Gold nanoparticle based immunochromatography using a resin modified micropipette tip for rapid and simple detection of human chorionic gonadotropin hormone and prostate-specific antigen

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Abstract

A novel bioanalysis system based on immunochromatography was developed in connection with a nitrocellulose resin-modified micropipette tip, namely as ZipTip®. The sandwich-type immunoassay was applied to our bioanalysis system. The first antibodies that were aspirated by the micropipette were immobilized on the immunochromatographic resin at the edge of micropipette tip. The blocking solution was also aspirated in the same fashion. The measurement operation was performed by aspirating the sample solution, and then the gold colloidal nanoparticles (Au naps) conjugated secondary antibody solution. Since this bioanalysis system utilizes a micropipette, it is possible to increase the sample volume, which would enable the detection of antigen at low concentrations. In addition, the washing procedure can also be performed easily to reduce the background level. After the antigen-antibody reaction, the color intensity of Au naps could be observed by the naked eye. For analytical evaluation, the color intensity was captured by a scanner, and processed by analysis software. We have achieved the detection of human chorionic gonadotropin hormone (hCG) and total prostate-specific antigen (TPSA), which are well-known as fundamental indicators of pregnancy and cancer. The limit of detection (LOD) for hCG was 8 ng/ml (0.8 ng/tip), which is comparable to that of other conventional systems based on immunochromatography. Moreover, the LOD for TPSA was improved over the existing systems with the application of different sample volumes, such as 5 ng/ml (1 ng/tip) in 200 µl sample volume, and 2 ng/ml (0.6 ng/tip) in 300 µl sample volume. Since our bioanalysis system requires a small amount of immobilized antigen, it would be greatly useful in basic research for screening the antigen–antibody reactions. Besides, our bioanalysis system can be applied to on-field screening, since its operation is simple, and the visual results can be obtained rapidly.

Keywords: Immunochromatography; Human chorionic gonadotropin (hCG); Prostate-specific antigen (PSA); Gold colloidal nanoparticles; Biosensor

1. Introduction

The development of biosensors is spreading widely in various fields including clinical, environmental and forensic applications [1]. An immunochromatographic test strip is a good candidate for the development of decentralized biosensors. Various kinds of bioanalytical systems based on immunochromatographic test strips have been developed in the past 40 years, as simple-handling systems [2]. Since the test strips based on immunochromatography can be widely used in order to detect the presence of specific analytes visually in a short analysis time, they have been applied to design several biosensors.

Test strips for bioanalysis based on gold colloidal nanoparticles (Au naps) as the visual indicator have already been commercialized. The resonance absorbance of Au naps in visible spectroscopy has made them suitable labels for molecular detection in immunochromatography, microscopy and electrophoresis blotting techniques [3,4]. Au naps are more stable and easy to use than the conventional systems utilizing fluorescence or enzymatic labels. Moreover, nano-scale surfaces of Au naps are appropriate for accelerating the antibody-antigen recognitions, which enhances the
immunoassay signals [5]. The rapid observation of results directly by naked eyes ensures the convenience of performing bioassays on-field. Therefore, colloidal gold-based immunochromatographic assays have provided attractive means for developing biosensors without the handling of toxic reagents, while allowing an easy and rapid procedure [2,6–9].

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 Au naps immunocomplex and the primary antibody immobilized on the membrane. After the antigen–antibody reaction, a red color, caused by the accumulation of Au naps at that location, appears on the membrane [10]. Through these detection processes, the results come out in approximately 10 min after the sample introduction. On the other hand, the conventional test strips based on immunochromatography have several disadvantages. In the fabrication procedure, huge volumes of antibody solution are needed for the immobilization onto the nitrocellulose membrane. In addition, the antibody needs to be immobilized onto the membrane uniformly; therefore, a precise dispensing system for exclusive use is required. Furthermore, the antibody-immobilized nitrocellulose membrane is obtained after several processes involving washing and blocking. It is, therefore, desirable to develop a simple method to reduce the use of excessive antibody.

Test strips for pregnancy diagnosis in urine based on the immunochromatographic detection of human chorionic gonadotropin hormone (hCG), are already commercially available. The hCG is produced by the developing placenta shortly after the fertilized egg. This glycoprotein is composed of α- and β-subunits. The α-subunit, which is 116-amino acid sequence, is identical with that of glycoprotein hormones’ α-chain family; luteinizing hormone, follicle-stimulating hormone (FSH) and thyroid-stimulating hormone. The β-subunit, a 132-amino acid sequence, is unique to hCG, and specific tests for it are not subject to hormonal cross-reactivity.

Recently, several detection kits based on immunochromatography have become commercially available for the detection of cancer marker, prostate-specific antigen (PSA), or some of the allergens in foods [11]. We have also employed total PSA (TPSA) as a measurement subject in our bioanalysis system. PSA is an intracellular glycoprotein (34,000 Da kallikrein-like protease) synthesized only by the prostate gland. PSA is a normal constituent of prostatic tissue, and is also found in metastatic prostatic carcinoma, prostatic fluid, and seminal plasma. PSA in serum exists both as free PSA (fPSA) and antichymotrypsin complexed with PSA (ACT–PSA) [12–14]. Total PSA (TPSA) is defined as the combination of both fPSA and ACT–PSA. The cut-off limit of TPSA between prostate hyperplasia and cancer is 4 ng/ml [15].

In order to improve the complicated fabrication procedure and reduce the sample volumes, we developed a simple bioanalysis system based on immunochromatography using ZipTip® in this report. The ZipTip® is usually used for concentration and washing of protein solutions. In this study, the ZipTip® is applied in a different way from its ordinary usage. Namely, the immunochromatographic resin, which is placed at the edge of the tip, was used as the solid support for antibody immobilization.

2. Materials and methods

2.1. Materials

Monoclonal anti-human α-subunit of follicle-stimulating hormone (Mab-FSH) and monoclonal anti-human chorionic gonadotropin (Mab-hCG) were purchased from Medix Biochemica (Kauniainen, Finland). The recombinant human chorionic gonadotropin (hCG) was purchased from Rohto Pharmaceutical Co., Ltd (Tokyo, Japan). Monoclonal anti-human total prostate specific antigen (Mab-TPSA) antibody 4D10 and N0.56 were purchased from Japan Clinical Laboratories, Inc. (Kyoto, Japan). The recombinant human total prostate-specific antigen (TPSA) was purchased from CosmoBio Japan. For fabrication of the bioanalysis 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 of antibody-immobilized nitrocellulose resin, which was filled into the ZipTip® edge, was purchased from Sigma Aldrich Japan (Tokyo, Japan). Sodium azide (NaN3) was purchased from Nakarai Tesque (Kyoto, Japan) for preserving the proteins in blocking and diluting solutions. Au naps with different diameters were purchased from British BioCell International (Cardiff, UK). ZipTip®MC was obtained from Nihon Millipore (Tokyo, Japan).

2.2. Apparatus for the quantitative determination of the antigens

After the antigen–antibody reaction on ZipTip®, the color density images were scanned by EPSON EU-34 from Seiko Epson (Nagano, Japan). The scanned images were then converted into gray scale readings by Adobe™Photoshop™5.5. The intensities of each signal were quantified with Image SMX vol. 1.74. At a random point of color intensity, measurements were taken for more than six times per one tip. The average of the color intensities were calculated for each concentration of hCG and TPSA.

2.3. Immobilization of primary antibody onto nitrocellulose resin

ZipTip® is attached to the micropipette like a disposable tip for aspirating and dispensing the solution. Monoclonal anti-human α-subunit of follicle-stimulating hormone (Mab-FSH) solution at 100 µg/ml was prepared by diluting with 50 mM phosphate buffered saline (PBS, pH 7.4). By using the micropipette, 10 µl of diluted antibody solution was aspirated and dispensed for 10 times (ZipTip®MC: absorption capacity 400 ng/tip) (Fig. 1a). For TPSA detection experiment, 100 µg/ml of Mab-TPSA solution...
was prepared by diluting with PBS, and treated in the same way as described above. The immobilization of antibodies was carried out simply by this pipetting operation. Afterwards, antibody-immobilized ZipTip\textsuperscript{®} was kept on ice for 1 h. Blocking solution (1% (w/v) BSA and 5% (w/v) sucrose in PBS) was aspirated, and dispensed for 10 times to block the non-specific adsorptions (Fig. 1b). Finally, antibody-immobilized and blocked ZipTip\textsuperscript{®} was kept at 4°C before use.

**2.4. Preparation of Au naps-conjugated Mab-hCG and antigen solutions**

Mab-hCG solution at 50 μg/ml was prepared by diluting with 5 mM KH\textsubscript{2}PO\textsubscript{4} solution (pH 7.5) in ultra pure water (18.3 MΩ cm) to a final volume of 250 μl. The diluted Mab-hCG antibody solution at 200 μl was added in 1.8 ml of Au naps solution (0.01%, w/v) and mixed immediately. The mixture was kept for 10 min at room temperature (RT) for the immobilization of antibody onto the Au naps’ surfaces. After immobilization, 100 μl of 1% (w/v) PEG in 50 mM KH\textsubscript{2}PO\textsubscript{4} solution (pH 7.5) and 200 μl of 10% (w/v) BSA in 50 mM KH\textsubscript{3}PO\textsubscript{4} solution (pH 9.0) were added for blocking the non-coated surfaces. After the immobilization and blocking procedures, Au naps-conjugated Mab-hCG was obtained by centrifugal operation (8000 g for 15 min at 4°C). The Au naps were pulse-sonicated for a few seconds, and added to 2 ml of preserving solution (1% (w/v) BSA, 0.05% (w/v) PEG 20000, 0.1% (w/v) NaN\textsubscript{3} and 150 mM NaCl that were dissolved in 20 mM Tris–HCl buffer, pH 8.2). After mixing, Au naps-conjugated Mab-hCG was collected by the same process as described above. After pulse-sonication, the Au naps-conjugated Mab-hCG solution was diluted with the preserving solution to OD\textsubscript{520} 6. To compare the visual sensitivity, the Au naps with diameters of 15, 40 and 80 nm were selected. The hCG was diluted with 1% (w/v) BSA and 0.01% (w/v) NaN\textsubscript{3} in PBS to 0–50 ng/ml. The volumetric ratio of Au naps-conjugated Mab-hCG solution to the solution of hCG was 1:10 (v/v) (100 μl/tip). Au naps-conjugated Mab-hCG solution and hCG were aspirated and dispensed slowly for 10 times (Fig. 1c).

**2.5. Preparation of Au naps-conjugated Mab-TPSA and antigen solution**

In this research, the Au naps-conjugated Mab-TPSA solution was prepared by the same procedure as the preparation of Au naps-conjugated Mab-hCG solution. The concentration of Mab-TPSA solution used in this procedure was 65 μg/ml diluted with 5 mM KH\textsubscript{2}PO\textsubscript{4} solution (pH 7.5) to a final volume of 250 μl. The Au naps-conjugated antibody solutions were applied to the antigen–antibody reaction. Different concentrations (0–100 ng/ml) of TPSA solutions were also used for the optimization of recognition reactions.

**3. Results**

**3.1. Evaluation of the sensitivity using Au naps with different sizes**

The different diameters (15, 40 and 80 nm) of Au naps were used for the evaluation of the sensitivity of hCG. The calibration curves are shown in Fig. 3. The color density increased with the increasing antigen concentration. We detected that the color density became higher when the Au naps with the diameter of 40 nm. In view of these results, we pursued further experiments by using 40 nm Au naps for the conjugation of antibodies. The results of hCG analysis using 40 nm diameter of Au naps are shown in Fig. 2. The calibration curves are shown in Fig. 3. The minimum antigen concentration, which made the color of detection line disappear, but...
caused a strong red band at the control line, was determined to be the limit of detection (LOD) for our visual tests. The LOD of our bioanalysis system for hCG at a concentration of 8 ng/ml, (0.8 ng/tip) was almost similar to that of the commercially available test strips.

3.2. Detection of TPSA

We have applied our bioanalysis system to the detection of TPSA. The Au naps of diameter 40 nm was used for conjugation. The result of color density for TPSA is shown in Fig. 4. As seen in the case of hCG, the color density increased with the increasing antigen concentration. In TPSA detection, a severe sensitivity level is required. Our proposed bioanalysis system can be applied in such a situation by simply increasing the sample volume. The density results for different sample volumes at trace TPSA concentrations are compared in Fig. 5. We applied different volumes of samples: at 100 µL (black diamond), 200 µL (gray circle) and 300 µL (white triangle) for TPSA, and 100 µL for hCG (black circle). We clearly observed that the detection ability got significantly improved to 2 ng/ml (0.6 ng/tip) for TPSA, when the sample volume increased up to 300 µL.

4. Discussion

In this research, the LOD of our bioanalysis system is compared with that of conventional test strips (~5 ng/ml). For this purpose, we employed clinically important model proteins, such as hCG and TPSA. First, sensitivity of the result was compared using gold colloids with different diameter in a case of hCG detection (Fig. 3). Au naps at 40 nm was also the preferred size used in the development of commercially available test strips. The tendency of color intensity variation was similar to the result of a test strip (data not shown). It was reported that the larger the size of Au naps, the higher the signaling efficiency [2]. In addition, it was reported that the intensity might have appeared weak, if the color of small size Au naps had become blue [16–18]. Au naps at 80 nm, which were conjugated with the secondary antibody, might have less space to bind with the antigen, and accumulate at an enough amount in that location for us to observe with the naked eye, whereas 40 nm Au naps could accumulate far better than the rest of the Au naps, and cause a rapid color change in the antigen binding location.

The affinities of the antibodies (Mab-hCG and Mab-TPSA) towards their antigens show a discrepancy. Therefore, the sensitivity of our system was directly affected by their affinity. 

![Fig. 3](image1.png)

Fig. 3. (A) Dependence of color density on the concentration of hCG by using different sizes of Au naps in conjugation with Mab-hCG. (B) Color density dependence for the detection of hCG at low concentrations by using different sizes of Au naps in conjugation with Mab-hCG.

![Fig. 4](image2.png)

Fig. 4. (A) Dependence of color density on TPSA concentration. Mab-TPSA was conjugated with 40 nm Au naps. (B) Color density dependence for the detection of TPSA at low concentrations.

![Fig. 5](image3.png)

Fig. 5. Comparison of color densities for each TPSA concentration while increasing the sample volume at 100 µL (black diamond), 200 µL (gray circle), 300 µL (white triangle), hCG at 100 µL (black circle). The density result for TPSA was greatly improved, when the sample volume was increased up to 300 µL.
levels. TPSA exists in male serum at about 1 ng/ml, and the value of TPSA increases along aging even in healthy male [19]. Clinically, TPSA value 4–10 ng/ml is called as gray zone, which is suspected as a symptom of early prostate cancer [20]. After all, TPSA assay requires detection of low concentration antigen [21], while for hCG assay detection of antigen (3 μg/ml) is considered to be enough even in early pregnancy female urine or none in non-pregnant (on/off format). Therefore, the LOD of TPSA was pursued in this report. The sample volume was increased in order to be able to detect TPSA under 5 ng/ml concentrations (Fig. 5). By increasing the sample volumes, a visible increase in the color density was observed with 5 ng/mL TPSA. In our system, it was quite easy to increase the sample volumes, in contrast to using conventional test strips. Note that there was only a negligible change in the background density level, even when the sample volume was increased significantly. The LOD of our bioanalysis system at 2 ng/ml is comparable with that of conventional test strips for TPSA detection.

This bioanalysis system has several advantages in comparison with the conventional immunochromatographic bioanalysis systems. First advantage is the short fabrication time. According to the protocol of ZipTip®, the whole fabrication procedure involves the adsorption of primary antibodies on the resin inside of ZipTip®, which takes place rapidly by simple pipetting. The test results in which ZipTip® was used immediately after immobilization of antibody and blocking procedures showed almost similar results as those obtained by the procedure described in Section 2. This enables us to visually check antigen–antibody reactions in short time, which would be crucial for the screening procedures during antibody production. Secondly, one ZipTip® needs only 10 μl of 100 μg/ml of antibody solution for immobilization onto the resin surface. This feature is also convenient in checking antigen–antibody reaction with a limited antibody sample volume in research. Moreover, the sample volume is easily variable, since ZipTip® is attachable to a micropipette. In the case of measurements with low concentration solutions like environmental and/or clinical samples, to aspirate more volume of the sample solutions would make the visual judgment easy. Furthermore, the washing procedure can also be performed by pipetting. By using other types of ZipTip® with less resin filled inside, we are planning to detect antigens in less volume of sample solutions.

Our bioanalysis system is applicable to many kinds of sensing systems, in which test strips have been used to detect certain infectious diseases or cancer [22,23]. Other promising areas would be drug monitoring, food safety, and on-field screening [23]. As the need for rapid, sensitive, user-friendly on-field testing grows, easy handling and rapid response of our system would be a promising candidate for the development of a sensitive biosensor.

5. Conclusions

This study demonstrates the high applicability of immunochromatographic micropipette tip (ZipTip®) for antigen detection based on a sandwich-type assay. This novel bioanalysis system is likely to find a variety of applications in clinical diagnosis, environmental measurements, and forensic research. The major advantage of this bioanalysis system is the ability of immediate preparation with a small amount of primary antibody. Research in our laboratory is in progress towards the detection of clinically important biomolecules by using our immunochromatographic system.

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