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Quantitative Lateral Flow Immunoassay FOR Total Prostate Specific Antigen in Serum

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Abstract

A simple method for the rapid determination of prostate-specific antigen (PSA) in serum is reported using a lateral flow immunoassay with gold nanoparticles as the label. The method uses the intensity of colored test lines to determine PSA from 0.3 to 30 ng/ml. The limit of detection was 0.3 ng/ml and the coefficient of variation was less than 10%. The analysis time was approximately twenty minutes. The novel method showed good correlation with enzyme linked immunosorbent assay (ELISA) measurements of prostate-specific antigen concentration in human serum with a linear regression coefficient of 0.985. The developed system was stable for at least twelve months when stored from +4 to 30°C and has potential application for clinical practice.

Keywords

INTRODUCTION

For successful treatment of oncological diseases, early diagnosis is important, allowing therapy to begin at the early stages. One approach uses total screening of the population for the detection and quantification in human blood with tumor markers that indicate the presence of cancer (Duffy 2013). For these assays simple, rapid, highly sensitive, and reliable diagnostic methods are needed.

Prostate specific antigen is a serine protease (glycoprotein) with a molecular mass of about 33 kDa produced by prostate gland cells. Currently in medical practice it is considered as the generally accepted marker of prostate cancer in men. Serum protein exists in two forms – free and complexed with other proteins, mainly with α_1-antichymotrypsin (Lilja, Cockett, and Abrahamsson 1992). An increase in the concentration of total PSA level above 3–4 ng/ml indicates the possible presence of prostate cancer (Gann et al. 2002). In this case, additional

study is needed, for example, to determine ratio of total and free PSA concentrations in the blood. When serum total PSA level is higher than 30 ng/ml, the probability of prostate cancer is almost 100%.

In recent years, in order to rapidly identify biologically active compounds and diagnose disease, visual immunochromatographic assay techniques have been widely used. These methods are characterized by a short analysis time (10–20 min) and a simple determination procedure that does not require expensive equipment or highly trained personnel, allowing analysis in residential locations with high sensitivity, visual characterization of the results, and low cost (Rosen 2009; Posthuma-Trumpie, Korf, and van Amerongen 2009; Drygin et al. 2009). This approach is based on using a membrane carrier containing the reaction components needed for analysis. The most common of these methods is the lateral flow immunoassay, also known as immunochromatographic assay, in which gold nanoparticle labels are often used to provide a visual detection of the results (Dykman et al. 2008).

Currently available various test-systems of immunochromatographic determination of PSA are mainly based on using gold nanoparticles as the label (Liubavina et al. 2007; Fernández-Sánchez et al. 2005). Fluorescent labels are also used in these assays (Yoo et al. 2010). Most of the existing express tests only allow qualitative assessment of the results; quantitative analysis results generally requires special, proprietary, and expensive equipment. The goal of this study was to develop a simple, sensitive method for the determination of prostate specific antigen concentration in human serum by a lateral flow immunoassay for detecting prostate cancer risk.

EXPERIMENTAL

MATERIAL AND METHODS

Biochemical and general chemical reagents, bovine serum albumin (BSA), sodium citrate, and pH test strips were purchased from Sigma (USA); chloroauric acid was obtained from Fluka (Switzerland). Two types of monoclonal antibodies against prostate specific antigen (capture – Ab150 and detecting – Ab30), PSA, sheep antimouse IgG antibodies were provided by JSC NVO Immunotek, Russia). All membranes for the immunochromatographic assays were purchased from MDI (India) including an analytical nitrocellulose membrane – CNPC (15 μ), glass fiber conjugate pad membrane – PT-R5, sample pad membrane – GFB-R7L (0.6), and absorbent pad – AP-045.

Standard PSA solutions with concentrations of 0, 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10, and 30 ng/ml were prepared in PSA-free blood serum. Serum was provided by JSC NVO Immunotek; ELISA kits were used for quantitative determination of total PSA concentration according to the manufacturer instruction (ZAO "NVO Immunotek", Russia, reg. Nr. TY 9398-341-11361534-2004).

Preparation of Colloidal Gold Nanoparticles

Solutions of colloidal gold nanoparticles of a given size were obtained according to Frens (1973). Briefly, 100 ml of 0.01% chloroauric acid were heated to boiling in a flask with a magnetic stirrer; 1, 1.6, 2 or 2.4 ml of 1% aqueous solution of sodium citrate (depends on desired size of gold nanoparticles) were rapidly added with stirring and heated for an additional 15 minutes, and cooled to room temperature in the dark. The absorption spectra were recorded from 400 to 700 nm.

Determination of Optimal Conditions for Antibody Adsorption on Gold Nanoparticles

Optimization was performed in ninety-six well polystyrene plates (Greiner, Austria). Each row and each column corresponded to a certain pH value and specific antibody concentration, respectively. The pH values of the aliquots (2.5 ml) of the colloidal gold suspension were adjusted from 5.5 to 8.5 by increments of 0.5 with 0.2 M sodium carbonate. Colloidal gold solution (200 μL aliquots) and antibody solutions with concentrations of 0, 30, 60, 120, 180, and 360 μg/ml prepared in deionized water (20 μL aliquots) were added to the wells. After the incubation of the plate for 15 minutes with stirring at room temperature, 50 μL of 10% NaCl solution were added to each well. The absorption in the wells of the plate was measured at 520 and 580 nm.

Preparation of Gold Nanoparticles Conjugated with Antibodies

The solution of detecting antibodies (Ab30, 1 ml aliquots) with a specific concentration was added dropwise to 10 ml of colloidal gold solution (pH 6.0–6.5) under stirring, and the mixture was incubated for 30 minutes at room temperature under stirring. The resulting solution was supplemented with bovine serum albumin (final concentration, 0.1%), sucrose (final concentration, 10%), and sodium azide (final concentration, 0.01%), and stored at 4°C. To remove unbound antibodies, the conjugate solution was centrifuged at 11,000 × g for 20 minutes at 4°C. The supernatant was discarded and the pellet was redissolved in the required volume of 0.01 M potassium phosphate buffer, pH 7.0 supplemented with 0.1% bovine serum albumin, 10% sucrose, and 0.01% sodium azide.

Immunochromatographic Composite Assembly

A solution of specific capture antibodies (Ab150) in 0.01 M potassium phosphate and 0.15 M NaCl, pH 7.2–7.4 was applied on the analytical nitrocellulose membrane CNPC

(15 μ) glued on a plastic backing card (260 × 75 mm), using a BioDot XYZ 3050 programmable automatic dispenser (BioJet Quanti 3000, BioDot, United States) to generate the analytical zone. To form the control zone of the immunochromatographic system, a solution of sheep antimouse antibodies (1 mg/ml in the same buffer) was added 5 mm above the analytical zone. Samples were applied using a BioJet Quanti 3000 pump with the following parameters: droplet size, 30 nL; pitch, 0.3 mm; velocity, 50 mm/s; and deposed amount 1 μL/cm . The strips were dried at 37°C and relative humidity of 25 to 30%) for 24 hours and stored at room temperature in a sealed package.

The solutions of antibodies conjugated with colloidal gold nanoparticles (A_{520} ~ 2 optical units) were applied on a glass fiber membrane PT-R5 (260 × 5 mm) and air dried at room temperature. The test cassette (260 × 75 mm), assembled in accordance with the scheme shown in **Figure 1** was prepared with an Index Cutter-I (A-point Technologies, Gibbstown, USA), and the resulting test strips (4 × 75 mm) were stored from 4 to 30°C in a sealed package with silica gel.

Immunochromatographic Assay Procedure

Standards of PSA solutions or serum to be analyzed (30 μL) were applied on a sample pad GFB-R7L(0.6) of ready-to-use test strips and placed in the wells of a polystyrene plate for ELISA containing 200 μL of 0.01 M potassium phosphate, 0.15 M NaCl, and 0.1% Tween 20, pH 7.4. After 15 minutes of incubation, the test strips were placed on a horizontal surface and evaluated. To quantify the results of the analysis, the test strips were scanned with an Epson Perfection V700 Photo scanner (Seiko-Epson, Japan). Images obtained in the form of graphic files (.tif, RGB, 24 bits, 600 dpi) were processed using the Scion Image program. The calibration

curves of band intensity (as arbitrary units) as a function of the concentration of standard PSA solutions were constructed.

For the detection limit evaluation, a series of PSA standards were measured. The detection limit of the method (ng/ml) was deemed to be the minimal concentration of PSA resulting in the appearance of a stained band in the test zone that may be reliably visually registered.

RESULTS AND DISCUSSION

A sandwich type immunochromatographic assay for the determination of prostate-specific antigen (PSA) in serum was developed. The immunochromatographic test strip for the assay represents successively glued membranes assembled on a plastic backing card (**Figure 1**). The sample moves along the strip due to capillary forces. In the presence of PSA, the complex of the antigen with specific antibodies labeled with gold nanoparticles, which are adsorbed on a glass fiber membrane, begins to form. Then, the formed immunocomplex binds to the specific antibodies immobilized in a narrow line in the test zone, which is accompanied by the appearance of a stained band. The intensity of the band is proportional to the analyte concentration in the sample. The unbound labeled antibodies migrate further and form the second colored line in a control zone. In the absence of PSA, gold labeled antibodies are only retained by the secondary antibodies in the control zone.

Colloidal Gold Preparation

Gold nanoparticles were prepared according to the Frens (1973) method, based on chloroauric acid reduction with sodium citrate. The approach is easy to implement and provides homogenous gold nanoparticles (from 8 to 40 nm in diameter). Depending on the ratio of the

initial concentrations of the reaction components, four sizes of gold nanoparticles were obtained (**Table 1**). The average size of the resulting nanoparticles were characterized by scanning electron microscopy (Carl Zeiss, Germany) and had diameters of 12, 15, 20, and 35 nm. As an example, **Figure 2** shows a micrograph of the 12 nm colloidal gold nanoparticles. The particles were spherical with a rather narrow size distribution. With increasing size of the nanoparticles, the absorption peak of the colloidal solution shifted to longer wavelengths from 517 to 528 nm, which is consistent with the literature (Khlebtsov et al. 2007). The color of the colloidal gold solution was pink-red for the smallest particle size (12 nm) and purple for the largest particle size of (35 nm).

Colloidal gold Conjugates with Antibody Preparation

The production of antibodies labeled with gold nanoparticles remains quite a complex process, due to the need for retaining the antigenic activity of antibodies upon the adsorption on the gold surface and the stability of the nanoparticles themselves (Horisberger 1990). The flocculation of gold nanoparticles and nonadsorption of proteins are solved through varying of protein concentrations and pH values to determine the optimal adsorption conditions. At acidic pH, proteins are charged positively and the attraction forces with negatively charged gold nanoparticles are sufficiently strong for one protein molecule to be bound by several nanoparticles, forming large aggregates that precipitate. At pH values around the pI of a given protein, the attraction of the protein for gold nanoparticles based on charge has decreased. Assuming the lowest solubility of an aqueous solution for a given protein to be near to its pI, the weakly hydrated protein may more readily adsorb to the hydrophobic surface of gold nanoparticles. As the pH values increase above the pI, repulsion between the negatively charged

proteins and gold nanoparticles and an increase in the hydration of the proteins combines to favor dissolution rather than adsorption of the solute (Geoghegan and Ackerman 1977).

First, it was necessary to select the optimal minimum stabilizing concentration and pH of adsorption for obtaining stable detecting antibodies (Ab30), labeled with gold nanoparticles, to effectively interact with PSA. The optimal pH values and the concentration of antibodies were selected by cross titration. A series of colloidal gold solutions with pH values from 5.5 to 8.5 was prepared in increments of 0.5 units, to which specific antibodies at concentrations from 0 to 30 μg/ml were added. An increase in ionic strength as a result of the addition of NaCl led to the aggregation of gold nanoparticles, which was accompanied by a change in the color of the solution from red to gray–blue or to colorless. For stabilized gold nanoparticles, the characteristic plasmon resonance peak was near 520 nm, and the optical density at 580 nm corresponds to the aggregated state (Geoghegan 1988). Therefore, to determine the quantitative effect of stabilization, it was necessary to calculate the difference in the absorbance of the solution at 520 and 580 nm. **Figure 3** shows the data obtained for the Ab30 monoclonal antibody specific to PSA. The results obtained for the other gold nanoparticles of different size were similar. As the concentration of antibodies increased, the effect of pH (starting from 7.0) on stabilization decreased. To obtain labeled antibodies, a pH range of 6.0–6.5 was selected as optimal.

In the studied range of the sol load with antibodies (0–30 μg/ml), the minimum saturating concentration of antibodies that ensured the maximum resistance of the sol to the coagulating effect of the electrolyte corresponded to the point at which the curve reached a plateau; for 12, 15, 20 and 35 nm diameter gold nanoparticles, the value was 10 μg/1 ml of sol (**Figure 4**). These concentrations were used to prepare the conjugate for detecting Ab30 antibodies with gold

nanoparticles. Excess unbound antibodies were removed by centrifugation. To prevent coagulation of labeled gold nanoparticles during centrifugation, and further the process of applying and drying on a glass fiber membrane, 10% sucrose was added. The stabilizing effect of sucrose is associated with additional protection of hydration shells around gold nanoparticles by retaining water molecules.

Immunochromatographic Assay

The resulting complexes of gold nanoparticles with detecting antibodies were analyzed by a standard immunochromatographic assay by determining the concentration of PSA. The highest measured assay signal was observed for the 35 nm gold nanoparticles (**Figure 5**). This agrees with literature data, where an increase in the extinction coefficient of gold nanoparticle solutions was reported with increasing particle size (Liu et al. 2007). Visually the test lines on the strips using such gold nanoparticles most clearly appeared. This may be due to the fact that human eye perceives a better contrast for the purple color of the larger particles. On the other hand, the higher intensity coloration test strip zones may be due to an increase in the number of IgG molecules bound to the larger diameter gold nanoparticles (Safenkova, Zherdev, and Dzantiev 2010). In this regard, all the following experiments were carried out with a colloidal gold solution with a particle diameter of 35 nm.

In the course of further work, the influence of the concentration of specific immunoreagents on the analysis results was studied. It was shown that with an increase in concentration of the Ab150 capture antibodies and the amount of labeled Ab30 antibodies, the intensity of the signal and the slope of the calibration curve were increased. This behavior is typical for sandwich immunoassays. However, the increase in concentration of capture

antibodies was limited to the capacity of an analytical membrane (~2 mg/ml for IgG according to manufacturer's data), as well as the appearance of the background determined in the absence of the antigen. So, the following concentrations were used as optimal: 1 mg/ml Ab150 capture antibodies and Ab30 detecting antibodies labeled with gold nanoparticles – 7 μl solutions (A_{520} ~ 2 optical units) per strip. A typical calibration curve using the system is shown in **Figure 6**.

The detection limit of the method was 0.3 ng/ml PSA in serum. This corresponds well to the sensitivity of commercial ELISA kits (0.1–03 ng/ml) used in clinical practice. The developed method allowed the determination of PSA from 0.3 to 30 ng/ml. The method offers good accuracy and reproducibility of the results. The coefficients of variation for PSA concentrations of 0.6, 2.5, 5, and 10 ng/ml were 1.67, 4.31, 4.82, and 5.55% within one day (n = 10, P = 0.95) and 3.13; 6.57; 9.80 and 8.05% between days (n = 3, P = 0.95), respectively. The analysis time was 15–20 minutes.

The stability of the developed test system was characterized. The system was stable for at least 12 months at room temperature. A comparison of the calibration curves obtained for a freshly prepared test-system and after storage showed they were nearly identical (**Figure 6**).

To validate the method, comparative determination of PSA concentrations in patient's blood serum using a standard commercial ELISA kit reagents was performed. Fifty samples were analyzed, including 28 with a concentration ≤4 ng/ml (normal), eleven samples with a concentration of 4–10 ng/ml (suspicious region), and eleven samples with a high concentration of PSA (>10 ng/ml). The results by the developed system were in good agreement with the

ELISA measurements. The Pearson's correlation coefficient (R) was 0.985, demonstrating the reliability of determining PSA.

CONCLUSIONS

A sensitive test system for the determination of prostate specific antigen by lateral flow immunoassay with gold colloidal nanoparticles as the label was designed and optimized. The method is simple to use with an analysis time of approximately 15 minutes. Quantitative evaluation of the results does not require special equipment and highly qualified staff, and may be easily performed by using a household scanner and computer. Particular attention was paid to theoretical and practical aspects of general approach to the creation of stable and active gold colloidal labeled proteins. Good correlation between the results of PSA determination by the developed method with those obtained for the same samples by ELISA suggests potential applicability of this system in clinical and laboratory practice with conventional techniques.

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Table 1. Characterization of gold colloidal nanoparticles as a function of size.

Volume (ml) of 1% (w/v) trisodium citrate added to 100 ml of 0.01% HAuCl4	Diameter of gold nanoparticles (nm)	Absorption maximum (nm)	Color
2.4	12	517	Pink-red
2.0	15	518	Red
1.6	20	520	Intensive red
1.0	35	528	Purple

Figure 1. Lateral flow test scheme.

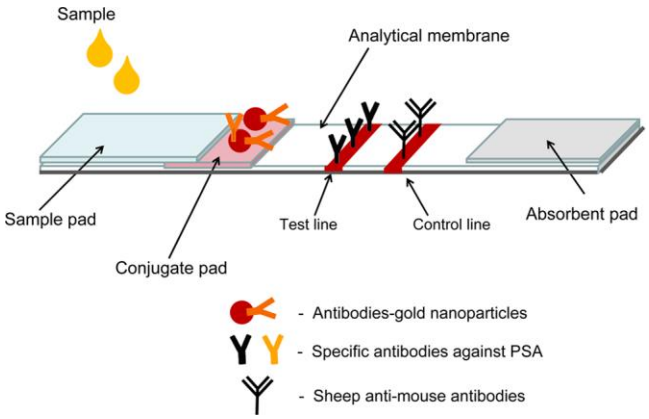

Figure 2. Scanning electron micrograph of 12 nm average diameter gold nanoparticles.

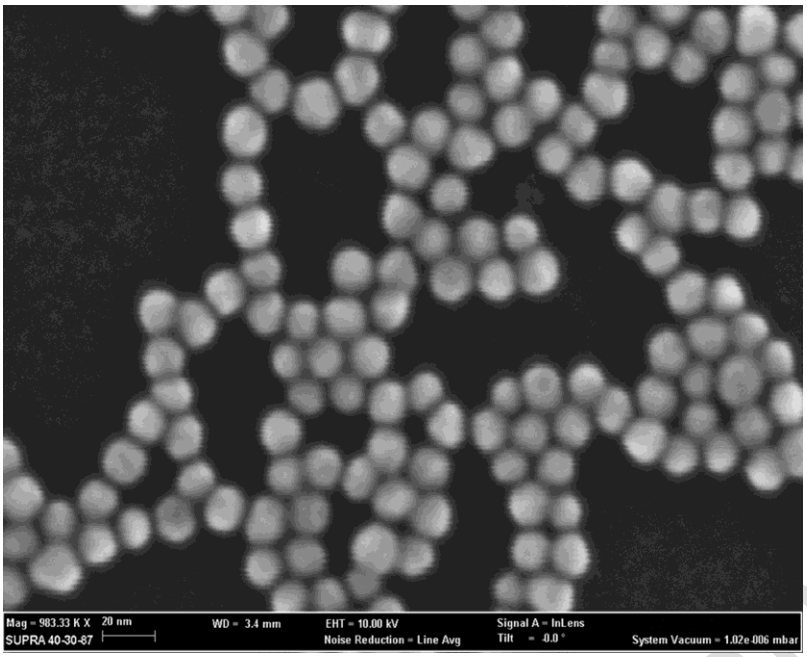

Figure 3. Dependence of absorbance difference at 520 and 580 nm as a function of pH using 12 nm gold nanoparticles for Ab30 antibody concentrations of (1) 8 µg/ml, (2) 10 µg/ml, and (3) 20 µg/ml.

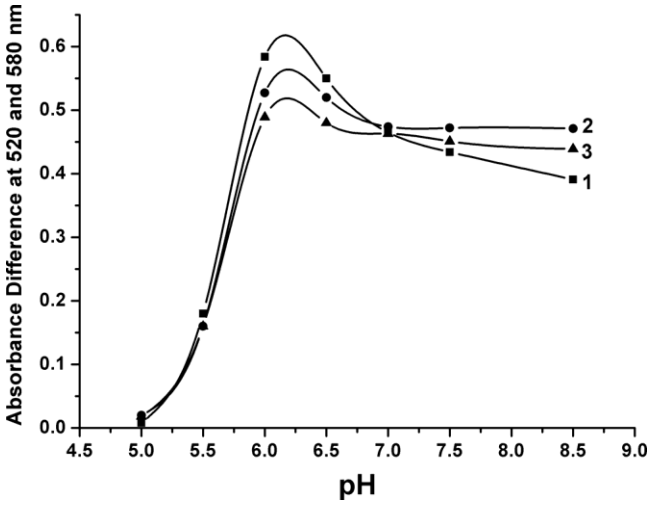

Figure 4. Dependence of absorbance difference at 520 and 580 nm at pH 6.0 as a function of Ab30 antibody concentration for gold nanoparticles with diameters of (1) 12 nm, (2) 15 nm, (3) 20 nm, and (4) 35 nm.

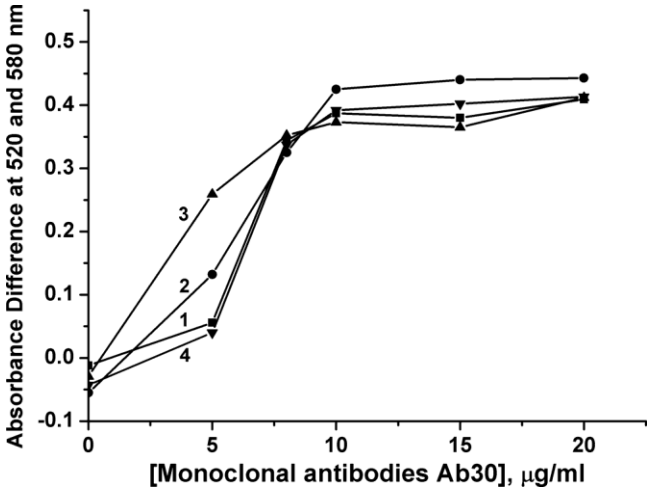

Figure 5. Calibration curves for Ab30 antibodies labeled with gold colloidal nanoparticles with diameters of (1) 12 nm, (2) 20 nm, and (3) 35 nm. Conditions: nitrocellulose membrane, 1 mg/ml Ab150 capture antibodies.

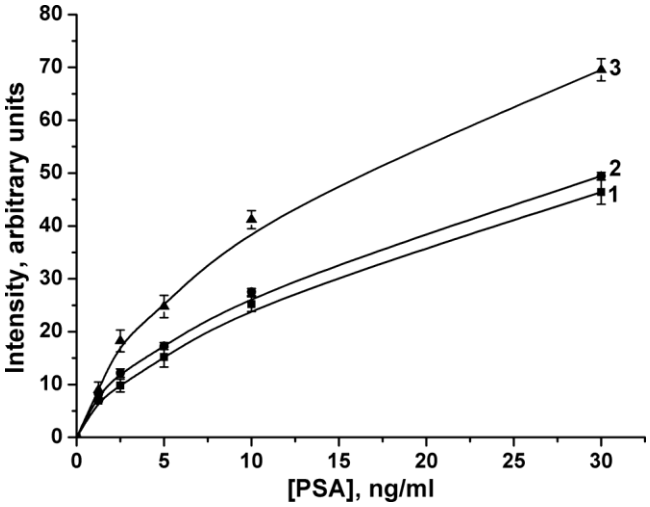

Figure 6. Calibration curves for prostate specific antigen (PSA) using a (1) freshly prepared system and (2) system stored for 12 months at room temperature. Standards were prepared using PSA-free serum.