Label-free electrochemical aptasensing of the human prostate-specific antigen using gold nanospears

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ABSTRACT

Gold nanospears were electrodeposited with the assistance of arginine as a soft template and precise selection of experimental parameters. The nanospears were then employed as a transducer to immobilize an aptamer of prostate-specific antigen (PSA) and fabrication of a label-free electrochemical aptasensor. The aptasensor was employed for the detection of PSA with a linear concentration range of 0.125 to 200 ng mL⁻¹ and a limit of detection of 50 pg mL⁻¹. The aptasensor was successfully applied to detect PSA in blood serum samples of healthy and patient persons.

Keywords: Human prostate-specific antigen; Aptasensor; Gold; Nanospears

1. Introduction

Nowadays, improving the life needs prediction and control of different diseases, and early diagnosis of hard diseases, such as cancer, is one of the challenges in this regard. Fast and sensitive monitoring methods are required to detect disease-related biomarkers in both basic medical researches and clinical diagnostics [1, 2]. Prostate cancer (PCa) is the second leading cause of cancer mortality in men [3]. Prostate-specific antigen (PSA) is part of the proteolytic secretion and contributes to the liquefaction of the ejaculate [4] with a low concentration in the blood in normal men. Any disruption of the basal membrane or of the normal prostatic architecture causes to release PSA into the blood [5, 6]. On the other hand, PSA exists in the serum in various molecular forms including free PSA and its complex with the protease inhibitor α_1 -antichymotrypsin [7]. A total PSA level of higher than 4 ng mL^{-1} [8, 9] or 10 ng mL^{-1} [10] in the blood is often suspected to PCa; a PSA level in this range is considered as a danger zone. However, diagnosis prostate cancer in the postsurgery recurrence [9] and detection of PSA as a diagnosis of breast cancer [11] need detection of lower levels of PSA. Therefore, development of highly sensitive detection methods of the serum PSA concentration is essential in early diagnosis of PCa and breast cancer, determination of actual effectiveness of surgery and other therapeutic treatments, and monitoring of disease recurrence after treatment.

DNA and RNA aptamers can bind different biological targets such as proteins, peptides and cells with a high selectivity and nanomolar to micromolar dissociation constants [12, 13]. Application of aptamers as recognition elements in biosensors can provide advantages over the immunosensors due to their ability to denature and re-nature several times, higher stability and lower cost than antibodies, and because that aptasensors are faster, simpler, and more stable than the immunosensors. Electrochemical biosensors have many advantages of high sensitivity, low fabrication

cost, fast response time, mechanical flexibility, miniaturization ability, and automation capability [14-19]. In this regard, electrochemical aptasensors using single strand DNA or RNA for different analytes have been developed [20-23].

Nanotechnology provides new routes and promising opportunities to fabricate novel sensors and biosensors with enhanced characteristics. Nanostructured materials have been widely employed to fabricate ultra-high sensitive sensors due to the high ratio of surface area to volume, and chemical, electrochemical, electronic and optical properties depend on their shape, size and morphology [24-29].

Up to now, a lot of methods and tools have been proposed for determination of PSA including fluorescence [30, 31], surface enhanced Raman scattering (SERS) [32], surface plasmon resonance [33, 34], field-effect transistors [35], capillary electrophoresis [36], white-light reflectance spectroscopy [37], mass spectrometry [38], lateral flow [39], electrochemical [40-45], chemiluminescence and electrochemiluminescence [46-53], ELISA and immunoassay [54-56], and a point-of-care detection chip [57]. PSA detection in the blood by most of these methods includes routine immunoassays. Despite the advantages and widely utilization of immunoassays in analysis of a variety of analytes, they can be complicated, inactive, inconvenient, expensive, and suffer from the drawbacks of antibodies related to the production, stability, and manipulation [58-60]. There are also some reports on the development of aptamer-based PSA biosensors with optical [61-64] or electrochemical [43, 65-68] transductions. These electrochemical PSA aptasensors suffers form complex steps of fabrication [65, 66], and multiple materials and binding steps employed for the immobilization of aptamers [43, 65, 67], or needing high-cost impedance measurement device [68]. Therefore, developments of novel biosensors for PSA detection are highly desirable. In the present study, an electrochemical

aptasensor based on a transducer of gold nanospears for a rapid, and highly sensitive detection of PSA is presented.

2. Experimental section

2.1. Materials

All chemicals were of analytical grade from Merck (Germany) or Scharlau (Spain), and were used without further purification. All solutions were prepared by redistilled water. A 32-base thiolated aptamer probe (Apta) to capture and quantify PSA was selected with the following sequence:

5' SH-(CH₂)₆ TT TT TA AT TA AA GC TC GC CA TC AA AT AG CT TT-3' and purchased from Bioneer (Korea). PSA, bovine serum albumin and hemoglobin were purchased from Sigma (USA). The Apta stock solutions were prepared with a 20 mmol dm⁻³ Tris-HCl buffer, pH 7.4 solution (Tris) and kept frozen.

2.2. Apparatus

Electrochemical measurements were carried out in a conventional three-electrode cell powered by a μ -Autolab potentiostat/galvanostat (the Netherlands). An Ag/AgCl, 3 mol dm⁻³ KCl, a platinum wire, and a gold disk (Au electrode, 2 mm of diameter) or the Au electrode covered with gold nanospears (nsAu electrode) were employed as the reference, counter and working electrodes, respectively. The system was run on a PC by GPES 4.9 software.

Field emission scanning electron microscopy (FESEM) was performed using a Zeiss, Sigma-IGMA/VP (Germany) equipped with energy-dispersive X-ray spectroscopy (EDS). The samples were coated by a 2-5-nm thin film of gold by sputtering.

2.3. Preparation of nsAu electrode

Before electrodeposition of gold nanospears, the Au electrode was polished on a sand paper and then on a polishing pad with 50 nm-alumina powder lubricated by water. Polishing was continued to attain a mirror-like surface. The electrode was then cleaned by immersion in a 1:3 water/ethanol mixture and ultrasonication for 8 min in an ultrasound bath. The electrode was further electropolished by immersion in a 500 mmol dm⁻³ H₂SO₄ solution and applying potential in the range of cathodic to anodic edges of the electrolyte stability in a regime of cyclic voltammetry for 20 consecutive cycles. Upon this pretreatment, clean and stable gold surface was attained. The Au electrode was then placed in the cell containing the synthesis solutions comprising 20 mmol dm⁻³ HAuCl₄ + 500 mmol dm⁻³ H₂SO₄ + 150 mmol dm⁻³ arginine. Electrodeposition of gold nanospears was performed at 0 mV for 600 s. The nsAu electrode was then rinsed thoroughly with distilled water.

2.4. Immobilization of Apta

Lyophilized Apta was dissolved in appropriate distilled water. Then, 10 µL dithiothreitol (DTT) solution (containing 10 mmol dm⁻³ sodium acetate, pH 5.2 and 500 mmol dm⁻³ DTT) was added, mixed, and incubated at room temperature for 15 min. Excess DTT and unwanted thiol fragments were removed from the mixture by three times extracting with ethyl acetate (total volume of 150 µL), and the upper layer was discarded. The next step was then immediately followed because the free sulfhydryl group is unstable.

Immobilization of Apta was performed by dropping 10 µL of 10.0 µmol dm⁻³ Apta solution dissolved in Tris on the nsAu electrode surface and kept refrigerated at

4 °C for 8 h [19, 69]. Then the electrode was rinsed with Tris. The Aptamodified nsAu electrode was further treated with 1.0 mmol dm⁻³ 6-mercapto-1-hexanol at room temperature for 30 min to obtain a well aligned Aptam monolayer [69]. Then the electrode was washed again with Tris and double distilled water, respectively, to remove unspecific absorbed thiols. The obtained electrode was denoted as the aptasensor.

2.5. PSA capturing

The capturing process was performed by immersing the aptasensor into Tris containing various concentrations of PSA for 1 h at 37 °C [70-72]. Then, the aptasensor was rinsed with Tris to remove non-bonded PSA, and then it was incubated in a solution of Tris + 20 mmol dm⁻³ NaCl + 20 µmol dm⁻³ methylene blue (MB) for 5 min at 37 °C [19, 73]. Finally, the aptasensor was rinsed with Tris to remove the physically adsorbed MB.

2.6. Electrochemical measurements

The real surface areas of the Au and nsAu electrodes were measured electrochemically. The Au or nsAu electrode was transferred to a solution of 0.5 mol dm⁻³ KCl containing 0.5 mmol dm⁻³ K₄[Fe(CN)₆] and cyclic voltammograms at different potential sweep rates were recorded. Using the Randles-Sevcik equation [74] and the value of 7.60×10⁻⁶ cm s⁻¹ for the diffusion coefficient of [Fe(CN)₆]⁴⁻ [75], the real surface areas of the Au and nsAu electrodes were obtained.

Electrochemical detection of PSA binding to the aptasensor was performed in an electrochemical cell containing 10 mL Tris by recording cyclic voltammograms (CVs) and differential pulse voltammograms (DPVs) for the reduction peak of bonded

MB. DPVs were recorded with a pulse width of 25 mV, a pulse time of 50 ms, and a scan rate of 10 mV s⁻¹. The concentration of PSA was quantified as the MB reduction peak current. All electrochemical measurements were performed at room temperature.

2.7. Human samples

Blood samples of healthy and patient persons were obtained from Faghihi Hospital, Shiraz, Iran, and store frozen until assay. The blood serum samples were separated, and the values of PSA concentrations in the samples were firstly determined by an immunoradiometric assay. These samples were then diluted with distilled water (1:9) and assayed by the aptasensor.

3. Results and discussion

Fig. 1 presents FESEM images (A, B) of the nsAu electrode surface with different magnifications and an EDS spectrum (C). The images show a complex morphology, and the EDS spectrum confirms that the surface is covered by pure gold. At low magnification, the gold nanostructure is javelin-like comprising small serrate structure, while, at high magnification, the nanospears comprise nanowedges with sawtooth-shaped surfaces. This special nanostructure provides a favorable substrate for immobilization of Apta, and can promote the binding of Apta-PSA efficiency [76]. The real surface areas of the Au and nsAu electrodes were obtained as 0.05±0.01 and 0.99±0.05 cm², respectively. The geometric surface area was 0.0314 cm² indicating roughness factors of 1.62 and 31.4 for these electrodes. The high roughness factor of the nsAu electrode indicates that the surface texture of this electrode provides a large overall surface area needed for the immobilization of a high surface concentration of Apta resulting in a high sensitivity of the aptasensor.

For the formation mechanism of gold nanospears, it can be mentioned that gold atoms or clusters are initially and rapidly formed, followed by promoted nucleation by the Au electrode surface [77]. These processes are fast due to the high negative potential (compared to the formal potential of gold) employed for the electrodeposition, and cause to formation of many randomly distributed gold nuclei. Simultaneously, arginine, as a shape-directing agent, is adsorbed flat on the (1 1 1) plane surface of the nuclei [78], due to the high value of interaction free energy of the arginine with the gold surface [79]. The arginine adsorption process causes to formation of a meta-stable and densely packed arginine layer in which arginine is attached to the surface via the zwitterions functional group, and its positively charged guanidinium functional group tends outward into the solution [78]. This causes a further electrostatic adsorption of AuCl_4^- ions on this plane and preferential growth of gold along the (1 1 1) directions by the diffusion-limited aggregation mechanism [80].

Fig. 2 shows DPVs of MB recorded in Tris interacted with the aptasensor, before and after binding with various concentrations of PSA. MB binds to DNA and RNA sequences (including Apta) through different modes including electrostatic attractions and intercalation [81]. At the ionic strength of the working solution (Tris), MB binds to the free guanine bases, rather than electrostatic interaction with Apta [82] through hydrophobic forces of guanine with the fused aromatic rings in MB structure. Upon binding PSA with Apta, the Apta structure is changed in a manner that double stranded is formed with simultaneous binding to (and covering with) PSA. Therefore, the bonded MB is expelled and released, and the current in DPVs will be decreased. The dependency of the current of MB on the PSA concentration is represented in Fig. 2, inset. The plot is linear in the range of 0.125 to 200 ng mL^{-1} of PSA with a regression equation of $y = -(0.6856 \pm 0.0121)x + (2.0839 \pm 0.0153)$. Using

the plot, a limit of detection (LOD, $3\delta/m$) of 0.05 ng mL^{-1} of PSA was attained. Scheme 1 represents the fabrication protocol and the mechanism of the aptasensor and detection of PSA, and a comparison between LOD values reported for the PSA detection methods is presented in Table 1.

To inspect the selectivity of the aptasensor, DPVs were recorded with the non-specific proteins of bovine serum albumin and hemoglobin (Fig. 3). No noticeable current changes observed for these proteins. On the other hand, it should be mentioned that blood serum includes different biological compounds (such as proteins, enzymes, antibodies and hormones) with high concentrations. Because the aptasensor can directly analyze PSA in the serum samples with small constant error values (*vide infra*), it can be considered the aptasensor to be selective.

In order to employ the aptasensor for the detection of PSA in real samples, blood serum samples of healthy and patient persons (confirmed by immunoradiometric assay) were analyzed. DPVs recorded using the aptasensor for the healthy samples revealed that the peak current was the same as when there was no PSA (data not shown). In addition, the values of the PSA concentrations in the patient samples were obtained, and compared with the immunoradiometric assay in Table 2. Therefore, the aptasensor can detect PSA in patients, and can be used in clinical diagnosis.

In order to inspect the reproducibility of the aptasensor fabrication, it was placed in the Piranha solution for 30 s and then re-fabricated ($n=5$). Fig. 4 shows DPVs of the aptasensor for the repeating fabrications. Based on the results, a relative standard deviation (RSD) of 5.5% in the peak current was attained.

To inspect the regeneration of the aptasensor, after its binding with 1.0 ng mL^{-1} PSA for 1 h at 37°C , it was immersed in hot water of 70°C for 5 min to release PSA

from the Apta, and then cooled down slowly to room temperature. Then, the aptasensor was re-bonded with the same PSA concentration ($n=5$). Fig. 5 presents DPVs recorded after repeated binding with 1.0 ng mL^{-1} PSA. A RSD of 4.5% for the regeneration of the aptasensor was obtained.

The stability of the aptasensor was investigated by recording DPVs for the aptasensor capturing 1.0 ng mL^{-1} PSA over 40 days, while the aptasensor was stored in Tris at 4°C and measured twice in a day in the same conditions. No regular change was observed at least for 25 days.

4. Conclusion

A green electrodeposition method was developed for the synthesis of gold nanospears. The method can be expanded to the deposition of other gold nanostructures using other amino acids. The nanostructure provided a high surface area to immobilize a high surface concentration of the aptamer sequence. The fabricated aptasensor represented a high sensitivity to detect PSA in patients' samples and would be applicable for clinical analysis of PCa.

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Figure legends:

Fig. 1: FESEM images (A, B) and an EDS spectrum (C) of the nsAu electrode surface.

Fig. 2: DPVs of MB recorded using the aptasensor in Tris before and after binding with PSA of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32, 64, 128, 200 ng mL⁻¹. The arrow shows the direction of PSA concentration increment.

Inset: The dependency of the MB reduction peak current on the PSA concentration (the calibration curve).

Fig. 3: DPVs of MB recorded using the aptasensor in Tris before (blue) and after binding with 50 mg mL⁻¹ bovine serum albumin (red) or 17.5 mg mL⁻¹ hemoglobin (green).

Fig. 4: DPVs of MB recorded using the aptasensor in Tris before for the repeating fabrication.

Fig. 5: DPVs of MB recorded using the aptasensor in Tris before (red curves) and after (blue curves) reparative binding with 1.0 ng mL^{-1} PSA.

Table 1: A comparison between LOD values reported for the PSA detection methods.

Detection method	Recognition element	Linear range (ng mL ⁻¹)	LOD (pg mL ⁻¹)	Reference
Electrochemical	Aminated aptamer	1-100	1000	[43]
Electrochemical	Biotinated aptamer	0.25-200	250	[65]
Electrochemical	Aptamer-MIP hybrid receptor	0.1-100	1	[66]
Electrochemical	Aptamer/AuNPs-graphitized mesoporous carbon	0.25-200	250	[67]
Electrochemical impedance	Aptamer	0.007-6	1000	[68]
Electrochemical immunoassay	Label-free PSA antigen	0.05-5	13	[83]
Electrochemical immunoassay	Graphene/cobalt hexacyanoferrate nanocomposite	0.02-2	100	[84]
Electrochemical immunoassay	Magnetic beads enzyme linked immunosorbent	0-1	<100	[85]
Voltammetric ELISA	DAB-H ₂ O ₂ -HRP	0.2-32	100	[86]
Electrochemical immunoassay	Ab ₁ -Ag-Ab ₂ -HRP	0-15	250	[87]
Electrochemical dual sensor	Enzyme/antibody	1-10	1000	[88]
Colorimetric assay	A specific PSA peptide	0.1-100	1.0×10 ⁴	[89]
RLS assay	Aptamer-modified AuNPs	0.13 - 110	320	[64]
Optical	Antibody	0.0005-5	10	[90]
Duplexed immunoassay	Functional microbeads	0.01-5	50	[91]
Cathodic ECL immunoassay	Antibody/luminal/graphene	0.01-8	8	[92]
Sandwich-type immunoassay	Antibody on PDMS microfluidic chips	4-10	520	[93]
Fluorescence	Dye-labeled aptamer/MoS ₂ nanosheets	0.5-300	200	[61]
Chemiluminescence	Dye-labeled aptamer/Fe ₃ O ₄ -graphene oxide nanoparticles	1.6-50	500	[62]
Chemiluminescence	Dye-labeled aptamer	1.9-125	1.0×10 ³	[94]
Surface acoustic wave detection	Aptamer beacon	10-1000	1.0×10 ⁴	[63]
Electrochemical	Aptamer (Apta)	0.125-200	50	This work

Abbreviations:

LOD: Limit of detection

MIP: Molecularly imprinted polymer

RLS: Resonance light scattering

AuNPs: Gold nanoparticles

DAB-H₂O₂-HRP: 3,3'-diaminobenzidine-H₂O₂-horseradish peroxidase

ECL: Electrochemiluminescence

PDMS: Poly(dimethylsiloxane)

Ab₁-Ag-Ab₂-HRP: Antibody1-antigene-antibody 2- horseradish peroxidase

Table 2: Values of the PSA levels in the patients' blood serum obtained by the aptasensor.

Nominal value ^a (ng mL ⁻¹)	Obtained value (ng mL ⁻¹)	Bias (%)
5.31	5.69	-7.20
6.78	6.56	3.22
6.87	6.39	6.95
8.48	8.86	-4.51
9.19	8.92	2.95
12.31	11.67	5.15
13.31	12.87	3.27
19.00	18.94	0.29
27.06	28.79	-6.40
53.08	50.22	5.39

^aNominal values were obtained by the immunoradiometric assay.

2 μm

(A)

200 nm

(B)

(C)

Fig. 1

Figure 2

Figure 3

Figure 4

Figure 5

Scheme 1