3. Confirmation of binding to secondary antibody and sensitizer by SPR

We confirmed that the sensitizer could bind to the secondary antibody by using SPR (BIACORE). We described the image of conformation of binding to secondary antibody to sensitizer (Fig 5C). We confirmed two different sizes Φ5 and 40 nm of gold nanoparticle conjugated sensitzers to bind (Fig. 5A and B). At first, we immobilized the primary antibodies on the gold surface of sensor chip. The primary antibody immobilized sensor chip was docked into the BIACORE. The gold nanoparticle-conjugated MabhCG and hCG complex solution was flowed. We could observe the binding to primary antibody on gold surface and antigen of gold nanoparticle-conjugated MabhCG. Next, we injected the sensitizer after the sensorgram was stable. We could also observe the bonding the sensitizer and gold nanoparticle-conjugated MabhCG. The both sizes Φ5 and 40 nm gold nanoparticle-conjugated sensitizer were similar to bind to gold nanoparticle-conjugated MabhCG. The Φ5 nm gold nanoparticle-conjugated sensitizer was difficult to enhance the sensitivity of immunochromatographic test strip. However, we could confirm the binding of Φ5 nm gold nanoparticle-conjugated sensitizer by BIACORE.
4. Conclusion

The sensitizer could enhance the sensitivity of immuno-chromatographic assay for detecting hCG as a model case. The hCG at 25 pg/ml could not be detect by using normal method. After normal method assay, the sensitizer of the gold nanoparticle size over $\Phi 60 \text{ nm}$ was added to the test strip by capillary force, and then we could detect the hCG at 25 pg/ml. The test line intensity of hCG at 25 pg/ml treated with sensitizer was almost equal to the density that we observed at 1 ng/ml with the normal method. The sensitivity could be increased $\sim 40$-fold while observing the test line intensity. When the sensitizer was applied to measure the control sample that contained only gold nanoparticle-conjugated secondary antibody, we could not observe the non-specific binding of the sensitizer. We also tested binding event of antigen and sensitizer by BIACORE. The binding of gold nanoparticle with different sizes was also detected by SPR. Such a low concentration of antigen detection by using immunochromatographic assay is applied to ELISA. The detectable concentration of hCG by the ELISA kit using horseradish peroxidase conjugated antibody was around 300 pg/ml in serum. In this research, we could detect 25 pg/ml of hCG in PBS using sensitizer. Immunochromatographic assay is spread to many fields as easy handling method. Our new method can be applied to measure very low antigen concentrations that would be impossible to detect by using a normal sandwich-type immunochromatographic assay.

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