

Surface plasmon resonance biosensor for detection of pregnancy associated plasma protein A2 in clinical samples

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Abstract Pregnancy associated plasma protein A2 (PAPP-A2) is a metalloproteinase that plays multiple roles in fetal development and post-natal growth. Here we present a novel surface plasmon resonance (SPR) biosensor for the rapid and quantitative detection of PAPP-A2 in blood samples. This biosensor uses a single surface referencing approach and a sandwich assay with functionalized gold nanoparticles for signal enhancement. We demonstrate that this SPR biosensor enables the detection of PAPP-A2 in 30 % blood plasma at levels as low as 3.6 ng/mL. We also characterize the performance of the biosensor and evaluate its cross-reactivity to a PAPP-A analogue. Finally, we utilize this SPR biosensor for the detection of PAPP-A2 in blood serum from two groups of subjects: pregnant women and healthy non-pregnant women and men.

Keywords Pregnancy associated plasma protein A2 · Surface plasmon resonance · Blood sample · Nanoparticles

Introduction

Pregnancy associated plasma protein A2 (PAPP-A2) is a metalloproteinase from the metzincin family that regulates the biological activity of insulin-like growth factor binding proteins. PAPP-A2 plays multiple roles in fetal development and post-natal growth and it is believed to be connected to disturbances during pregnancy (e.g., pre-eclampsia or HELLP syndrome [1]). Although the physiological functions of PAPP-A2 are still under investigation, it is believed that they could be related to PAPP-A and, furthermore, that functions of PAPP-A2 and PAPP-A are linked to one another.

In order to reveal the diagnostic potential of PAPP-A2, a reliable and sensitive method for the detection of circulating PAPP-A2 needs to be developed. To date, only a few studies concerned with the quantification of PAPP-A2 in blood samples have been published. Klooverpris et al. [1] used ELISA to monitor levels of PAPP-A2 in the blood serum of women during their pregnancy, reporting a limit of detection (LOD) for PAPP-A2 in buffer of 71 pg/mL. ELISA was also employed for the determination of PAPP-A2 levels in blood serum from both women with pre-eclampsia and those with an uncomplicated pregnancy, reporting a LOD in buffer of 1.25 ng/well [2]. It should be noted, however, that both of these studies did not include calibration in blood serum, and thus PAPP-A2 quantification in clinical samples might have been affected by effects originating from the serum matrix. To date no biosensor for the detection of PAPP-A2 in blood samples has been reported.

In this work, we present a SPR biosensor for the detection and quantification of PAPP-A2 in blood samples. This biosensor utilizes a single surface referencing method and sandwich assay involving functionalized gold nanoparticles (AuNPs), which provides a sensitive, yet robust, tool for the measurement of low levels of PAPP-A2 in serum samples.

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Material and methods

Reagents

11-Mercapto-tetra(ethyleneglycol)undecanol (HS-C₁₁-(EG)₄-OH; Prochimia, Gdynia, Poland), 11-mercapto-hexa(ethylene glycol) undecyloxy acetic acid (HS-C₁₁-(EG)₆-OCH₂-COOH, Prochimia), *N*-hydroxysuccinimide (NHS, PharmaTech, Prague, Czech Republic), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; PharmaTech), ethanolamine hydrochloride (EA; Sigma-Aldrich, Prague, Czech Republic), Tween®20 (Sigma-Aldrich), albumin from bovine serum (BSA, Sigma-Aldrich), recombinant human PAPP-A2 (PAPP-A2; R&D Systems, Minneapolis, United States), monoclonal antibody against PAPP-A2 (Ab-1; R&D Systems), biotinylated antibody against PAPP-A2 (Ab-2; R&D Systems), commercial human blood plasma in sodium citrate (mixed gender, pooled; BioChemed Services, Winchester, United States). All other chemical reagents were purchased in molecular biology grade or higher. Buffers PBNa (10 mM phosphate, 2.9 mM KCl, 750 mM NaCl, pH 7.4), PBS_{BSA} (10 mM phosphate, 2.9 mM KCl, 137 mM NaCl, 250 µg/mL BSA, pH 7.4), and PBST_{BSA} (PBS_{BSA} + 0.05 % Tween®20, pH 7.4) were prepared using deionized water (18 MΩ/cm resistivity, Direct-Q from Millipore, Prague, Czech Republic).

Samples

Sixteen serum samples were used in this study: eight samples from pregnant women and eight samples from non-pregnant women and men. Blood drawn into collection tubes without anticoagulant was centrifuged at 1450 *g* at 4 °C for 15 min to obtain serum.

Surface plasmon resonance biosensor and detection assay

In this study, we used a four-channel SPR sensor platform based on the wavelength spectroscopy of surface plasmons (Plasmon IV) developed at the Institute of Photonics and Electronics, CAS (Czech Republic) [3]. In this SPR biosensor the sensor response is expressed as a shift in the wavelength for which the resonance occurs and is directly proportional to the mass of biomolecules attached to the surface of the sensor. For a resonance wavelength of around 750 nm, a shift of 1 nm in the resonance wavelength represents a change in the protein surface coverage of 17 ng/cm² [4]. Sample delivery to the sensor was carried out using a microfluidic flow cell in a near dispersionless manner [5]. A schematic of the detection assay is shown in Fig. 1: PAPP-A2 is first captured by primary antibodies (Ab-1) immobilized on the SPR sensor surface, followed by the binding of biotinylated secondary antibodies (Ab-2) to previously captured PAPP-A2, and finally by the binding of (signal enhancing) streptavidin-coated AuNPs to

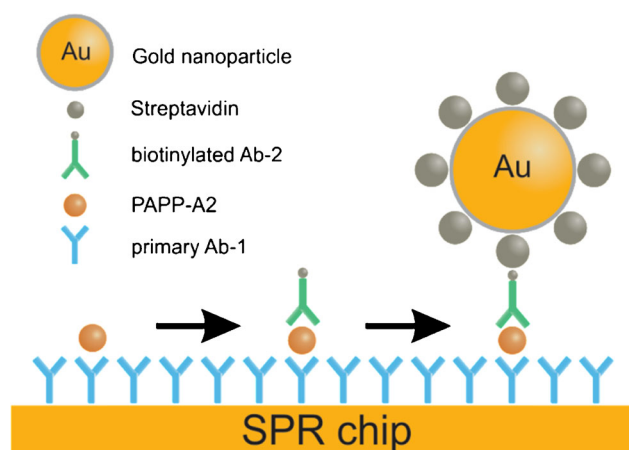


Fig. 1 The principle of sandwich detection format with functionalized AuNPs for signal enhancement

the secondary antibodies via the streptavidin–biotin interaction.

Immobilization of primary antibody against PAPP-A2 (Ab-1) on the surface of the sensor chip was achieved via covalent attachment to a ω -carboxyalkylthiolate self-assembled monolayer (SAM). The details of both the preparation of mixed SAM of HS-C₁₁-(EG)₄-OH and HS-C₁₁-(EG)₆-OCH₂-COOH alkylthiols as well as the in situ immobilization of the primary antibodies are described in [3]. In order to generate sensor chips with reproducible sensing characteristics, the same amount of Ab-1 (240 ng/cm², corresponding to a SPR sensor response of 14 nm) and BSA (50 ng/cm², corresponding to a SPR sensor response of 3 nm) was immobilized on the surface of each SPR chip prior to each detection experiment. The reproducibility of the immobilization procedure was determined to be better than 98 % (obtained from 20 independent channels on five different chips). Forty nm diameter AuNPs were prepared and purified according to the procedure described in [6]. All SPR detection experiments were performed at a flow rate of 20 µL/min and a temperature of 25 °C.

The PAPP-A2 detection assay was performed via the following protocol. After immobilization of Ab-1, the sensor surface was washed with PBST_{BSA} buffer until a stable baseline was established, after which the sample with PAPP-A2 was injected for 15 min. This was followed by a series of short injections of PBST_{BSA}, high-ionic strength PBNa (to remove nonspecifically adsorbed molecules), and PBST_{BSA}. Then, biotinylated Ab-2 at a concentration of 1 µg/mL in PBST_{BSA} was injected for 10 min, followed by a washing step with PBST_{BSA}, then an injection of PBS_{BSA}. Finally, a PBS_{BSA} buffer containing streptavidin-coated AuNPs (optical density of 0.1) was injected for 10 min, after which the sensor surface was washed with PBS_{BSA}.

In order to compensate for interfering effects, we used a reference channel in each experiment. This reference channel was exposed to the same sequence of solutions *except* for the solution containing PAPP-A2 (direct detection step), in which a sample without PAPP-A2 was injected. The specific sensor

response to PAPP-A2 was then determined from the reference-compensated final level of bound AuNPs.

Detection of PAPP-A2 in buffer and pooled blood plasma

The sensor response to known concentrations of PAPP-2 in both buffer and 30 % blood plasma was measured to establish calibration curves pertaining to each respective matrix. Prior to each measurement, stock solutions of PAPP-A2 in buffer were prepared. These stock solutions were further diluted by a factor of 20 to obtain respective final concentrations in both buffer and 30 % plasma. For reference purposes, the plasma samples were diluted with PBST_{BSA} in a volume ratio of 19:1 (plasma:PBST_{BSA}).

Cross-reactivity to PAPP-A and PSA and CEA biomarkers

In order to test the cross-reactivity to PAPP-A analogue, the experiments in both buffer and 30 % blood plasma were performed, in which samples with PAPP-A (instead of PAPP-A2) at a concentration of 100 ng/mL were analyzed. To assess the cross-reactivity of the biosensor to CEA and PSA biomarkers, 30 % blood plasma was spiked with CEA and PSA at concentrations of 100 ng/mL and analyzed by the biosensor.

Detection of PAPP-A2 in serum samples

For the detection of PAPP-A2 in serum samples, we used a single surface referencing (SSR) approach [7]. Briefly, each serum sample was diluted to 30 % with PBST_{BSA} and divided into two parts. One part was used as the detection sample (diluted with PBST_{BSA} in a volume ratio of 19:1), whereas the other was used as the reference sample (diluted with PBST_{BSA} with Ab-1 (10 µg/mL) in a total volume ratio of 19:1).

Prior to detection, the samples were incubated for 10 min in order for Ab-1 to bind to PAPP-A2 molecules present in the reference sample. The concentration of Ab-1 added to the reference sample (10 µg/mL) was optimized beforehand in blood plasma; this concentration represents a 100-fold excess compared with the spiked PAPP-A2, which was sufficient to bind to the majority of PAPP-A2 present in the blood plasma sample such that the effect of the remaining free PAPP-A2 binding to the surface was negligible.

To examine the effect of potential matrix effects on the sensor response, we performed detection experiments in which both 30 % blood serum and citrated blood plasma were spiked with the same concentration of PAPP-A2. We did not observe a significant difference between the two sample matrices, in terms of the specific sensor response as well as the absolute value of the nonspecific sensor response.

Results and discussion

Detection of PAPP-A2 in buffer and pooled blood plasma

The SPR biosensor was used for the detection of PAPP-A2 in both phosphate buffer and blood plasma spiked with PAPP-A2. Typical sensor responses to the respective assay steps in buffer are shown in Fig. 2. The calibration curves established from sensor responses obtained for different concentrations of PAPP-A2 (ranging from 0.1 to 1000 ng/mL) are shown in Fig. 3; these results are reference-compensated, where standard deviations were calculated from at least three measurements.

In buffer, the use of functionalized AuNPs offered an enhancement in sensor response by a factor of ~400 compared with the response for the direct detection of PAPP-A2 (determined for the concentration range of 25–250 ng/mL). The enhancement obtained in pooled blood plasma was six times lower; however, this is only a rough estimate as the sensor response to the direct detection of PAPP-A2 is strongly influenced by high nonspecific interactions.

The LOD for the data shown in Fig. 3 was calculated as the concentration that corresponds to twice the critical value of the sensor response, which is related to that from a blank sample (Student's *t*-test, 10 replicates, significance level of 5 %), and was determined to be 100 pg/mL in buffer and 3.6 ng/mL in 30 % blood plasma, respectively. The LOD achieved in buffer is in good agreement with that demonstrated recently by Kloverpris et al. using ELISA [1]. The higher LOD obtained in blood plasma can be attributed to the nonspecific interaction between surface of the biosensor and blood plasma matrix.

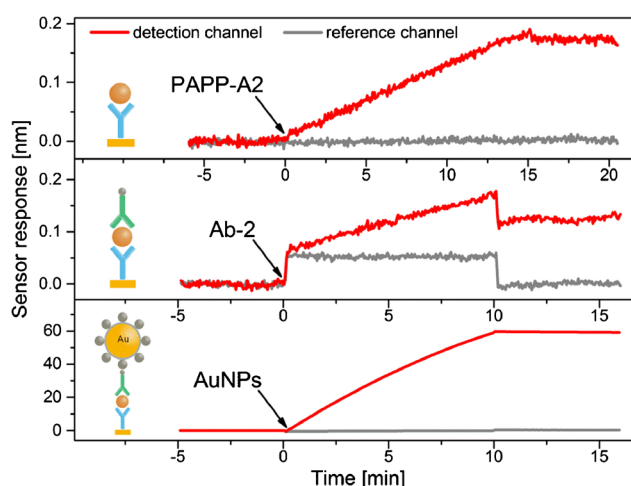


Fig. 2 Temporal sensor response corresponding to respective steps of the assay for detection of PAPP-A2 in buffer. (Top) Direct detection of PAPP-A2. (Middle) Binding of biotinylated Ab-2. (Bottom) Binding of streptavidin-coated AuNPs for signal enhancement

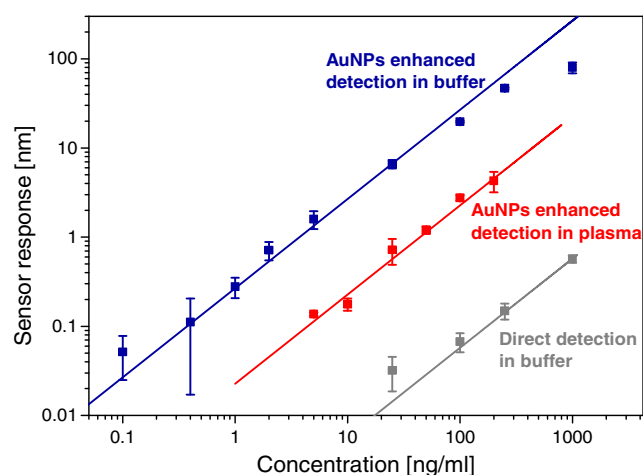


Fig. 3 Calibration curve for direct (grey) and enhanced (blue) detection of PAPP-A2 in buffer and enhanced detection in 30 % blood plasma (red)

Cross-reactivity to PAPP-A and PSA and CEA biomarkers

We tested the robustness of this method and its potential cross-reactivity to PAPP-A (which shares the same five-domain structure and 45 % of the amino acid residues with PAPP-A2) in both buffer and 30 % blood plasma. We found that the sensor response to PAPP-A was less than 2 % of the specific (AuNP-enhanced) sensor response to the binding of PAPP-A2, both in buffer and in 30 % blood plasma (data not shown). The cross-reactivity of the biosensor to PSA and CEA cancer biomarkers was evaluated by comparing the biosensor response to blood plasma spiked with PSA and CEA and to non-spiked plasma. The difference originating from the presence of PSA and CEA was found to be negligible, as it fell within the standard deviation of biosensor response to the non-spiked plasma.

Detection of PAPP-A2 in serum samples

This biosensor was applied to the measurement of PAPP-A2 levels in 30 % blood serum samples obtained from two groups of subjects: pregnant women (eight samples, first trimester) and healthy non-pregnant women and men (eight samples). The sensor response from each sample was converted to a PAPP-A2 concentration using the calibration curve obtained in 30 % blood plasma (Fig. 3). The average concentration of PAPP-A2 in the blood serum of healthy non-pregnant women and men was 8 ± 8 ng/mL. As anticipated, the blood serum from pregnant women showed much higher levels of PAPP-A2, with an average concentration of 195 ± 140 ng/mL. This is in very good agreement with previous research [1] and, thus, demonstrates the functionality of this SPR biosensor-based method for the detection of PAPP-A2 in clinical samples. The large data variability can be attributed to the fact that even though all tested women were in the same trimester of

pregnancy, PAPP-A2 levels are known to vary dramatically even on a time scale of weeks [1].

Conclusions

In this work, we have presented a surface plasmon resonance biosensor and detection assay for the rapid and sensitive detection and quantification of pregnancy associated plasma protein A2 (PAPP-A2). Through the use of model experiments with PAPP-A2 spiked in both buffer and 30 % blood plasma, we demonstrated that this biosensor is able to detect PAPP-A2 at levels down to 100 pg/mL (in buffer) and 3.6 ng/mL (in 30 % blood plasma). This biosensor was also used for the detection of PAPP-A2 in clinical samples; we demonstrated that the levels of PAPP-A2 in the blood serum of pregnant women were higher by a factor of more than 30 compared with a control group of non-pregnant women and men. These results suggest that the presented biosensor is applicable for the detection of PAPP-A2 in blood serum in pregnancy and can be used to further ascertain the potential function of PAPP-A2 in the pathology of pregnancy unrelated diseases.

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Compliance with ethical standards The authors declare that they have no conflict of interest. The study was performed in adherence to the principles of the Helsinki Declaration and approved by the Ethical Committee of the General University Hospital in Prague. All participants gave informed consent prior to entering the study.

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