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A novel enhancement assay for immunochromatographic test strips using gold nanoparticles

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Abstract The immunochromatographic assay is a well-known and convenient diagnostic system. In this report, the development of a novel enhancement assay for the test strips is described. Additionally, this highly sensitive immunochromatographic assay was applied to detect human chorionic gonadotropin hormone (HCG) as the model case. The primary antibody-conjugated gold nanoparticles were used as the enhancer of the standard method. The primary antibodies were immobilized within a defined detection zone (test line) on the diagnostic nitrocellulose membrane. The secondary antibodies were conjugated with colloidal gold nanoparticles. In combination with an effective sample pretreatment, the gold-conjugated antibodies and the primary antibodies formed a sandwich complex with the target protein. Within the test line, the sandwich complex was immobilized, and furthermore, concentrated by the enhancer resulting in a localized surface plasmon resonance (LSPR) phenomenon and a distinct red color on the test line. The intensity of

color of the red test line (signal intensity), which correlated directly with the concentration of the target protein in the standard or spiked samples, was assessed visually and by computer image analysis using a three-determination analysis. Under optimum conditions, the limit of detection (LOD) for HCG assay was 1 pg/mL. When using human serum, 10 pg/mL of HCG could be detected. We have also spiked total prostate-specific antigen (TPSA) in female serum. The LOD for TPSA was determined as 0.2 ng/mL. With this method, the quantitative determination of the target protein could be completed in less than 15 min. Our novel immunochromatographic strips using the enhancing method based on LSPR of gold nanoparticles are useful as a rapid and simple screening method for the detection of important analytes for medical applications, environmental monitoring, food control, and biosecurity.

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Introduction

The development of biosensors is spreading widely in various fields and includes medical, environmental and forensic applications [1]. The development of the immunochromatographic assay has been under especially intense investigation because of the assay's desired properties, such as user-friendly operation and short analysis time. The immunochromatographic assay, using a membrane strip as the immunosorbent, provides a unique analytical platform that permits a one-step, rapid and low-cost analysis [2–5]. The immunochromatographic assay system was devised in order to determine the concentration of the measured subjects simply and rapidly. Furthermore, concentration levels can be observed directly with the naked eyes. Because of these advantages, the immuno-

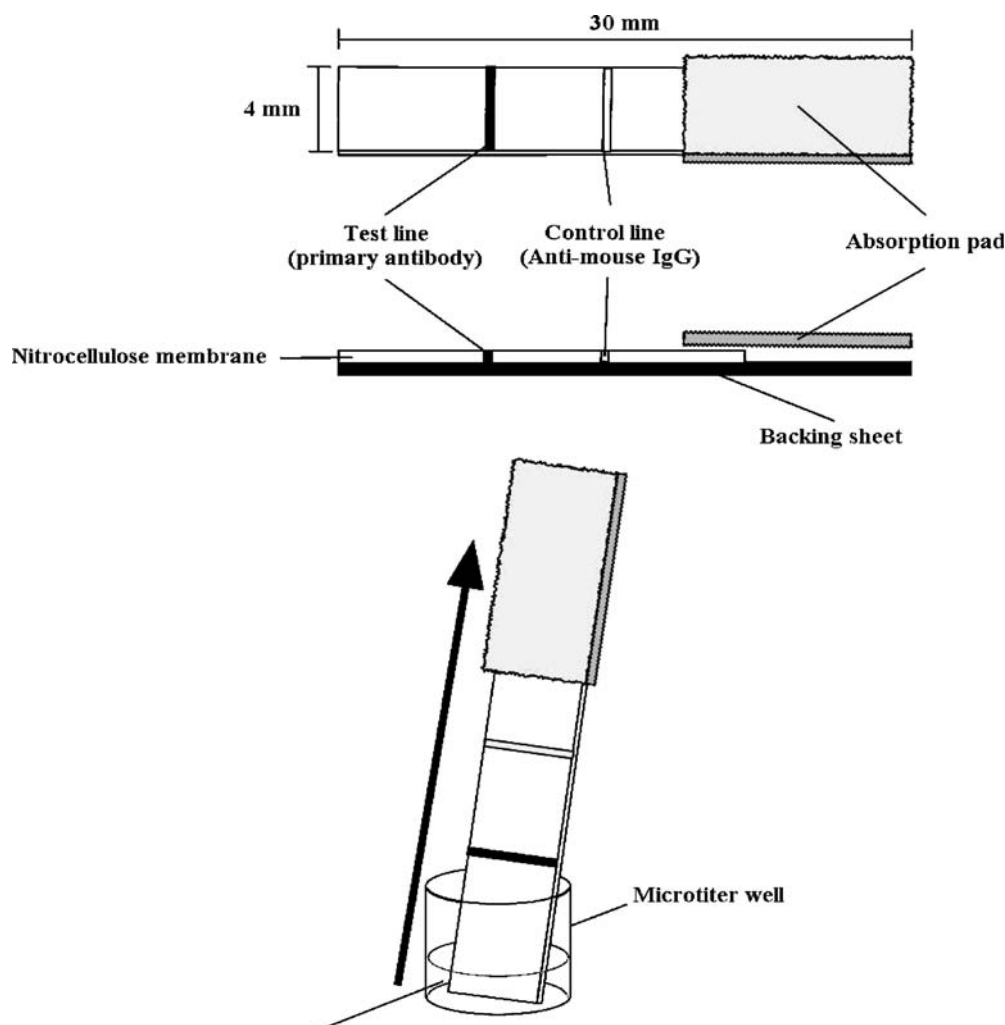
chromatographic assay has been widely used as a convenient assay system.

In the immunochromatographic assay (Fig. 1), the ligands, such as antibodies or DNA probes, are immobilized on the carrier colloidal gold. The analytical signals are observed after the specific interaction of the ligand and the analyte, such as antigen–antibody reaction and DNA–DNA hybridization [6–10]. These specific interactions take place in the membranes by capillary action of the medium. Generally, in the membrane, the sandwich assay is achieved between the antigen, the secondary antibodies that are conjugated with colloidal gold and the primary antibodies that are immobilized on the membrane. After the antigen–antibody reactions, the red color caused by the accumulation of the colloidal gold at the test and the control lines appears on the membrane. Monitoring the strength of the red color provides the basis for the quantitative determination of the target molecule. Therefore, the concentration of the measured subjects can be determined. Furthermore, colloidal gold is more stable than other kinds of labeling reagents such as fluorescent dyes and enzymes. As a result of these specific characteristics, colloidal gold is a suitable indicator for monitoring antigen–antibody reactions

easily. The results appear approximately 15 min after the sample introduction. Previously, colloidal gold was used as a labeling reagent for molecular detection in microscopy and electrophoresis-blotting techniques because of its localized surface plasmon resonance (LSPR) absorbance in the visible region [11–13]. We have also employed the LSPR absorbance of gold nanoparticles for the enhancement process on our immunochromatographic test strips.

However, the sensitivity of the conventional immunochromatographic assay is lower than the conventional enzyme-linked immunosorbent assay (ELISA). Silver precipitation on gold nanoparticles was reported to enhance the sensitivity of immunochromatographic assays [4]. Moreover, other methods have been described involving enzyme labeling in connection with fluorescence or electrochemistry [14–16]. However, these enhancing methods had several disadvantages, such as their time-consuming procedures with intensive liquid handling. Here, we report on a novel enhancing method that is mainly constructed with colloidal gold nanoparticles. This enhancing method was applied to detect the antigen–antibody reactions using an immunochromatographic test strip. This method requires no other kind of labeling

Fig. 1 Structure of immunochromatographic test strip and detection method. Antigen (40 μL) and gold nanoparticle-conjugated primary antibody (4 μL) are mixed. The mixture is applied to the membrane. With the help of the capillary force, gold nanoparticles accumulate, and the color of the test line becomes a distinct red as a result of localized surface plasmon resonance (LSPR)



The enhancer solution: Antigen and the gold nanoparticle conjugated primary antibodies

reagents, such as fluorescent dyes and/or enzymes. Furthermore, a monitoring procedure similar to that of the conventional immunochromatographic assay method can be applied. This method is applicable for detecting the measurement subjects easily with high sensitivity. Using this enhancement method, we detected the human chorionic gonadotropin hormone (HCG) and total prostate-specific antigen (TPSA) [17–19] as model cases.

Materials and methods

Materials

Monoclonal anti-human α -subunit of follicle-stimulating hormone (Mab-H α S, clone code: 5,503) and monoclonal anti-human chorionic gonadotropin (Mab-HCG, clone code: 5,506) were purchased from Medix Biochemica (Kauniainen, Finland). Polyclonal anti-rabbit IgG (code no: N1,508) was purchased from DakoCytomation (Glostrup, Denmark). The recombinant human chorionic gonadotropin (hCG) as a measurement subject for evaluation of the calibration characteristics was purchased from Rohto Pharmaceutical (Tokyo, Japan). Monoclonal anti-human total prostate-specific antigen antibody (Mab-TPSA, clone code: 4D10) was purchased from Japan Clinical Laboratories (Kyoto, Japan). The recombinant human total prostate-specific antigen (TPSA, catalog no: RDI-SCPI,923) was purchased from Research Diagnostics (Flanders, NJ). Gold nanoparticles for labeling the antibodies were purchased from Tanaka Kikinzoku Kogyo (Tokyo, Japan). High-flow nitrocellulose membrane (catalog no: FH180AK020) and the absorption pad were kindly donated by Nihon Millipore (Tokyo, Japan). For the fabrication of the immunochromatographic assay system, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), sucrose, polyethylene glycol (PEG 20,000), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA) for blocking of the antibody-immobilized nitrocellulose membrane was purchased from Sigma Aldrich Japan (Tokyo, Japan). Sodium azide (NaN_3) was purchased from Nakarai Tesque (Kyoto, Japan) for preserving the proteins in blocking and diluting solutions.

Apparatus

The dispensing system (Biojet Quanti 300) for the immobilization of the primary antibody on the nitrocellulose membrane was purchased from BioDot (CA, USA). After the immunochromatographic assay, the optical images were captured using a digital scanner (EPSON EU-34) from Seiko Epson (Nagano, Japan) for the evaluation of the color intensity of the test lines. The scanned optical images were converted to the gray scale using Adobe Photoshop 6.0 software. The intensities of each signal were quantified with Scion Image software.

Preparation of gold nanoparticle-conjugated Mab-HCG

Mab-HCG solution at 50 $\mu\text{g}/\text{mL}$ was prepared by diluting it with 5 mM KH_2PO_4 solution (pH 7.5) in ultra-pure water (18.3 $\text{M}\Omega \text{ cm}$) to a final volume of 250 μL . The diluted Mab-HCG solution at 200 μL was added to 1.8 mL of gold nanoparticles solution (i.d. 40 nm, 0.0065 wt%) and mixed immediately. The mixture was kept for 10 min at room temperature (RT) for the immobilization of antibodies onto the gold nanoparticles' surfaces by physical adsorption. After immobilization, 100 μL of 1% (w/v) PEG, which was dissolved in 50 mM KH_2PO_4 solution (pH 7.5), and 200 μL of 10% (w/v) BSA, which was also dissolved in 50 mM KH_2PO_4 solution (pH 9.0), were added to block the non-coated gold nanoparticle surfaces. After the immobilization and blocking procedures, gold nanoparticle-conjugated Mab-HCG was separated by a centrifugal operation (8,000 g for 15 min at 4 °C). The gold nanoparticle-conjugated Mab-HCG was pulse-sonicated for a few seconds and was added to 2 mL of preserving solution (1% (w/v) BSA, 0.05% (w/v) PEG 20,000, 0.1% (w/v) NaN_3 and 150 mM NaCl in 20 mM Tris-HCl buffer, pH 8.2). After mixing, gold nanoparticle-conjugated Mab-HCG was collected by the same process as described above. After pulse sonication, the gold nanoparticle-conjugated Mab-HCG solution was diluted with the preserving solution to $\text{O.D.}_{520} = 6$.

Preparation of the gold nanoparticle-conjugated Mab-H α S

In this research, the enhancer consisted of gold nanoparticle-conjugated Mab-H α S (primary antibody). The gold nanoparticle-conjugated Mab-H α S was prepared by the same procedure as the gold nanoparticle-conjugated Mab-HCG described above. For the estimation of the effect of particle diameter, different diameters of gold nanoparticles were used for the preparation of the Mab-H α S-conjugated gold nanoparticles. The diameters of gold nanoparticles were 15, 40, 60, and 80 nm. The Mab-H α S-conjugated gold nanoparticles that were prepared with different diameter gold nanoparticles were used as the enhancer. After the preparation, the concentration of the Mab-H α S-conjugated gold nanoparticles was prepared as $\text{O.D.}_{520} = 6$.

Preparation of the enhancer-based immunochromatographic test strip

The preparation of the antibody solution was carried out as shown in Table 1. Mab-H α S 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, 650 μL of the Mab-H α S solution was mixed with 20% sucrose solution diluted with 50 μL of 50 mM potassium dihydrogen phosphate buffer (pH 7.5) and

Table 1 The protocol for using enhancer-based immunochromatographic test strips**Steps**

- Prepare the nitrocellulose membranes on a backing sheet and paste the absorbent pad on the backing sheet.
- Cut the sheets to an adequate size (30×4 mm) for the test strips.
- Prepare the Mab-H α S solution (primary antibody) for test line and polyclonal anti-mouse IgG solution for control line.
- Prepare gold nanoparticle-conjugated primary antibody as the enhancer.
- Add a certain amount of the total test-line solution to Mab-H α S solution (primary antibody).
- Introduce solution (e) and the polyclonal anti-mouse IgG solution to the nitrocellulose membrane as test and control lines using the dispensing system.
- Dry at room temperature (RT) for 1 h.
- Apply the blocking procedure, and incubate for 30 min at RT.
- Wash the blocked membrane with the washing solution and incubate for 30 min at RT.

50 μ L of 2-propanol. For the preparation of the enhancer-based immunochromatographic test strip, different concentrations of the gold nanoparticle-conjugated Mab-H α S solution were introduced as 1, 3, and 5% of the total test-line solution. Additionally, different diameters of gold nanoparticles were used for optimization of sensitivity at 15, 40, 60, and 80 nm. For the preparation of the control line, 40 μ L of polyclonal anti-mouse IgG was mixed with 60 μ L of 2-propanol and 1,100 μ L of 50 mM potassium dihydrogen phosphate buffer. Mab-H α S and polyclonal anti-mouse IgG were introduced to the nitrocellulose membrane as test and control lines using the dispensing system. After immobilization, the antibody-immobilized nitrocellulose membrane was dried at RT for 1 h. Then to prevent nonspecific adsorption, the blocking procedure was applied by immersion in 50 mM boric acid buffer containing 0.5% (w/v) casein (pH 8.5) and incubation for 30 min at RT. Then the blocked membrane was washed by immersion in 5.0 mM phosphate buffer (pH 7.5) containing 0.01% (w/v) sodium dodecyl sulfate for 30 min at RT. After the blocking procedure, the antibody-immobilized nitrocellulose membrane was dried at RT overnight. The membranes were prepared on a backing sheet, and the absorbent pad was also pasted on the backing sheet. The sheet was cut to an adequate size for the test strip.

Preparation of the human serum

The present work with human samples was carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1996. The human blood was transferred into a test tube. After incubation at 40 °C for 3 h, the test tube was centrifuged for 10 min at 3,000 rpm. The supernatant was loaded into a vial, and stored frozen at -20 °C until use.

Preparation of gold nanoparticle-conjugated Mab-TPSA

The gold nanoparticle-conjugated Mab-TPSA antibodies were prepared using the same procedure as for the

preparation of the gold nanoparticle-conjugated HCG antibodies. The concentration of Mab-TPSA used in this procedure was 1 mg/mL diluted with 5 mM PBS. The gold nanoparticle-conjugated Mab-TPSA antibodies were applied to the antigen-antibody reaction. The sensitivity of the procedure for detecting Mab-TPSA antigen was first investigated with serial dilutions (5, 1, 0.2, 0.04 ng/mL) of Mab-TPSA antigen solutions in 5 mM PBS. In addition, we examined its performance with human serum samples. We prepared serial dilutions (5, 1, 0.2, 0.04 ng/mL) of Mab-TPSA antigen solutions in human female serum.

Evaluation of the characteristics of the enhancer-based immunochromatographic test strip

We prepared different concentrations of antigen solutions (1, 0.1, 0.01, 0.001 ng/mL) and the gold nanoparticle-conjugated Mab-HCG. Gold nanoparticle-conjugated Mab-HCG at 4 μ L and antigen at 40 μ L were mixed together in a dry microtiter plate. The mixed solution was then 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 Mab-HCG on the test line, was measured by an image analyzer. The detection of TPSA was also performed following the same procedure as described above for Mab-HCG.

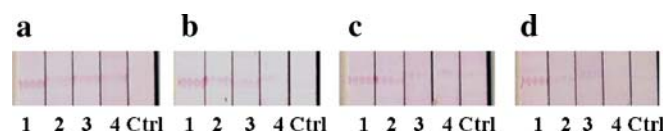
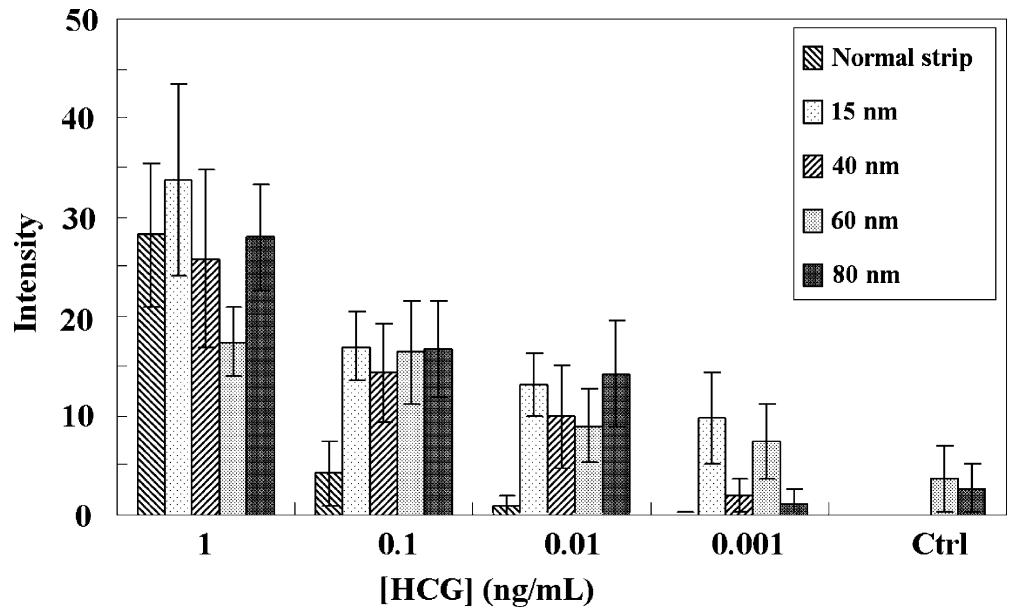
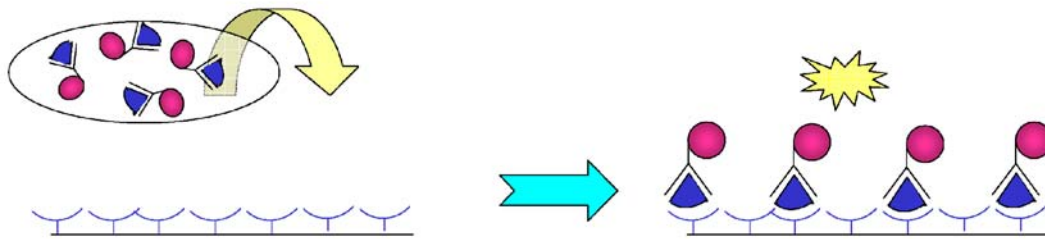


Fig. 2a–d The effect of gold nanoparticles with different diameters on the performance of the enhancer-based immunochromatographic test strips. Intensity of the enhancer-based immunochromatographic test strips was determined using gold nanoparticles with diameters of **a** 15 nm, **b** 40 nm, **c** 60 nm, **d** 80 nm using HCG antigen concentrations of 1 1 ng/mL, 2 0.1 ng/mL, 3 0.01 ng/mL, 4 0.001 ng/mL, Ctrl 0 ng/mL

Fig. 3 Comparison of the intensity responses of the normal strips and the enhancer-based strips at several concentrations of HCG (*Ctrl*=0 ng/mL). We changed the diameter of the gold nanoparticles for the enhancer-based strips. The changes in intensity were measured by an image analyzer (*n*=5)



a Conventional test strip



b Enhanced test strip

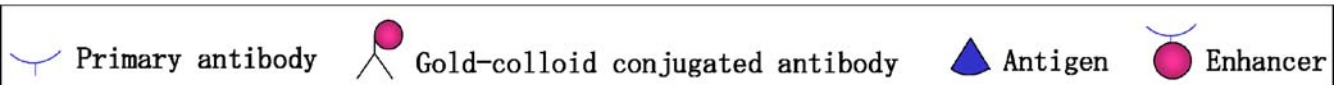
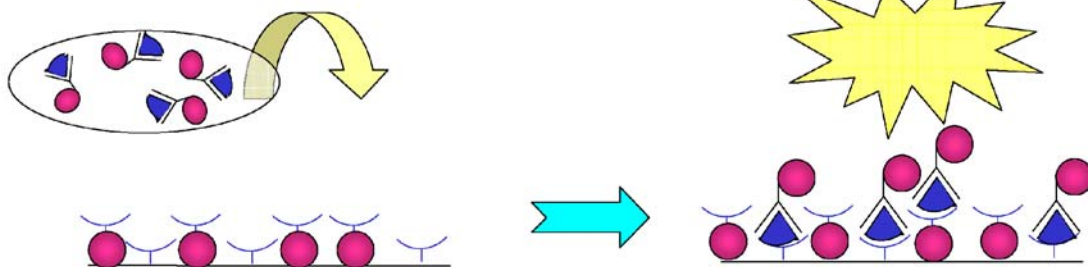
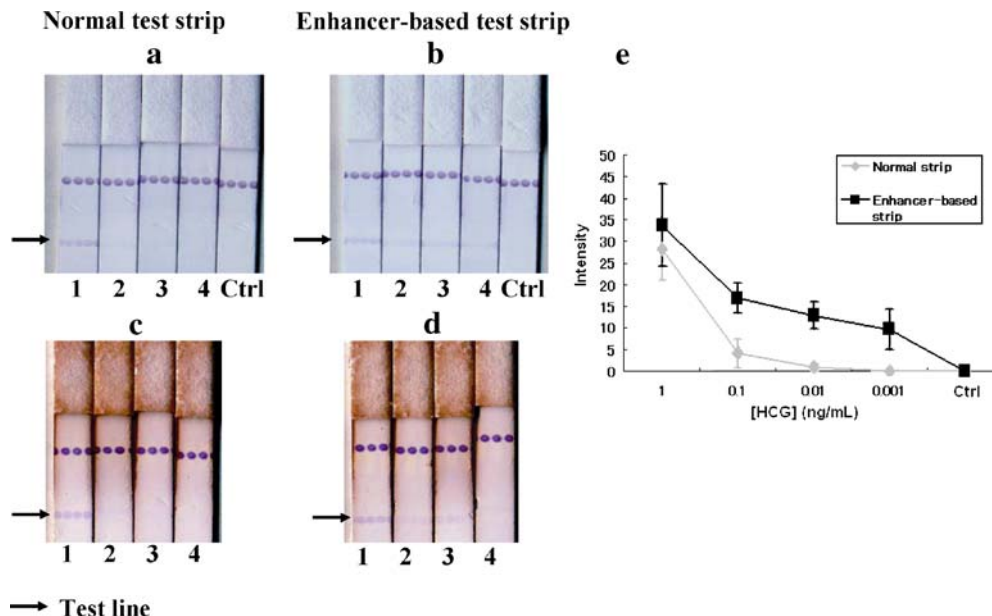


Fig. 4a,b Schematic illustration of the principle of the enhancer-based detection method. The primary antibody-conjugated colloidal gold and the non-conjugated primary antibody solutions were mixed. Then, the mixture of these solutions and the sample solution was immobilized on the nitrocellulose membrane surface. After the immobilization procedure, the antigen and gold colloid-conjugated secondary antibody mixture was introduced onto the conventional (a) and the enhancer-based test strips (b). The gold colloid-

conjugated primary antibodies and gold colloid-conjugated secondary antibodies accumulated as a result of the immunogenic recognition reactions. The localized surface plasmon resonance (LSPR) absorbance of the colloidal gold in the visible region depends on the distance between the gold nanoparticles. Since the antibody-antigen reaction brought a high amount of gold nanoparticles in close proximity, a distinct red color could be easily observed with the naked eye

Fig. 5a,b Normal (a) and the enhancer-based strips (b) for the detection of HCG in PBS, and normal (c) and enhancer-based strips (d) for the detection of HCG in serum. HCG concentrations: 1 1 ng/mL, 2 0.1 ng/mL, 3 0.01 ng/mL, 4 0.001 ng/mL, Ctrl 0 ng/mL. The test line is marked with a black arrow. The enhancer-based strips (b and d) contained $\Phi 15$ nm gold nanoparticles as the enhancer. **e** Dependence of the intensity values ($n=3$) on the concentration of HCG



Results and discussion

Optimal conditions for the enhancer-based immunochromatographic test strips

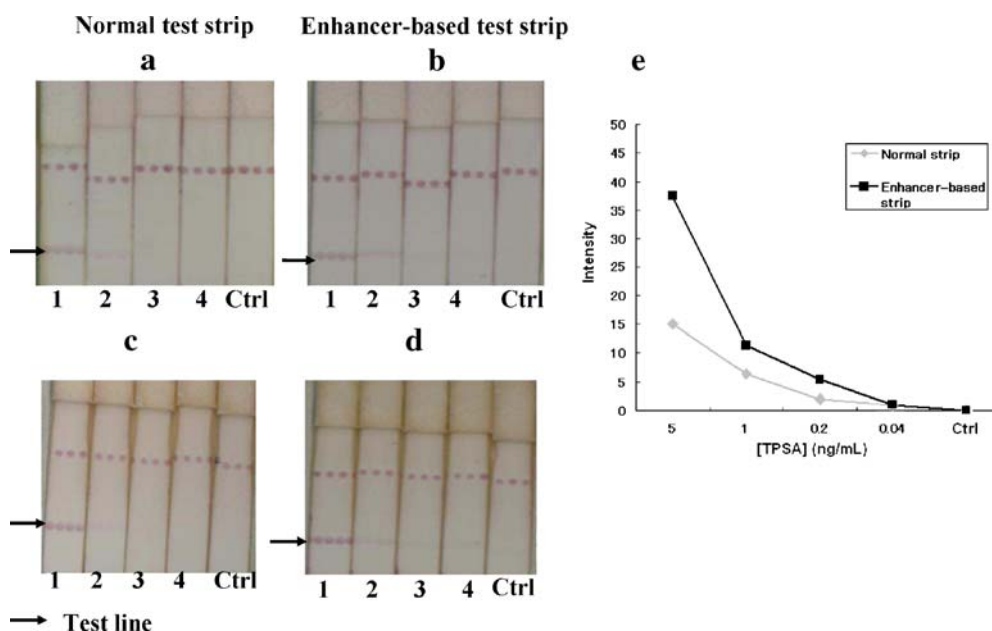
First, we checked the effect of gold nanoparticles with different diameters on the performance of the enhancer-based immunochromatographic test strip. Figures 2 and 3 show the comparison of color intensity based on concentration according to the size of the gold nanoparticles that were conjugated with Mab-H α S. The diameters of the gold nanoparticles were 15, 40, 60 and 80 nm. When Mab-H α S solution was created with 3% concentration of the gold nanoparticle-conjugated Mab-H α S for immobilized test

line, the red color of gold nanoparticles on the test line was not observed (data not shown).

Detection of HCG

Figure 4 shows the principle of our newly developed method for enhancing the sensitivity of immunochromatographic test strips. The primary antibody-conjugated colloidal gold and the non-conjugated primary antibody solutions were mixed. Then, the mixture of these solutions and the sample solution was immobilized on the nitrocellulose membrane surface. After the immobilization procedure, the antigen and gold colloid-conjugated secondary

Fig. 6 Normal (a) and enhancer-based strips (b) in the detection of TPSA in PBS and normal (c) and enhancer-based strips (d) in the detection of TPSA in serum. TPSA concentrations: 1 5 ng/mL, 2 1 ng/mL, 3 0.2 ng/mL, 4 0.04 ng/mL, Ctrl 0 ng/mL. The test line is marked with a black arrow. The enhancer-based strips (b and d) contained $\Phi 15$ nm gold nanoparticles as the enhancer. **e** Dependence of the intensity values on the concentration of HCG



antibody mixture was introduced onto the antibody-immobilized nitrocellulose membrane. From these experimental procedures, the gold colloid-conjugated primary antibody and gold colloid-conjugated secondary antibody were accumulated by the antigen-antibody reactions. The localized surface plasmon resonance (LSPR) absorbance of the colloidal gold depends on the distance between the gold nanoparticles [20]. As a result of our procedure, a higher absorbance could be observed.

The experiments involved the normal sandwich-type method in comparison with the enhancer immunochromatographic test strip (Fig. 5). The detection of HCG at 1~0.1 ng/mL was possible with the naked eye following the normal sandwich-type method. We could also detect a weak signal at 0.001 ng/mL of HCG with the naked eye. When we used the gold nanoparticles with diameters of 15–40 nm, we could detect low concentration of HCG antigen (0.001 ng/mL). As the result, it was suggested that this method was 1,000-fold more sensitive than the normal sandwich method (1 ng/mL). HCG antigen solutions (1, 0.1, 0.01, 0.001 ng/mL) were used to check the effect with human serum in the same way. The results were obtained visually based on the appearance of the red color. Red color on the enhancer-based strip was observed with a lower limit of detection at approximately 0.01 ng/mL. Figure 5e shows the dependence of the signal intensity values on the concentration of HCG for the normal and the enhancer-based strips. The enhancer-based strips provided significantly higher intensity values than the normal strips.

Detection of TPSA

When using TPSA solutions in 5 mM PBS, the detection limit was determined as 0.04 ng/mL. The control results involved the normal sandwich-type method in comparison with the enhancer-based immunochromatographic test strip. In the normal sandwich-type immunochromatographic test strip, the limit of detection was 1 ng/mL. Similarly, we examined the performance of our system with human serum samples. When we prepared serial spiked dilutions (5, 1, 0.2, 0.04 ng/mL) of TPSA in human female serum, the detection limit was 0.2 ng/mL (Fig. 6). Figure 6e shows the dependence of the signal intensity values on the concentration of TPSA for the normal and the enhancer-based strips. The enhancer-based strips clearly showed higher intensity levels than the ones observed from the normal strips. However, in the normal sandwich-type immunochromatographic test strip, the limit of detection was 1 ng/mL of TPSA in human female serum. The detection of TPSA from the serum samples with high

sensitivity is very promising for rapid diagnosis in the presence of the patient.

Conclusions

The enhancer-based immunochromatographic assay was applied for the visual detection of HCG and TPSA with a higher sensitivity than the normal test strips. The rapid observation of clear results that can be seen with the naked eye ensures the convenience of performing these tests in the presence of the patient. Therefore, our enhancer-based immunochromatographic assay provides an attractive biosensor without the handling of toxic reagents, while allowing an easy and rapid procedure.

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