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RESEARCH ARTICLE

Rapid Bispecific Antibodies Based Homogeneous Immunoassay for Detection of Prostate-Specific Antigen (PSA)

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Abstract

Development of rapid and economical method for detection of prostate-specific antigen (PSA) in human blood.

Methods: The usual procedure for the detection of prostate cancer markers in human is prostate-specific antigen (PSA) in blood (normal level ≤ 4 ng/mL) using heterogeneous immunoassay enzyme linked immunosorbent assay (ELISA). However, a rapid homogeneous immunoassay for the detection of PSA in serum, based on bispecific antibodies, is more convenient due to its speed, accuracy and obviating the need of multiple washing steps. The assay using bispecific antibody P57 (against PSA and peroxidase) and monospecific antibody B87 (against PSA) conjugated with glucose oxidase was developed in the presence of excess catalase. Similarly, in solid phase homogeneous immunoassay the monospecific antibody B87 (against PSA) and glucose oxidase were immobilized onto a solid support (plastic) and other reagents, bio-chemicals, and bispecific antibody P57 were taken in homogeneous solution. All variables, *viz.,* glucose oxidase, peroxidase and catalase were optimized at different PSA concentrations.

Results: Homogeneous immunoassay (HIA) showed linearity of PSA detection 1-10 ng/mL whereas, solid phase homogeneous immunoassay (SPHIA) showed in the range of 1-50 ng/mL suggesting SPHIA has a broader operating range, thus much better than HIA. Detection of PSA in a homogeneous solution can be completed in 90 min without involving any washing and incubation steps.

Conclusions: Homogeneous assay is a rapid, economical method that eliminates all washing and incubation steps of conventional ELISA.

Key Words: *Bispecific antibody; PSA assay; Prostate cancer; Prostate-specific antigen; Homogeneous assay*

Introduction

The enzyme linked immunosorbent assays are heterogeneous immunoassays that are usually more sensitive than homogeneous format and less susceptible to interference by sample matrix [1]. Nevertheless, homogeneous immunoassays have a major advantage that they do not require any washing steps which leads to convenience of application with respect to simplicity and short analysis time [2]. Therefore, there is still a need to develop a rapid and practicable method i.e., rapid onsite analysis of multiple samples. Homogeneous immunoassays for detection of different analytes are reported by

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many researchers [3-6]. Whole blood analysis of prostate-specific antigen spotted on a solid support is patented by Savajani [7]. Prostate-specific antigen (PSA), an important tumor associated marker [8,9] is produced by the prostatic epithelium.

PSA is a glycoprotein with molecular weight of 34 KD comprising a single polypeptide chain. It is a normal antigen of the prostate gland which is not found in other normal or malignant tissues. It is present in benign, malignant, and metastatic prostate cancer [10]. Prostate cancer is the second most prevalent form of male malignancy and early diagnosis is the key to potential cure and provide predictive modeling [3,11]. Mostly, the diagnosis of prostate cancer at late stage (spread beyond prostate, metastasis) is done like other cancers such as chest x-ray, bone scan or imaging tests [12]. Digital rectal examination and serum prostatic acid phosphatase (PAP) enzyme test are less effective than PSA blood test [8,13]. The fine needle biopsy is suitable for localized examination of prostatic tumors for clinico-morphological correlations [14]. However, among all techniques, biosensors may help in screening and diagnosis [15-18]. Normally, healthy people have PSA concentrations in blood ≤ 4 ng/mL [10]. Elevation of serum PSA above 4 ng/mL level is found only in prostate cancer which increases with the progression of the disease. PSA is a good marker for establishing prognosis in prostate cancer [19,20]. Presently, there are many different assays for the measurement of PSA [21-27]. All of them require monoclonal or polyclonal antibodies labeled with an enzymatic, fluorometric or colorimetric label (gold nanoparticles, latex, etc.) [28-32]. In our laboratory a bispecific antibody P57 was made for rapid and sensitive detection of PSA [33-36].

In this paper, we report the development of a homogeneous as well as a solid phase homogeneous assay for the detection of PSA. This is based on the oxidation of glucose to gluconic acid and hydrogen peroxide catalyzed by glucose oxidase (conjugated with monospecific antibody B87 against PSA or immobilized onto solid support).

Materials and Methods

Materials

Glucose oxidase (GOD) from *Aspergillus niger* (activity:128 units/mg solid), Peroxidase from horseradish (HRP activity:118 units/mg solid), Catalase from bovine liver (activity:10,900 units/mg solid), Bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO), Glutaraldehyde, Protein-G Sepharose, Prostate-specific antigen (PSA) from human semen, Tris(hydroxymethyl)aminomethane and 3,3′,5,5′-tetramethyl benzidine (TMB) were purchased from Sigma-Aldrich Chemical Co. USA. A monoclonal monospecific antibody (MAb) B87 against PSA was kindly provided by Biomira Inc., Edmonton, Canada, and bispecific monoclonal antibody (BsMAb) P57 against PSA and peroxidase was developed in our laboratory. Glycine, Potassium chloride, Potassium dihydrogen phosphate, di-sodium hydrogen phosphate, Sodium acetate and other chemicals were purchased from BDH, Inc., Toronto, Canada. TMB peroxidase substrate kit was purchased from Kirkegaard and Perry Laboratories, Maryland, USA. Dialysis tubing (diameter 15.5 mm, MW cut off 8000 Daltons) was purchased from BioDesign Inc., Carmel, New York, USA. Millipore filter was obtained from Millipore Products Division, Bedford, USA. ELISA plates were purchased from Nunc, Naperville, USA.

Methods

Chemical cross-linking of monoclonal antibody B87 with glucose oxidase

Glutaraldehyde was used as a cross-linking reagent for the preparation of antibody-enzyme conjugate. Glucose oxidase (1600 units/mL) in 0.1M potassium phosphate buffer, pH 6.0 was added to the solution of monospecific antibody B87 (0.8 mg/mL) in an Eppendorf tubes. Glutaraldehyde (25%, v/v) 30 µl/mL was added slowly to the above mixture with constant shaking. The reaction mixture was incubated for 4 h at 4°C. The excess glutaraldehyde in the solution (1.0 mL) was removed by dialysis against three changes of 100 mL of 0.1M potassium phosphate buffer, pH 6.0 at 4°C. The dialyzed

solution was filtered through 0.45 µm Millipore filter to remove the insoluble polymer formed during cross-linking.

Purification of conjugate

The cross-linked conjugate monoclonal antibody (MAb) B87-GOD was again dialyzed against 0.1 M sodium acetate buffer, pH 5.0. Protein G-Sepharose was packed in a small column (8 mm x 12 mm) and equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The dialyzed conjugate (0.7 mL) was loaded onto the column and unbound fractions (0.5 mL each) were collected. The column was washed 4 times using 0.1 M sodium acetate buffer, pH 5.0 (10 mL). Then, bound antibody-conjugate was eluted with 0.1 M glycine-HCl buffer, pH 2.8 (0.5 mL fraction of each). To minimize the exposure at low pH 10 µL of 1 mM Tris-HCl buffer, pH 8.0 was added in each tube. The absorbance of each fraction was recorded in Beckman DU-64 spectrophotometer at 280 nm. The unbound fractions 2 and 3 only showed the absorbance at 280 nm whereas, other fractions had no absorbance at all. All the fractions were analyzed for glucose oxidase activity and it was observed that only unbound fraction contained activity. This showed that conjugate, antibody, and enzyme did not bind to the matrix in the column.

Assay of glucose oxidase

The enzyme activity of glucose oxidase in all collected fractions was estimated according to the modified method of Bergmeyer [33]. In the glucose oxidase assay, the glucose oxidase catalyzes oxidation of glucose to produce gluconate (D-Glucono-1,5-lactone) and hydrogen peroxide (H_2O_2). The liberated H_2O_2 in the presence of peroxidase oxidizes *o*-dianisidine dye. The change in absorbance per minute at 500 nm was recorded until it became constant. The enzyme activity (units/mL) of glucose oxidase was calculated as described in the protocol reported by Bergmeyer [33]. The amount of glucose oxidase in each fraction was determined by mixing 200 µL of the fraction with *o*-dianisidine (2.5 mg/mL, 20 µL), glucose (100 mM, 100 µL) and horseradish peroxidase (10 U/mL, 200 µL) in a final volume of 1.0 mL with 50 mM sodium acetate buffer, pH 5.0. The activity of glucose oxidase in each fraction was expressed as micromole of glucose oxidized per minute.

The unbound fractions 2 and 3 only showed the enzyme activity whereas other fractions (washed and eluted) had no activity at all. The absorbance of unbound fraction at 280 nm also supported that conjugate, antibody and glucose oxidase did not bind to the column. This is because the Fc portion of the antibody (B87) is not free to bind protein G-Sepharose. It may be possible that Fc portion of the antibody is conjugated with glucose oxidase and binding site of antibody which mostly binds to protein-G is occupied by glucose oxidase. The schematic representation of conjugate of antibody B87 and glucose oxidase is shown in (Figure 1). The results also confirmed that binding was very strong because there was no free antibody which could be retained by the column and subsequently eluted.

Figure 1) *Schematic representation of conjugation of MAb B87 and glucose oxidase in the presence of cross-linking reagent glutaraldehyde.*

Preparation of TMB Solution

A solution of (3,3′,5,5′ tetramethyl benzidine (TMB, 1.0 mg/mL) was made in dimethyl sulfoxide (DMSO) and diluted twenty times in 0.1 M potassium phosphate buffer, pH 6.0. The final concentration of TMB (0.05 mg/mL) was used in the experiment.

Preparation of phosphate buffered saline, pH 7.3 (PBS)

The PBS solution was made using 8.0 g sodium chloride (MW 58.4), 200 mg potassium chloride (MW 74.5), 1.44 g di-sodium hydrogen phosphate (MW 141.96) and 245 mg potassium dihydrogen phosphate (MW 136.08) in 800 mL Milli-Q water and adjusted pH 7.3 with hydrochloric acid. The final volume was made 1L with Milli-Q water.

Homogeneous immunoassay (HIA)

The ELISA 96 well plate was blocked with 200 µL of 1% BSA in phosphate buffered saline pH 7.3 (PBS) and incubated overnight at 4°C. Next day, the plate was washed 4 times (2 min) with PBS to remove unbound BSA. After washing, the plate can be stored at 4°C for further use. The bispecific monoclonal antibody (BsMAb) P57 (≈ 1mg/mL) was diluted ten times in PBS and 10 µl was added in each well. Different concentrations of PSA (0-20 ng/mL) were added to different wells. 0.17 units of horseradish peroxidase (HRP) was added in each well and incubated for 30 min. After that, 20 units of catalase and 0.2 µg conjugate B87-GOD was added in each well and incubated for another 30 min. Finally, 10 mM glucose and 20 µl TMB (0.05 mg/mL) was added in each well. The volume of each well was maintained 100 µL by adding PBS. The plate was kept for shaking and after 30 min (optimized time) the developed blue colour was measured at 650 nm on an ELISA plate reader. The control contained all reagents and antibodies except PSA. The same experiment was carried out in triplicate. All parameters (time, temperature, biochemicals and reagent concentrations) were optimized before using their final concentrations in homogeneous immunoassay.

Validation of homogeneous immunoassay (HIA)

The assay system was same as described above for HIA. The ELISA plate was blocked with 200 µL 1% BSA (in PBS) and incubated overnight at 4°C. The plate was washed completely with PBS. The BsMAb P57 (approx. 1.0 µg), HRP (0.17 units) and spiked PSA 50 µL of different samples (0,2,4 and 8 ng/mL in serum) were added in wells and incubated for 30 min. After incubation, catalase (20 units) and conjugate B87-GOD (0.2 µg) were added in each well and incubated for another 30 min. Finally, the reaction was started after adding 10 mM glucose and 20 µL TMB in assay system. The final volume of each well was maintained 100 µL with PBS and kept on shaking. The control has all reagents and antibodies except PSA (sample 1). After 30 min, the developed blue colour was measured at 650 nm.

Solid phase homogeneous immunoassay (SPHIA)

The solid phase homogeneous assay was carried out at variable concentrations of PSA after optimization of all the parameters. The ELISA plate was coated with 100 µL/well mixture of MAb B87 (1µg) and glucose oxidase (6.4 units). The plate was incubated overnight at 4°C. Next day, the plate was washed with 200 µL PBS (4 times, 2 min) and blocked with 200 µL 1% BSA (in PBS). The plate was kept for 1 h at room temperature (25°C). The plate was again washed 4 times (2 min) with PBS and different concentrations of PSA (0-100 ng/mL in 0.1% BSA in PBS) were added to different wells and incubated for 30 min. After incubation, the assay mixture [10 µL BsMAb P57(100 times diluted) and 0.17 units HRP, 20 units catalase and 20 µLTMB] were added in different wells and incubated for 30 min at room temperature. The reaction was started by adding 10 mM glucose and the final volume was maintained 100 µL with PBS. The plate was shaken for 30 min (optimized time) and the developed blue colour was measured at 650 nm. The experiment was carried out in triplicate and values plotted are the mean of the triplicate.

Optimization of SPHIA at variable concentrations of PSA and glucose oxidase

The ELISA plate was coated with 100 µL/well by mixture of different concentrations of glucose oxidase (0.1,0.5,1.2,6.4 and 12.8 units) and monospecific monoclonal antibody B87 (1.0 µg in PBS). The plate was incubated at 4°C overnight. Next day, the plate was washed 4 times (2 min) with 200 µL PBS. After complete washing, the wells were blocked with 200 µL of 1% BSA (in PBS) for 1h at room temperature. Again, washed 4 times (2 min) with PBS to remove all unbound BSA. After washing, the plate can be stored at 4°C for further use. Different concentrations of PSA 0-100 ng/mL (in 1% BSA in PBS) were added to different wells and incubated for 30 min at room temperature. Then, 30 µL of a mixture [BsMAb P57 10 µL (100 times dilution), HRP (0.02 units) and catalase (20 units) and TMB (20 µL)] was added in each well and incubated for another 30 min. Reaction was started by adding glucose (10 mM) and the final volume was maintained at 100 µL with PBS. The plate was shaken for 30 min and the developed blue colour was measured at 650 nm on ELISA reader.

Optimization of SPHIA at variable concentrations of PSA and peroxidase

The SPHIA was carried out at variable concentrations of horseradish peroxidase (HRP) to optimize the amount of HRP required for assay system. The ELISA plate was coated with 100 µL/well mixture of B87 (1µg) and glucose oxidase (6.4 units) and incubated overnight at 4°C. Next day, the plate was washed with PBS (200 µL/well 4 times, 2 min) and blocked with 1% BSA (in PBS) and incubated for 1h at room temperature. The plate was again washed with PBS (200 µL/well 4 times, 2 min) and 40 µL different concentrations of PSA (0-100 ng/mL in 1% BSA) were added to different wells and incubated for 30 min. After incubation, 30 µL assay mixture [10 µL (100 times diluted BsMAb P57), 20 units catalase and HRP (1.7,0.17,0.017 and 0.0017 units)] was added in each well and incubated for 30 min at room temperature. Reaction was started by adding 10 mM glucose and 20 µL TMB and the final volume of each well was maintained at 100 µL with PBS. The plate was shaken for 30 min and the developed blue colour was measured at 650 nm.

Optimization of SPHIA at variable concentrations of PSA and catalase

The SPHIA was carried at variable concentrations of catalase to optimize the amount of catalase required for the assay system. The ELISA plate was coated (with MAb B87 and glucose oxidase), blocked (with BSA) and washed (with PBS) as described above for optimization of the peroxidase. After washing, different concentrations of PSA 0-100 ng/mL (in 1% BSA in PBS) were added in different wells and incubated for 30 min. After incubation, 30 µL assay mixture [10 µl BsMAb P57 (x100 dilution), 0.17 unit of HRP, and catalase (0,2,5,20 and 40 units)] was added in each well and incubated for 30 min at room temperature. The reaction was started by adding 10 mM glucose and 20 µL TMB. The final volume was maintained at 100 µL with PBS. The plate was shaken and after 30 min, the developed blue colour was measured at 650 nm.

Validation of solid phase homogeneous immunoassay (SPHIA)

The validation of spiked PSA samples in serum were carried out as described in SPHIA assay system. The ELISA plate was coated with MAb B87 (1.0 µg) and GOD (6.4 units) and incubated overnight at 4°C. The plate was washed with 4 times (2 min) with PBS and blocked with 200 µL BSA (1% in PBS) for 1 h. Again, all wells were washed 4 times (2 min) with PBS. The spiked PSA 50 µL of different samples [0 ng/mL (control), 2 ng/mL (in PBS), 2 ng/mL (in serum), 4 ng/mL (in serum), 8 ng/mL (in serum) and 10 ng/mL (in serum)] were added in different wells and incubated for 30 min. Assay mixture 40 µL [10 µL BsMAb P57 (diluted x100), 0.17 units HRP, 20 units catalase and 20 µL TMB] was added in each well and incubated further 30 min. The reaction was started after adding 10 mM glucose and the final volume of each well was maintained at 100 µL with PBS. The control has all reagents and antibodies except PSA (sample 1). The plate was shaken for 30 min and the developed blue colour was measured at 650 nm.

Results

Homogeneous immunoassay (HIA)

For the homogeneous immunoassay of PSA, the monospecific antibody B87-glucose oxidase conjugate, bispecific monoclonal antibody (BsMAb) P57, catalase and other chemicals were taken up in a homogeneous solution (Figure 2). In control experiments (absence of PSA), both antibodies are separated from each other. In the presence of PSA, antibodies come closer to bind PSA. On addition of glucose, conjugated glucose oxidase with B87, liberates peroxide (H_2O_2) which is immediately captured by the peroxidase of bispecific antibody P57. Peroxidase catalyzes the oxidation of TMB (colorless) to blue colour by reducing peroxide (oxidizing agent). The blue colour was measured after 30 min (optimized time) at 650 nm (Figure 3). However, in the absence of PSA, both antibodies are apart from each other and the liberated hydrogen peroxide, before reaching to the peroxidase, is degraded by catalase. Therefore, no colour formation took place.

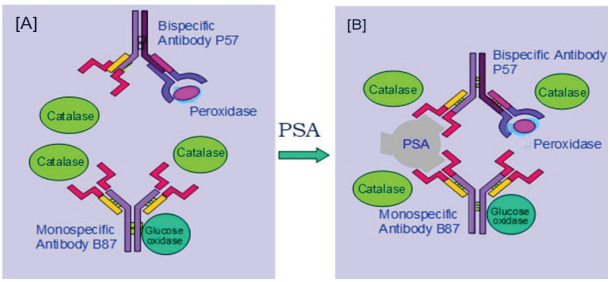

Figure 2) *Schematic representation of homogeneous immunoassay of PSA using BsMAb P57 and monospecific monoclonal antibody (MAb) B87 conjugated with glucose oxidase in the presence of excess catalase. [A] In the absence of PSA (control) and [B] in the presence of PSA.*

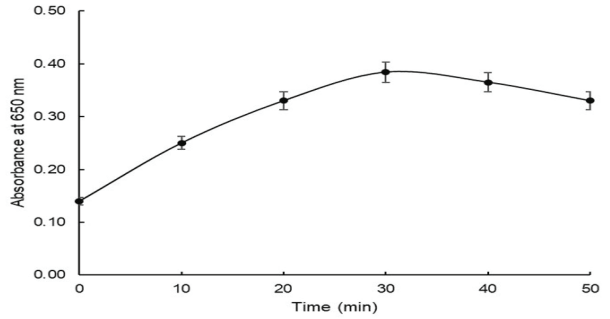

Figure 3) *Optimization of time for homogeneous immunoassay. The HIA was carried out as described above and the developed blue color was measured at 650 nm at different time.*

The homogeneous immunoassay at variable concentrations of PSA shows hyperbolic curve (Figure 4A) from 0 to 20 ng/mL. The linearity of the graph was up to 10 ng/mL and regression coefficient (R^2) 0.994 as shown in (Figure 4) inset [B]. From this standard graph, the concentration of unknown samples can be determined knowing their absorbance at 650 nm.

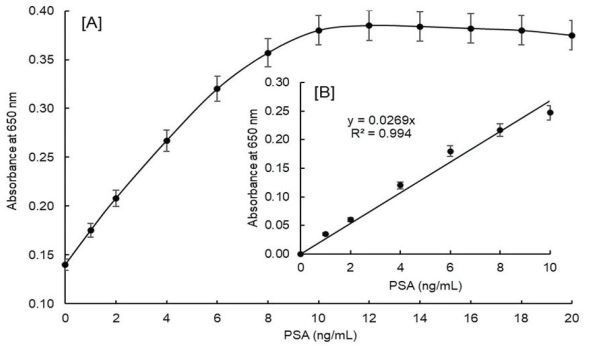

Figure 4) *Homogeneous immunoassay at variable concentrations of PSA (0-20 ng/mL). [A] Calibration plot between absorbance and increasing concentrations of PSA (0-20 ng/mL). The inset [B] shows 0-10 ng/mL PSA region of the linear standard graph (R^2=0.994) with respect to control for calibration of PSA concentrations of unknown samples.*

Validation of homogeneous immunoassay (HIA)

The validation of HIA was carried out using blood serum (woman 20 yrs, 110 mg/dL glucose and No PSA) spiked with different concentrations of PSA samples in triplicate. The absorbance of different spiked PSA samples [Sample 1 (control-No PSA, serum), sample 2 (PSA 2 ng/mL in serum), sample 3 (PSA 4 ng/mL in serum) and sample 4 (PSA 8 ng/mL in serum)] were multiplied by dilution factor and compared with standard calibration plot of HIA (Figure 4, inset [B]). The PSA values of different samples were found approximately same as spiked PSA concentrations (Figure 5).

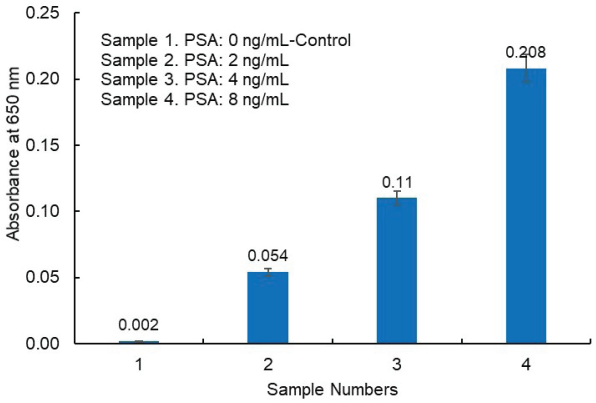

Figure 5) *Validation of spiked PSA samples in blood serum with homogeneous immunoassay for detection of prostate specific antigen (PSA). Sample 1 (control-No PSA, serum), sample 2 (2 ng/mL PSA in serum,), Sample 3 (4 ng/mL PSA in serum) and sample 4 (8 ng/mL PSA in serum) were used in triplicate. The all reagents and antibodies were same, and assay was carried out as described in HIA. The absorbance (after multiplication of dilution factor) at 650 nm was recorded and compared with standard plot (Figure 4B).*

Solid phase homogeneous immunoassay (SPHIA)

In solid phase homogeneous immunoassay, the monospecific antibody B87 (against PSA) and glucose oxidase were immobilized onto a solid support (plastic). Other chemicals, bio-chemicals and antibody were taken in a solution. The molecular weight of antibody B87 (MW 150,000) and glucose oxidase (MW 160,000) are very similar to each other. Therefore, after immobilization onto the solid support, these molecules got arranged in the same plane which acted as a bispecific antibody reaction and eliminated the requirement of a bispecific antibody (Figure 6). Thus, the solid phase homogeneous assay not only saved chemicals, biomaterials, and time but also labor for making bispecific antibody. The validation of

SPHIA at variable concentrations of PSA (Figure 7) showed hyperbolic curve between absorbance and PSA concentrations 0-100 ng/mL (Figure 7A) and linearity up to 0-50 ng/mL (R^2=0.9903). The concentrations of unknown PSA samples were determined using the standard graph (Figure 7B).

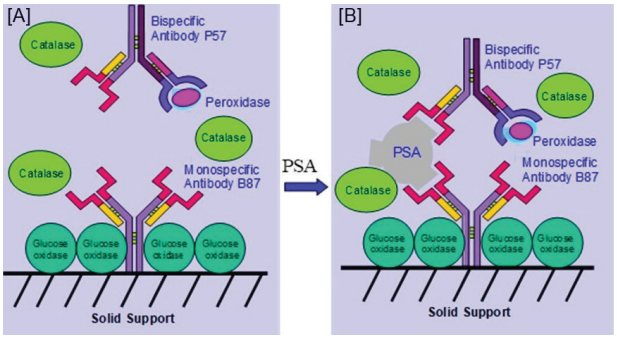

Figure 6) *Schematic representation of SPHIA. [A] In the absence of PSA (control) monospecific antibody B87 and glucose oxidase are immobilized onto the solid support whereas, the bispecific antibody P57 is in solution. On addition of glucose, glucose oxidase liberated peroxide is captured by catalase before reaching the peroxidase of bispecific antibody P57 and no colour occurs in the presence of TMB. [B] In the presence of PSA, the bispecific antibody P57 and monospecific antibody B87 bind to PSA and come closer to each other. On addition of glucose, glucose oxidase liberates hydrogen peroxide which is immediately captured by peroxidase (instead of catalase) of antibody P57 and oxidizes TMB to produce a blue colour.*

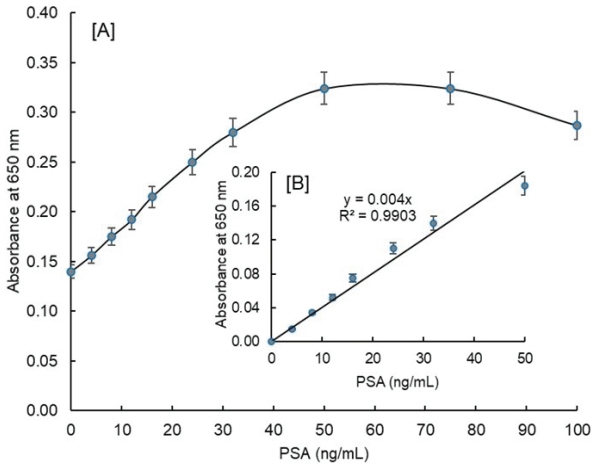

Figure 7) *SPHIA at variable concentrations of PSA. [A] The hyperbolic calibration curve between absorbance and increasing concentrations of PSA (0-100 ng/mL). The inset [B] shows 0-50 ng/mL PSA region of the linear standard graph (regression coefficient, R^2 = 0.9903) with respect to control for calibration of PSA concentrations of unknown samples.*

Optimization of SPHIA at variable concentrations of glucose oxidase

The results are shown in (Figure 8). The optimum concentration of glucose oxidase for SPHIA was found to be ~6.4 units/100 µL of assay system. The higher concentration of glucose oxidase (12.8 units/100 µL) was found inhibitory. This suggested that glucose oxidase attained the maximum velocity (V_{max}) at 6.4 units in the assay system at which all catalytic sites of immobilized glucose oxidase were saturated with glucose and caused no further increase in the velocity (V_{max}) of the reaction. At further higher concentrations, the product inhibition occurred due to which the rate of reaction decreased. As the PSA concentrations increased more HRP came closer and oxidized the dye (TMB) to blue color but at certain concentrations (saturation) no further oxidation occurred, hence, no increase in the absorbance. HRP and PSA concentrations may also be limiting factors. The enzymatic reaction shows hyperbolic curve and then decreases at higher concentrations due to product inhibition.

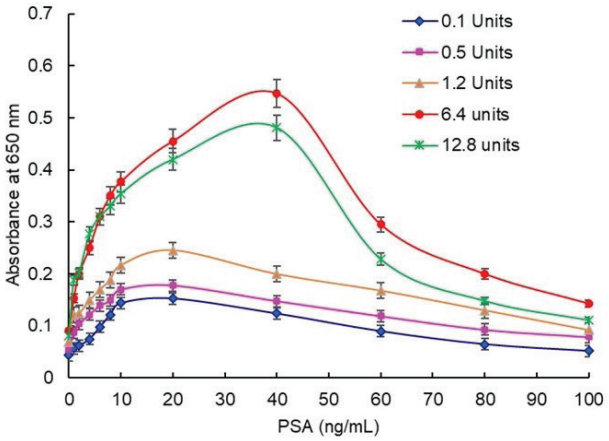

Figure 8) *SPHIA at variable concentrations of PSA and glucose oxidase. ELISA plate was coated with 1 µg B87 and different concentrations of glucose oxidase (0.1,0.5,1.2,6.4 and 12.8 units) per well. PSA (0-100 ng/mL) was used in different wells. The final concentration of HRP was 0.02 units and 10 µL BsMAb P57 (x100 dilution). The catalase was 20 Units/well and the colour was developed in the presence of 20 µL TMB and 10 mM glucose. The final volume of the assay system was 100 µL/well and the same experiment was performed in triplicate.*

Optimization of SPHIA at variable concentrations of peroxidase

The results are shown in (Figure 9). The optimum concentration of HRP for SPHIA was found to be ~0.17 units/100 µL of assay system. The higher concentration of HRP (1.7 units/100 µL) was found inhibitory.

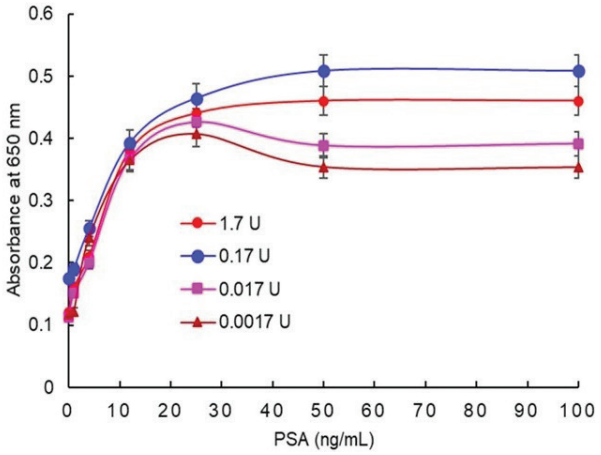

Figure 9) *SPHIA at variable concentrations of PSA and HRP. The ELISA plate was coated with 1.0 µg MAb B87 and 6.4 units of glucose oxidase/well. Different concentrations of PSA (0-100 ng/mL) were added to different wells. The catalase was 20 units/well and 10 µL BsMAb P57 (x100 dilution)/well. The HRP concentrations were 1.7,0.17,0.017 and 0.0017 units/100 µL. The reaction was started by adding 10 mM glucose and 20 µL TMB. The developed blue colour was measured at 650 nm. The same experiment was carried out in triplicate at same time and the final volume was maintained at 100 µL/well.*

Optimization of SPHIA at variable concentrations of catalase

The results are shown in (Figure 10). The optimum concentration of catalase for SPHIA was found to be ~20 units/100 µL of the assay system. However, the higher concentration of catalase (40 units/100 µL) was found inhibitory.

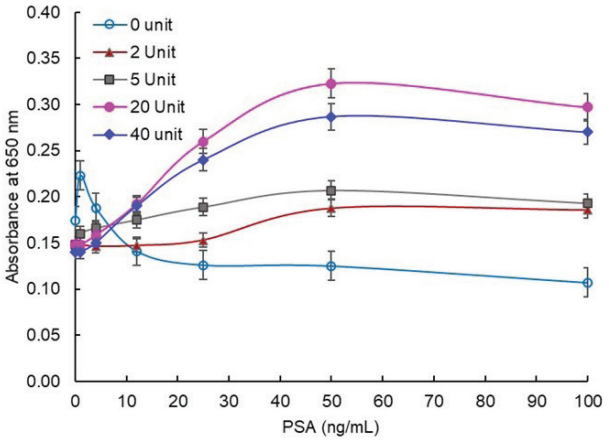

Figure 10) *SPHIA at variable concentrations of PSA and catalase. The ELISA plate was coated with 1 µg MAb B87 and 6.4 units of glucose oxidase/well. Different concentrations of PSA (0-100 ng/mL) were added to different wells. The final concentration of HRP was 0.17 units/well and BsMAb P57 (x100 dilution)/well. Catalase concentrations were 0,2,5,20 and 40 units/well. The reaction was started by adding 10 mM glucose and 20 µL TMB. The developed blue colour was measured at 650 nm. The same experiment was carried out in triplicate and the final volume was maintained at 100 µL/well.*

Effect of borate buffer on glucose oxidase and peroxidase

The bispecific monoclonal antibody (BsMAb) P57 was purified using peroxidase affinity chromatography and the antibody was eluted from the column with 50 mM borate buffer, pH 9.0. Here, in homogeneous assay system, BsMAb P57 was dialyzed against PBS, pH 7.3 to remove borate buffer, pH 9.0 and equilibrate with PBS, pH 7.3. The borate buffer, pH 9.0 was found inhibitory for glucose oxidase (GOD) as well as for HRP due to higher pH. The optimum pH for both glucose oxidase and HRP was found to be approximately in the range of pH 5.5-7.5. Hence, dialysis of antibody P57 against PBS, pH 7.3, was required before performing homogeneous immunoassay. The results are shown in (Table 1).

TABLE 1

Effect of borate buffer, pH 9.0 on glucose oxidase and peroxidase.

S. No.	Samples	Results
1.	12 Units GOD+50 mM Borate buffer, pH 9.0	No Colour
2.	12 Units GOD+PBS, pH 7.3	Colour developed
3.	0.034 Units HRP+50 mM Borate buffer, pH 9.0	No Colour
4.	0.034 Units HRP+PBS, pH 7.3	Colour developed

The qualitative assay was carried out by adding glucose (10 mM), HRP (0.002 units) and 10 µL TMB in samples 1 and 2 and in samples 3 and 4 by adding 20 µL TMB and 10 µL hydrogen peroxide (H_2O_2) solution. On completion of the reaction, the blue colour was developed in wells of the ELISA plate containing PBS, pH 7.3. Borate buffer, pH 9.0 was inhibitory of both GOD and HRP (No color).

Validation of solid phase homogeneous immunoassay (SPHIA)

The validation of SPHIA was carried out using human blood serum (woman 20 yrs, 110 mg/dL glucose and No PSA) spiked with different concentrations of PSA samples in triplicate [sample 1 (control-No PSA, serum), Sample 2 (PSA 2 ng/mL in 1% BSA in PBS), Sample 3 (PSA 2 ng/mL in serum), sample 4 (PSA 4 ng/mL in serum) and sample 5 (PSA 8 ng/mL in serum) and sample 6 (PSA 10 ng/mL in serum)]. The absorbance of each sample was multiplied by dilution factor and compared with the standard calibration plot of SPHIA (Figure 7B). The

respective PSA values were found approximately same as spiked PSA concentrations. Sample 2 (without endogenous glucose but contain 2 ng/mL PSA) and sample 3 (with serum glucose 110 mg/dL contain 2 ng/mL PSA) had not shown any significant changes due to very low concentration of endogenous glucose compared to the added glucose (Figure 11).

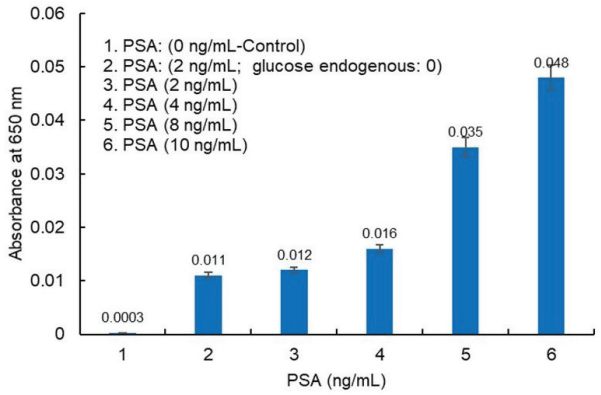

Figure 11) *Validation of spiked PSA samples in blood serum with solid phase homogeneous immunoassay. Sample 1 (control-No PSA, serum), Sample 2 (2 ng/mL PSA in 1% BSA in PBS), sample 3 (2 ng/mL PSA in serum), Sample 4 (4 ng/mL PSA in serum) sample 5 (8 ng/mL PSA in serum) and sample 6 (10 ng/mL PSA in serum) were used in triplicate. The all antibodies and reagents were same, and assay was carried out as described in SPHIA. The developed blue colour absorbance (after multiplication of dilution factor) at 650 nm was recorded and values were compared with standard plot (Figure 7B).*

Discussions

PSA is one of the most important serum tumor markers in patients with prostate cancer (PSA ≤ 4 ng/mL in healthy donors). Therefore, development of a simple, sensitive, and rapid assay system is required due to high throughput screening of samples in clinical laboratories. Monospecific antibody B87 (anti PSA) was procured from Biomira, Alberta and bispecific antibody P57 (anti PSA and HRP) developed in our laboratory [36]. After blocking with BSA followed by washings, the plate can be stored at 4°C, if not immediately required for further experiments. The linearity of PSA detection was 0-10 ng/mL (regression coefficient R^2=0.994) of the standard graph for homogeneous assay system (Figure 4). For this assay, glucose oxidase was cross-linked with monospecific antibody B87 (MAb B87) using glutaraldehyde (Figure 1).

The solid phase homogeneous immunoassay eliminated chemical conjugation of MAb B87 with glucose oxidase due to approximately same molecular weight of MAb B87 (MW 150,000) and glucose oxidase (MW 160,000). On immobilization, both MAb B87 and glucose oxidase were in the same plane of orientation and behaved as bispecific antibody [37,38]. The solid phase homogeneous immunoassay reduced the use of consumables, time, and labor in making bispecific antibody (against PSA and glucose oxidase). Monospecific antibody B87 and glucose oxidase on immobilization, in the presence of glucose, liberated hydrogen peroxide (H_2O_2) which was immediately captured by HRP of bispecific antibody P57 and produced a blue colour in the presence of TMB. In the absence of PSA, bispecific antibody P57 and monospecific antibody B87 were physically apart from each other, therefore, liberated H_2O_2 before reaching to HRP of bispecific antibody P57, was degraded by higher molecular weight (MW 250,000) of catalase (Figure 6 and Figure 7). Efforts were made to optimize the assay conditions by varying glucose oxidase, HRP and catalase concentrations at variable concentrations of PSA (Figures 8-10). The linearity of PSA detection in the standard graph was found 0-50 ng/mL (regression coefficient R^2=0.990) in SPHIA (Figure 7). The total time of homogeneous assay was found 90 min. In the conventional assay system, longer incubation and several washing steps with PBS are required. To overcome this problem, a homogeneous assay system was developed in our laboratory which was faster (reducing the assay time), eliminated several washing steps and allowed rapid on-site detection of PSA which could be performed even by non-trained personnel. The above PSA detection method using bispecific antibodies can be used as model system for analytical analysis. However, further experiments are required for clinical evaluation by analysis of patient's samples using the gold-standard method [39,40].

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Conflicts of Interest

The authors declare no conflict of interest.

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