Research Article

An Innovative Immunoanalysis Strategy towards Sensitive Recognition of PSA Biomarker in Human Plasma Samples Using Flexible and Portable Paper Based Biosensor: A New Platform towards POC Detection of Cancer Biomarkers Using Integration of Pen-on Paper Technology with Immunoassays Methods

Fatemeh Farshchi[1] [ID], Arezoo Saadati[2] [ID], Houman Kholafazad kordasht[3] [ID], Mohammad Hasanzadeh[1,4*] [ID]

[1]Pharmaceutical Analysis Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Drug Applied Research Center, Tabriz University of Medical Science, Tabriz, Iran
[3]Food and Drug Safety Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Nutrition Research Center, Tabriz University of Medical Science, Tabriz, Iran

Abstract

Background: The prostate-specific antigen (PSA) is one of the best markers for detecting prostate cancer. Rapid and real time recognition of PSA biomarker could be helpful in the early diagnosis and efficient treatment of prostate cancer. One of the usual methods to identify this biomarker is ELISA, which has a picomolar detection range but requires specialized personnel and also this technique is time-consuming and expensive.
Methods: Cost-effective POC devices are great solutions because they are very cost-effective, sensitive and simple, do not require expert operators and have a high response time in a short time. With that in mind, in this work, a novel and simple label-free paper-based electrochemical immunosensor were designed by using conductive Ag-ink and designed directly by pen on paper technique on the surface of photographic paper, which is a suitable substrate for antibody immobilization, for rapid detection of PSA.
Results: Based on the obtained results, under the optimum conditions, the synthesized Ag ink has a great substrate for antibody (Ab) and antigen (Ag) immobilization. The linear range was from 0.001 to 30 µg/L and the obtained low limite of quantification (LLOQ) was 0.001µg/L. This immunosensor also tested in human plasma samples, which had good analytical power.
Conclusion: The proposed paper-based immunoassay could be a hopefully new and cheap tool for the diagnosis of other biomarkers.

Introduction

The biosensor is a chemical sensing device in which a biologically derived recognition is coupled to a transducer, to allow the quantitative development of some complex biochemical parameter. The biological element can be an enzyme, an antibody or a nucleic acid. Depending on their particular application, biosensors are also known as immunosensors, aptasensors and genosensors.[1] One of the great applications of biosensors is point of care (POC) testing, which provides physicians with essential information.[2,3] The urgent need for these tests poses a new challenge for biosensor researchers. Microfluidic biosensors are a new type of bioassays that have attracted special attention due to their great features such as, good sensitivity, easy transportation and using a small number of samples.[4,5]

One of the most common cancers among men is prostate cancer, which is known as the sixth deadliest cancer.[5] The cancer-specific antigen is called PSA, which is one of the best markers for detecting prostate cancer. PSA is a 28.4 kDa serine protease secreted by epithelial cells of the glands around the urethra and healthy prostate cells. Normally, the concentration of this antigen is 0-4 ng/ml, which increases with cancer and ageing. According to studies, increasing it to 4-10 ng/ml increases the risk of cancer.[6,7] Therefore, rapid and timely detection of PSA with a low detection limit could be helpful in the early treatment of prostate cancer. One of the common methods to identify this biomarker is ELISA, which has a picomolar detection range but requires specialized

personnel and also this technique is time-consuming and expensive.[8] Cost-effective POC devices are great solutions because they are very cost-effective, sensitive and simple, do not require expert operators and have a high response time in a short time.[9] Recently, great advances have been made in the field of microfluidic biosensors, which has integrated and intelligent reading systems that have led to many advances in POC diagnostic devices.[10] Paper-based biosensors usually consist of one or more layers of paper composed of hydrophilic paper channels and designed by hydrophobic materials.[11] By employing these biosensors, the concentration of different fluids such as saliva, blood, urine, plasma and serum can be analyzed in one or two stages in a fast and accurate manner.[12] On the other hand, electrochemical paper-based biosensors have low design and manufacturing costs, high accuracy and sensitivity and can reduce in small size for easy movement, so they can be a great tool in identifying cancer biomarkers. In electrochemical paper-based biosensors, paper is valuable in point-off care tests because of its good electrical resistance and flexibility.[13–15] The use of conductive nano-inks that contain metal nanoparticles is also an excellent option in the design of paper in paper-based biosensors due to their low toxicity, high availability, biocompatibility and excellent conductivity. Metal nanoparticles such as gold and silver nanoparticles also have good oxidation stability and electrical conductivity, which makes them a strong element in paper-based biosensors.[13,16]

With that in mind, in this work, a novel and simple label-free paper-based electrochemical immunosensor were designed by using conductive Ag-ink, which is a suitable substrate for antibody immobilization and rapid detection of PSA. Unlabeled immunosensors are very fast and accurate enough due to the direct binding of antibodies to the surface compared to sandwich and labelled immunosensors.[17] The engineered immunosensor also tested in real samples, which had a high detection limit. Also, a Field scanning electron microscope (FE-SEM) and EDC used to study the morphology and element analysis of the prepared biosensor.

Materials and methods
Substances and chemicals
Human PSA ELISA kits, were gained from CanAg Diagnostic AB Technology Co. (Gothenburg, Sweden, with PSA antigen, antibody (anti-PSA) standard, standard buffer. N-Hydroxy succinimide(NHS), 1-Ethyl-3-(3-dimethylaminopropyl) (EDC), Potassium ferrocyanide (K_4 [Fe $(CN)_6$]), Potassium ferricyanide (K_3 [Fe $(CN)_6$]), Sodium hydroxide (NaOH), bovine serum albumin (BSA), Silver nitrate ($AgNO_3$), Diacetone Alcohol(DAA), Poly Acrylic acid (PAA), Diethanolamine (DEA), Human plasma samples were found from the Iranian Blood Transfusion Research Center (Tabriz, Iran).

Apparatuses
EDS (Energy scattering spectroscopy) was employed to study chemical elements. A High-resolution field emission scanning electron microscope (FE-SEM) Hitachi SU8020, Czech with a 3kV effectual voltage used for considering the morphology of the surface of electrodes. For electrochemical measurements, a Palm Sense 4c device (Palm Instruments, Utrecht, Netherlands) connected to a laptop and analyzed by PS Trace software was used. The three-electrode system (reference, counter and working electrode) drawn on the surface of the photographic paper by using synthesized conductive Ag-ink (Figure 1).

Synthesize of conductive Ag nano ink
First, DEA, PAA and DW (915.1-40-50 g, respectively) were mixed for 2 hours in a water bath. $AgNO_3$ solution was then added and stirred for 22 h (20 g in 15 ml DW). In the next step, the prepared solution was sonicated for 1.5 hours at 65 ° C and combined with 300 ml of ethanol and centrifuged (20 minutes, 9000 rpm). The resulting solution washed again three times with deionized water and centrifuged. Finally, 2% by weight of hydroxypropyl cellulose solution in a ratio of 1: 1 (v: v) methanol and water added to the above composite. Finally, the prepared mixture homogenized for three minutes (2000 rpm). Scheme 1 shows various steps of preparation of Ag ink.

Conductivity study of the synthesized Ag nano ink
To study the conductivity of synthesized Ag- ink, different lines and shapes with various thicknesses were drawn on the surface of photographic paper. A battery and an LED lamp (both three volts) placed on the surface of photographic paper, the cathode part of the battery was connected to the lines using a conductive wire and the anode part was connected to the lamp (Figure 2). When current flows from the anode to the cathode, an electrical

Figure 1. Indicates the connection of the designed three-electrode system by conductive Ag-ink (reference, working and counter electrode) to the electrochemical device
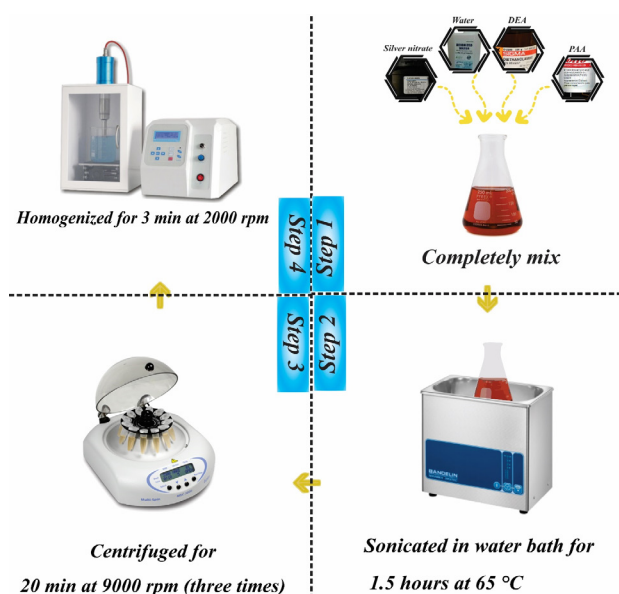
Scheme 1: Conductive ink preparation procedure

circuit was formed and the lamp turned on. Also, an ohmmeter was used to check the resistance of the lines.

Also, according to Figure 3, the conductive lines of Ag-ink drawn on paper have good flexibility and the prepared ink does not spread on the paper after drying, which indicates the stability of the synthesized conductive Ag-ink on the surface of photographic paper.

Characterizations

FE-SEM and EDS of bulk Ag nano-ink

FE-SEM imaging was used to study the morphology of the synthesized Ag nano ink. According to Figure 4, the uniform and spherical structure of silver nanoparticles have shown. There is no accumulation of nanoparticles. Also, as shown in Figure 4A, the average size of nanoparticles is about 50 nanometers. EDS spectrometer was also used to examine the elements and analyze the prepared assignment. As shown in Figure 5, the presence of silver nanoparticles in large quantities indicates the successful synthesis of conductive Ag-ink.

FE-SEM and EDS of various steps of prepared paper-based immunosensor

The morphology of each step of the immunosensor

preparation was recorded by FE-SEM imaging. In the first step, as shown in Figure 6 (A-C), the conductive Ag-ink nanoparticles are spherical and have created an uneven surface on the paper. After incubation of the antibody, the morphology of the surface completely changes and they have observed as crystalline sheets. Again, as shown in Figure 6 (G-I), after incubation of PSA antigen and BSA, the morphology changes indicate the successful stabilization of antibodies and antigens on the surface of the photographic paper designed with Ag ink.

According to Figure 7 and the results of EDC spectroscopy, in the first stage, the large amount of silver element is due to the presence of Ag- ink on the surface of the paper. In the second stage, after the antibody incubation, the amount of phosphate and sulfur elements
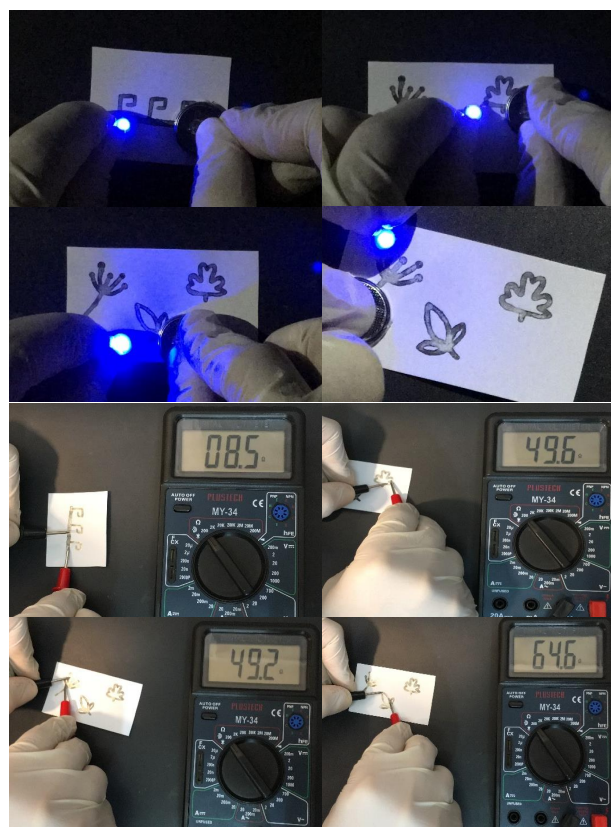

Figure 2. Images of different shapes with different thicknesses of synthesized Ag nano-ink on the surface of photographic paper
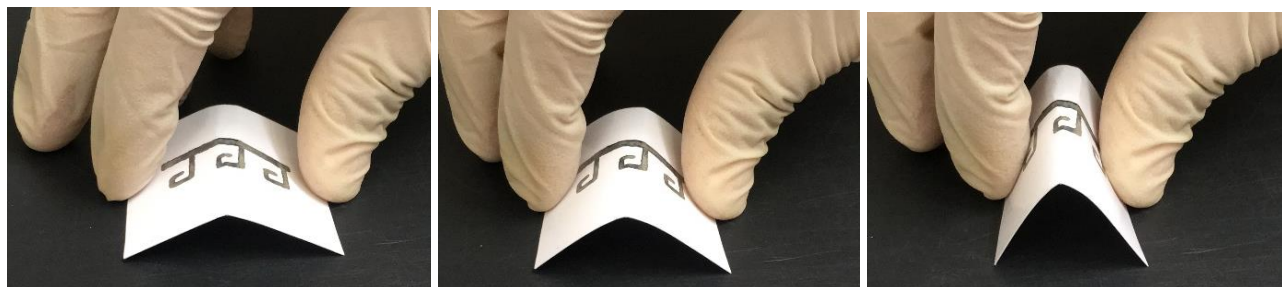

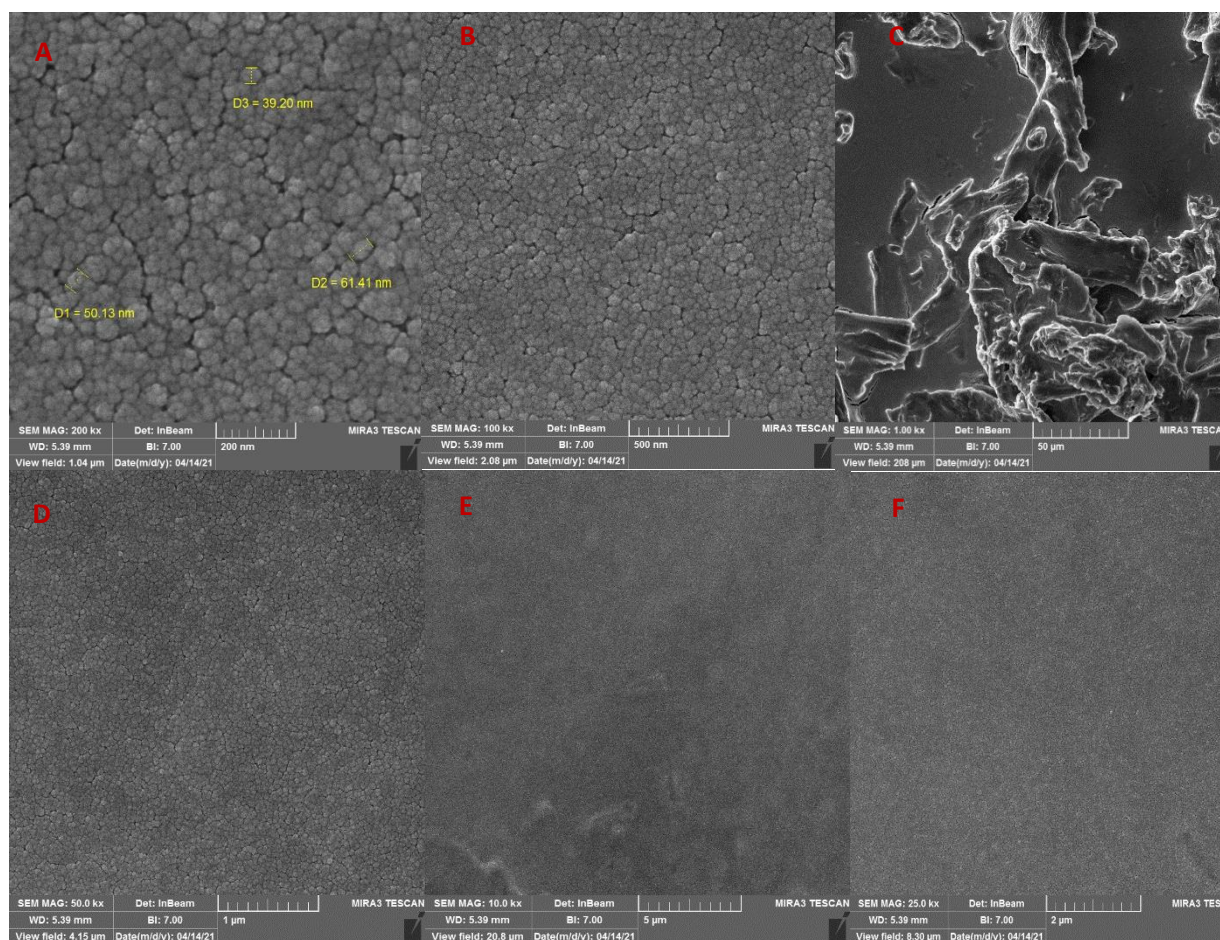**Figure 3.** Flexibility of the synthesized Ag-ink designed on surface of photographic paper

Figure 4. FE-SEM descriptions of the bulk Ag nano-ink. (A to F)
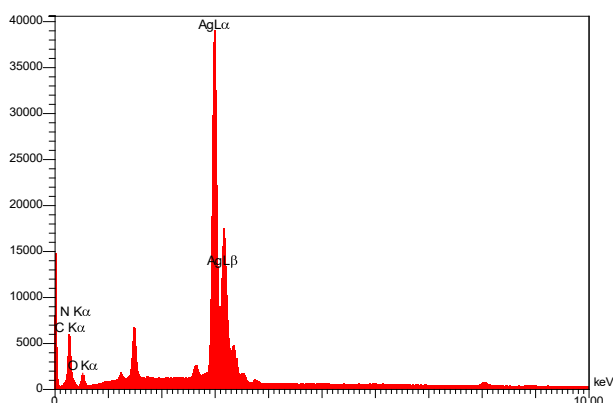

Figure 5. EDS descriptions of the bulk Ag nano-ink

increases, which indicates the successful incubation of biological materials on the electrode surface. In the last stage, after adding PSA antigen and BSA, the amount of Ag has decreased, i.e. the presence of antigen has overshadowed the amount of Ag.

Fabrication of the paper-based electrochemical immunosensor

To prepare the electrochemical paper-based immunosensor, the synthesized Ag nano-ink was designed on the surface of photographic paper and allowed to dry at room temperature for 5 minutes. Then, biotinylated-anti-PSA combined with NHS / EDC 1:1 (v/v). After 20 minutes, 5 μl of activated antibody was immobilized on the surface of the sensing zone of the electrode and incubated for 1 hour at 4 ℃. In the next step, after rinsing the electrode, the BSA solution was immobilized on the electrode for half an hour to block the remaining connection points. After incubation, the electrode surface washed again with deionized water to remove contamination. Finally, PSA antigen incubated on the electrode, after washing with buffer, it connected to the device for electrochemical evaluation. Scheme 2 displays the fabrication stages of the electrochemical paper-based immunosensor.

Results and discussion

Electrochemical evaluation of the prepared immunosensor

The electrochemical behavior performance of the prepared immunosensor in diverse steps has evaluated by using the DPV technique in 0.01 M aqueous $[Fe\ (CN)_6]^{3-/4-}$/ electrolyte containing KCl. According to Figure 8, the peak current intensity of the synthesized Ag-ink is equal to 500 μA, According to previous studies prepared Ag-ink have a good surface and current intensity and also have high oxidation stability and electrical conductivity.[18,19]
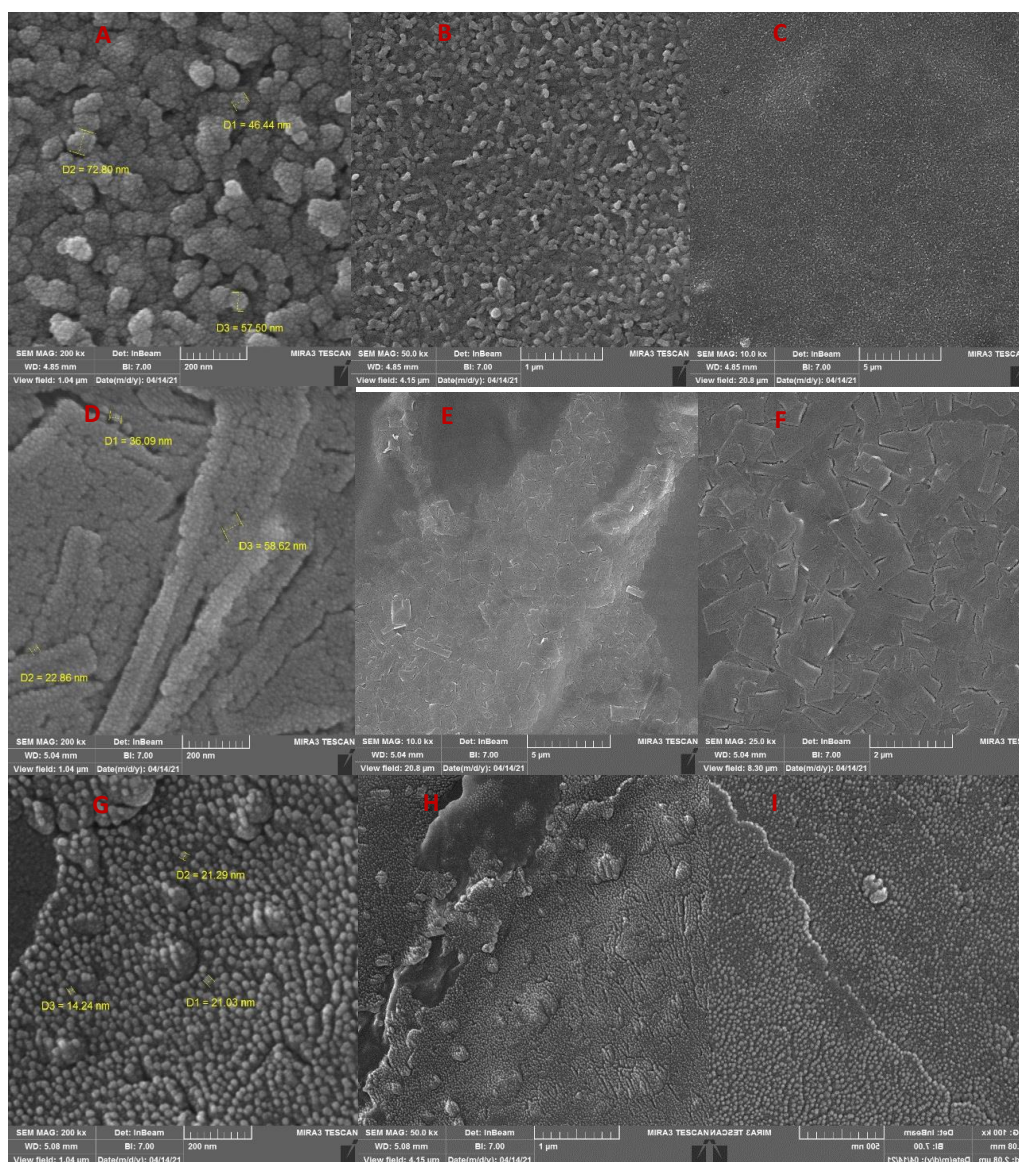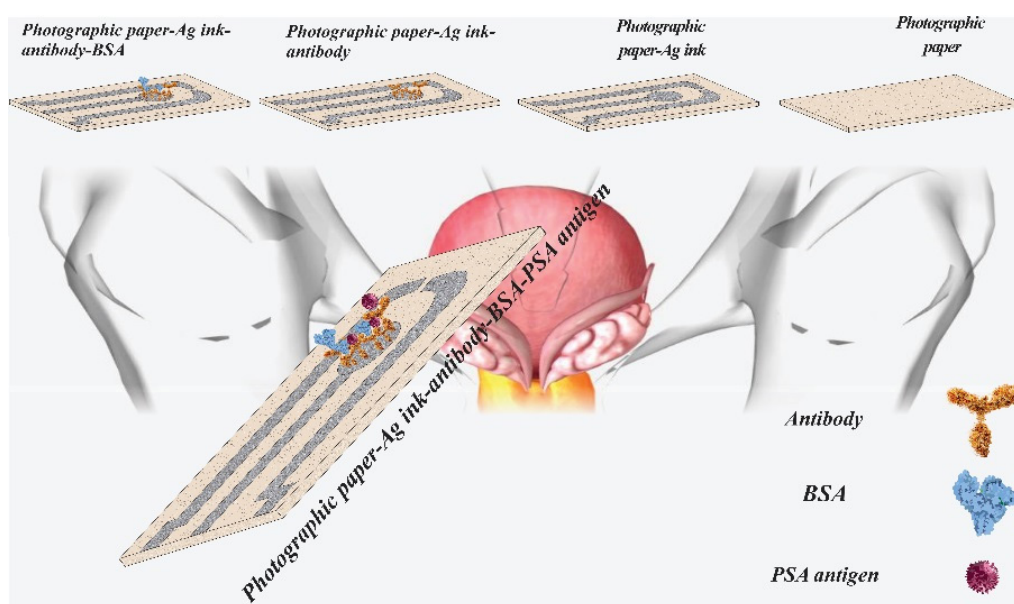
Figure 6. FE-SEM descriptions of the various steps of prepared paper-based immunosensor. (A to I)

Scheme 2. Schematic illustration of preparation steps of electrochemical paper-based immunosensor for detection of PSA
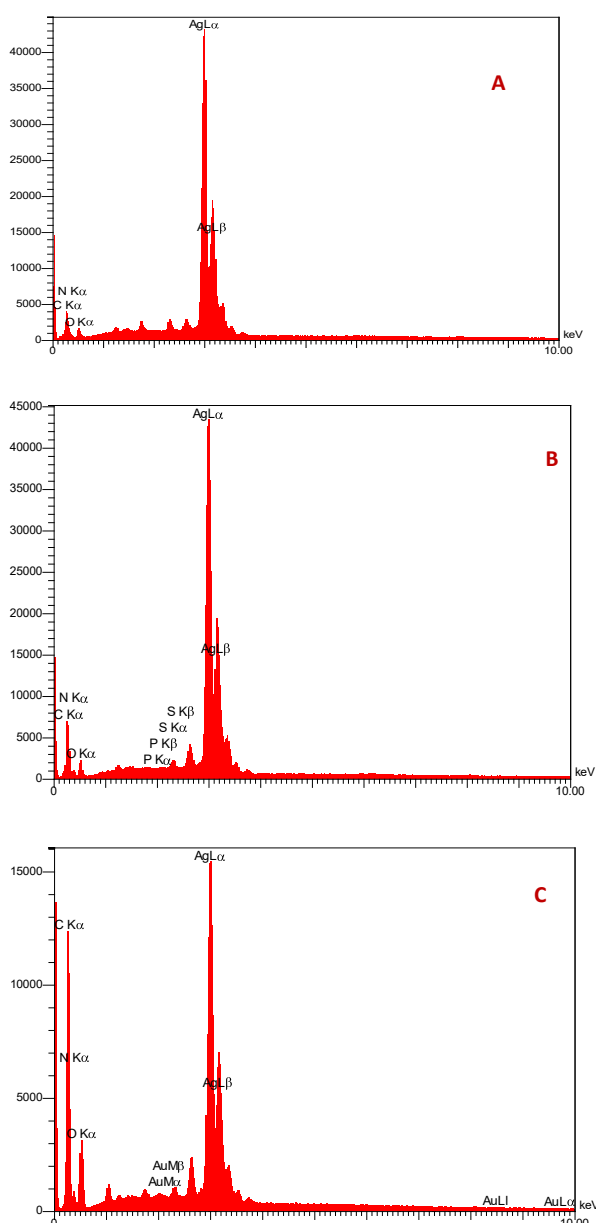
Figure 7. (A) EDC images of the Ag-ink. (B) EDC images of the Ab-Ag-ink, (C) EDC images of Ag/BSA/Ab-Ag-ink (D) on the surface of photographic paper

They provide an appropriate condition for immobilization of Ab against intended purpose analyte such as PSA biomarker.[20,21] After incubation of the antibody, the current increases to 547 μA which reveals a suitable appropriate substrate for the incubation of PSA antibody. Also, after immobilization of BSA and PSA, the current intensity increases to 625 μA, which shows the successful hybridization of the antigen-antibody complex.

Analytical approach
Analytical study of the prepared paper-based immunosensor in different concentrations of PSA biomarker

To investigate the effect of concentration, the DPV technique used. In this way, the various concentration of PSA (30-0.001 μg/L) immobilized on the surface of the

prepared immunosensor (BSA/Ab/Ag ink) and incubated at 4 °C for an hour. As shown in Figure 9 in the highest concentration (30 μg/L) the peak currency was 35 μA, when the concentration decreases to 0.001 μg/L, the current intensity increases to 143 μA. To recapitulate the previous concepts, it can be said that the current intensity is inversely related to the PSA concentration. In a way, as the concentration decreases, the current intensity increases. The cause of this reaction can be related to the relationship between the concentration of macromolecules and the intensity of the current, that results from the barrier behavior of the macromolecules against the electrochemical current.[22,23]

Also, the calibration curve and the standard curves have drawn for different concentration of PSA.

Under optimum conditions, the obtained lower limit of quantification (LLOQ) was 0.001μg/L.

The linear regression equations were as follow:

$$Ip\ (\mu A) = -0.0562C_{(PSA)} + 1.982,\ R^2 = 0.983.$$

The results show that the engineered immunosensor has a high ability for detection of PSA at low concentration. Based on the previous studies presented in Table 1, the prepared Ag-ink immune sensor is so simple, easy, disposable, cost-effective, sensitive, and present in a short time and does not require specialized personnel. Compared to conventional methods such as ELISA, these features are very important and could be used in clinical laboratories in the not-too-distant future.
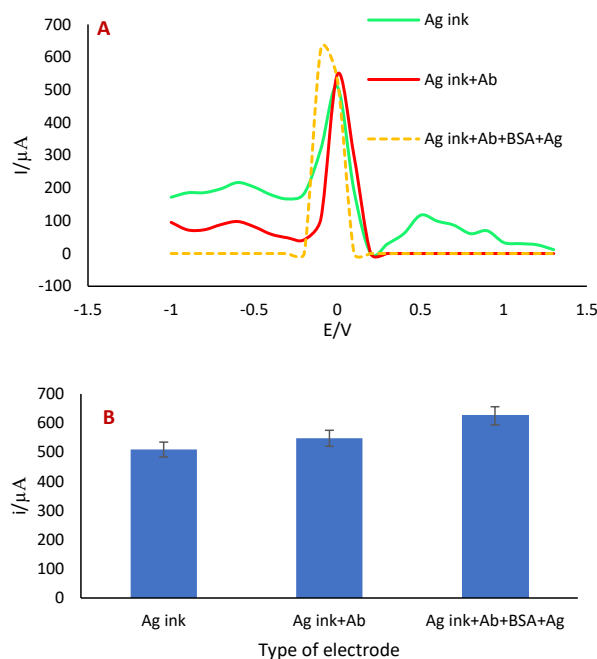

Figure 8. A) DPVs of immunosensor fabrication steps. (step potential= 1.0 V, E_{pulse}= 0.1, t_{pulse}= 0.2 s and scan rate= 10 mV/s). B) Histogram of peak current against the type of electrode. Supporting electrolyte was 0.01M [Fe (CN) 6]$^{[3-/4-]}$/KCl solution
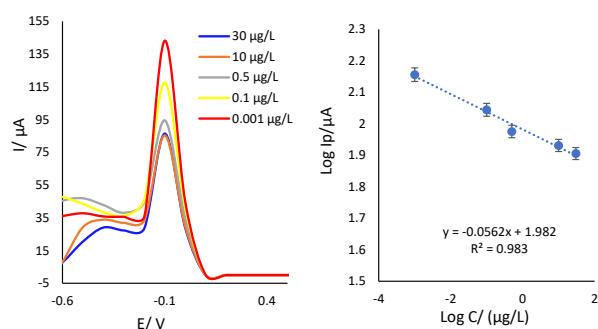
Figure 9. A) DPVs of the different concentration of PSA antigen (0.001 to 30) on the surface of photographic paper (Step potential= 1.0 V, E pulse= 0.1, t pulse= 0.2 s and scan rate= 10 mV/s). B) DPVs calibration curves. Supporting electrolyte was 0.01M [Fe (CN)₆]³⁻/⁴⁻/ KCl solution
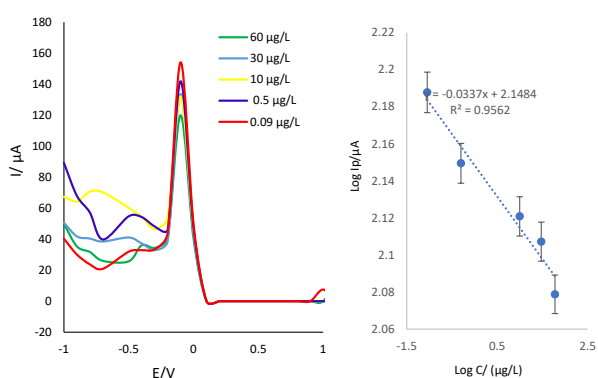

Figure 10. A) DPVs of the different concentration of PSA antigen (0.09 to 60) in human plasma samples on the surface of photographic paper (Step potential= 1.0 V, E pulse= 0.1, t pulse= 0.2 s and scan rate= 10 mV/s). B) DPVs calibration curves. Supporting electrolyte was 0.01M [Fe (CN)₆]³⁻/⁴⁻/KCl solution

Analytical performance of prepared paper-based immunosensor in Real sample

To investigate the effect of different concentration of PSA in human plasma samples as the real sample, PSA antigen was mixed with plasma 1:1 (v/v) and incubated on the surface of the engineered electrode (BSA/Ab-Ag ink). Figure 10 shows the DPV techniques diagrams in various concentration of PSA in human plasma samples. The intensity of the peak increases with decreasing the concentration. The obtained LLOQ was 0.09 µg/L and the attained linear range was from 0.09 to 60 µg/L.

The calibration curve plotted with the linear regression equivalence obtained from DPV in human plasma samples is as follows:

$$Ip\ (\mu A) = -0.0337\ C_{(PSA)} + 2.148,\ R^2 = 0.8944.$$

Most of the methods shown in Table 1 have good sensitivity and detection power. But the prepared label-free immunosensor is much simpler and more cost-effective. Due to its unlabeled, its preparation and analysis time is short (approximately 180 minutes). It also has acceptable stability and also, the conductive Ag ink used in this work has a higher conductivity. Therefore, it can be said that the proposed immunosensor can be an alternative to common methods in clinical laboratories and can use in the identification of other biomarkers.

Selectivity of the paper-based immunosensor

To study the detection power of the designed immunosensor, several interferers used as serum proteins that are also present in real human blood samples. In this

Table 1. Evaluation of sensitivity and performance of designed immunosensor to detect PSA in comparison with other studies

Strategy/nanomaterials	Detecting technique	LOD[a]/LOQ[b]/LLOQ[c]	Detection range	Ref
SP-modified rGO nanosheets/anti-PSA. Label free	Electrochemical (DPV)	0.053 ng.ml⁻¹ (LOD)	0.15-0.058 ng.ml⁻¹	24
Peptide biosensor based on in situ silver deposition Graphene (GR)-based gold (Au) composite	Electrochemical (DPV-CV)	0.001ng.ml⁻¹ (LOQ)	0.001–30 ng.ml⁻¹	25
Magnetic sandwich type immunosensor based on Fe3O4	Electrochemical (SWV)	0.001 µg. L⁻¹ (LOQ)	0.001-1 µg. L⁻¹	26
Label free based on SiO₂- AgNPs	Electrochemical (DPV)	0.001 µg. L⁻¹ (LOD)	0.03-0.001 µg. L¹	27
Label free immunosensor based on 6-mercapto-b-cyclodextrin SAM/anti-PSA.		0.0003 ng.ml⁻¹ (LOD)	0.001–1 ng.ml⁻¹	28
Label free immunosensor based on HDT SAM/AuNPs/anti-PSA..	Electrochemical (SWV)	0.01 ng.ml⁻¹ (LOQ)	0.2–200 ng.ml⁻¹	29
Magnetic sandwich type immunosensor based on KCC-1-npr-NH₂. KCC-1-npr-NH₂	Electrochemical (SWV-DPV)	0.01 µg. L⁻¹ (LOD)	1- 60 µg. L⁻¹.	30
Paper based immunosensor based on Citrate AgNPs-GQDs nano-ink/Cys A	Electrochemical (DPV)	0.05µg. L⁻¹ (LOD)	10-0.05 µg. L⁻¹	21
Paper based immunosensor based on conductive Ag-ink		0.09 µg. L⁻¹ (LLOQ)	60-0.09 µg. L⁻¹	This work

[a]LOD; limit of detection
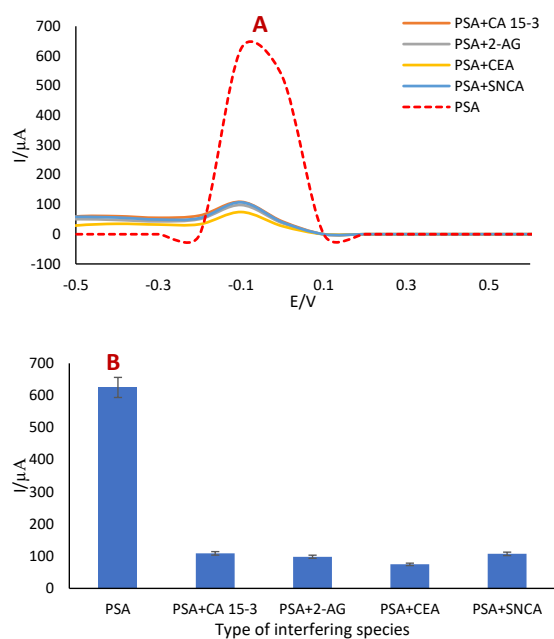[b] LOQ limit of quantification
[c] LLOQ; Low limit of quantification

Figure 11. A) DPVs of the prepared immunosensor in the presence of different interferes (Step potential= 1.0 V, E_{pulse}= 0.1, t_{pulse}= 0.2 s and scan rate= 10 mV/s). B) Histogram of peak potential and currents versus of interferes on the surface of photographic paper. Supporting electrolyte was 0.01M [Fe $(CN)_6]^{3-/4-}$/KCl solution

method, the DPV technique was employed. According to Figure 11, the current of the designed immunosensor is equal to 625 µA, which is drastically reduced in the presence of interferers. Therefore, the designed immunosensor does not have good selectivity and protein compounds interfere with the PSA detection process.

Reproducibility and stability of the paper-based immunosensor

Considering that stability and reproducibility are some of the important features for biosensors. Also, the cheapness of the prepared immunosensor is so important, too. So, the reproducibility and the types of stability for the designed immunosensor were investigated.

Cyclic stability

Cyclic voltammetry (CV) and DPV techniques used to evaluate the stability of the Ag-ink substrate on the surface of the paper. In this way, the number of 1-2-5 and 10 cycles was applied and then the DPV test performed after each. According to Figure 12 of the prepared Ag-ink on the surface of photographic paper, loses its function by increasing the number of cycles.

Long-term stability test

The prepared paper-based immunosensor stability evaluated in 72 hours. As shown in Figure 13, after 24 hours the intensity of the current greatly increases and the location of the peak shifted. After 72 hours it completely loses its efficiency. According, Figure 14 The stability

of the immunosensor also evaluated in one day with an interval of 1 hour. The immunosensor loses its function after almost an hour. Therefore, this immunosensor has poor stability.

Reproducibility

The DPV technique has used to evaluate the reproducibility of the immunosensor. Due to the difference in current recorded between the three electrodes, the prepared electrodes do not have good reprroducibilty (Figure 15).

Conclusion

In summary, the early detection of PSA helps in the early detection of prostate cancers. So, in this article, a novel and simple label-free paper-based electrochemical immunosensor developed by using conductive Ag-nano ink and designed directly by pen on paper technique on the surface of photographic paper. The prepared substrate was suitable for antibody and rapid detection of PSA. Although this immunosensor was label-free, it has a high detection limit. This immunosensor was evaluated by the DPV technique. Under the optimum conditions, the linear range was from 30-0.001µg/L and the obtained LLOQ was 0.001µg/L. This immunosensor also tested in human plasma samples, which had good analytical power. The developed immunosensor is so easy, sensitive, cost-
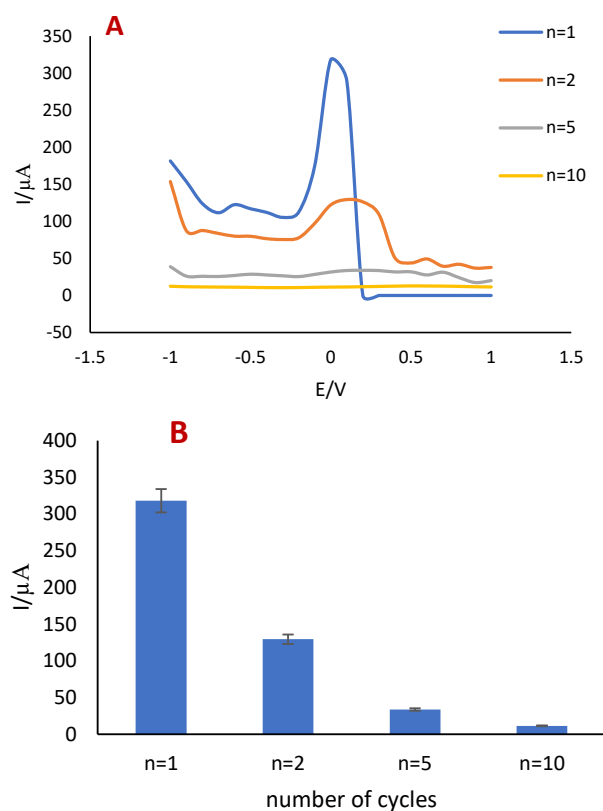

Figure 12. A) DPVs (Step potential= 1.0 V, E pulse= 0.1, t pulse= 0.2 s and scan rate= 10 mV/s). and B) histograms of engineered immunosensor stability on the surface of photographic paper. Supporting electrolyte was 0.01M [Fe $(CN)_6]^{3-/4-}$/KCl solution

Figure 13. A) DPVs of Intra-day stability of Ab/ Ag-ink electrode on the surface of photo graphic paper (Step potential= 1.0 V, E pulse= 0.1, t pulse= 0.2 s and scan rate= 10 mV/s). B) Histogram of intraday stability. Supporting electrolyte was 0.01M [Fe (CN)$_6$]$^{3-/4-}$/KCl solution

Figure 15. A) DPVs of Inter-electrode reproducibility of Ag ink on the surface of photographic paper. (Step potential= 1.0 V, E pulse= 0.1, t pulse= 0.2 s and scan rate= 10 mV/s). B) Histogram of Inter-electrode reproducibility. Supporting electrolyte was 0.01M [Fe (CN)$_6$]$^{3-/4-}$/KCl solution
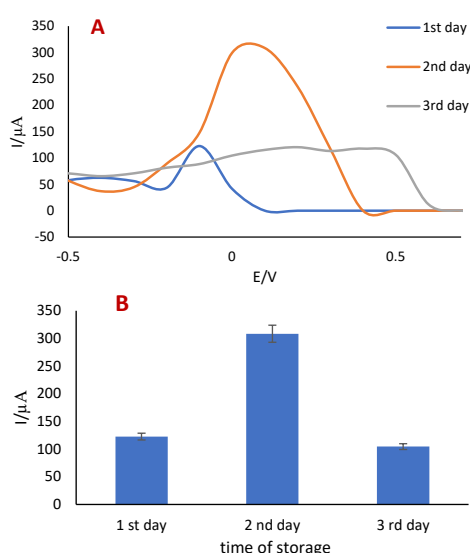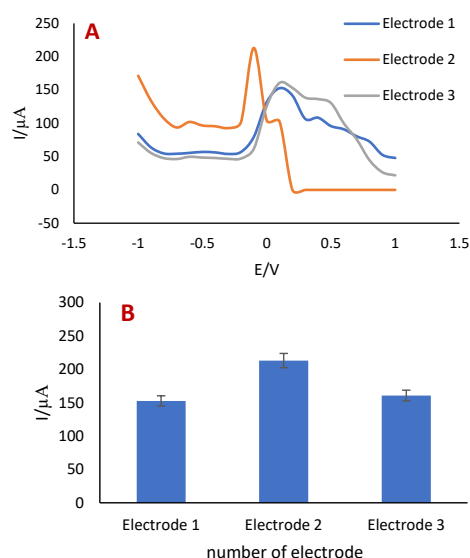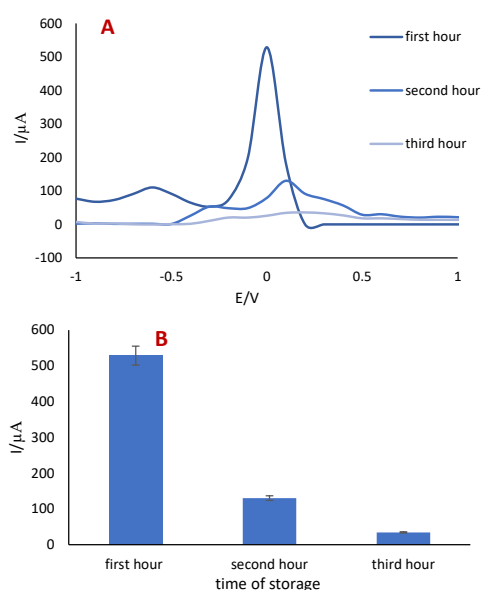

Figure 14. A) DPVs of Inter-day stability of Ab/ Ag-ink electrode on the surface of photo graphic paper (Step potential= 1.0 V, E pulse= 0.1, t pulse= 0.2 s and scan rate= 10 mV/s). B) Histogram of Interday stability. Supporting electrolyte was 0.01M [Fe (CN)$_6$]$^{3-/4-}$/KCl solution

effective, time-consuming and simple. Therefore, it can be an accomplished biodevice in clinical laboratories.

Conflict of Interest
The authors have no conflicts of interest to declare.

Ethical Issues
All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research.

References
1. Chambers JP, Arulanandam BP, Matta LL, Weis A, Valdes JJ. Biosensor recognition elements. 2008;
2. Kordasht HK, Hasanzadeh M. Aptamer based recognition of cancer cells: Recent progress and challenges in bioanalysis. Talanta. 2020;220:121436. doi:10.1016/j.talanta.2020.121436
3. Kholafazad Kordasht H, Hasanzadeh M. Biomedical analysis of exosomes using biosensing methods: recent progress. Anal Methods. 2020;12(22):2795–811. doi:10.1039/D0AY00722F
4. Farshchi F, Hasanzadeh M. Microfluidic biosensing of circulating tumor cells (CTCs): Recent progress and challenges in efficient diagnosis of cancer. Biomed Pharmacother. 2021;134:111153. doi:10.1016/j.biopha.2020.111153
5. Saadati A, Hassanpour S, Bahavarnia F, Hasanzadeh M. A novel biosensor for the monitoring of ovarian cancer tumor protein CA 125 in untreated human plasma samples using a novel nano-ink: a new platform for efficient diagnosis of cancer using paper based microfluidic technology. Anal Methods. 2020;12(12):1639–49. doi:10.1039/D0AY00299B
6. Singh P, Katkar PK, Patil UM, Bohara RA. A robust electrochemical immunosensor based on core–shell nanostructured silica-coated silver for cancer (carcinoembryonic-antigen-CEA) diagnosis. RSC Adv. 2021;11(17):10130–43. doi:10.1039/D0RA09015H
7. Díaz-Fernández A, Miranda-Castro R, De-los-Santos-Álvarez N, Lobo-Castañón MJ, Estrela P. Impedimetric aptamer-based glycan PSA score for discrimination of prostate

cancer from other prostate diseases. Biosens Bioelectron. 2021;175:112872. doi:10.1016/j.bios.2020.112872

8. Aldhaheri HN, AlSaimary IE, ALMusafer MM. The Estimation of prostate specific antigen (PSA) concentrations in patients with prostatitis by fully automated ELISA technique. J Med Res Heal Sci. 2020;3(11):1100–4. doi:10.15520/jmrhs.v3i11.279

9. Fathi N, Saadati A, Hasanzadeh M, Samiei M. Chemical binding of pyrrolidinyl peptide nucleic acid (acpcPNA-T9) probe with AuNPs toward label-free monitoring of miRNA-21: A novel biosensing platform for biomedical analysis and POC diagnostics. J Mol Recognit. 2021; doi:10.1002/jmr.2893

10. Mejía-Salazar JR, Rodrigues Cruz K, Materón Vásques EM, Novais de Oliveira Jr. O. Microfluidic Point-of-Care Devices: New Trends and Future Prospects for eHealth Diagnostics. Sensors. 2020;20(7):1951. doi:10.3390/s20071951

11. Solhi E, Hasanzadeh M, Babaie P. Electrochemical paper-based analytical devices (ePADs) toward biosensing: recent advances and challenges in bioanalysis. Anal Methods. 2020;12(11):1398–414. doi:10.1039/D0AY00117A

12. Loew N, Shitanda I, Kishiro K, Hoshi Y, Itagaki M. Paper-based Electrochemical Flow Biosensor Using Enzyme-modified Polystyrene Particles. Chem Lett. 2021;50(1):147–50. doi:10.1246/cl.200703

13. Yukird J, Soum V, Kwon O-S, Shin K, Chailapakul O, Rodthongkum N. 3D paper-based microfluidic device: a novel dual-detection platform of bisphenol A. Analyst. 2020;145(4):1491–8. doi:10.1039/C9AN01738K

14. Boonkaew S, Jang I, Noviana E, Siangproh W, Chailapakul O, Henry CS. Electrochemical paper-based analytical device for multiplexed, point-of-care detection of cardiovascular disease biomarkers. Sensors Actuators B Chem. 2021;330:129336. doi:10.1016/j.snb.2020.129336

15. Yakoh A, Pimpitak U, Rengpipat S, Hirankarn N, Chailapakul O, Chaiyo S. Paper-based electrochemical biosensor for diagnosing COVID-19: Detection of SARS-CoV-2 antibodies and antigen. Biosens Bioelectron. 2021;176:112912. doi:10.1016/j.bios.2020.112912

16. Farshchi F, Saadati A, Hasanzadeh M. Optimized DNA-based biosensor for monitoring Leishmania infantum in human plasma samples using biomacromolecular interaction: a novel platform for infectious disease diagnosis. Anal Methods. 2020;12(39):4759–68. doi:10.1039/D0AY01516D

17. Chen S, Xu L, Sheng K, Zhou Q, Dong B, Bai X, et al. A label-free electrochemical immunosensor based on facet-controlled Au nanorods/reduced graphene oxide composites for prostate specific antigen detection. Sensors Actuators B Chem. 2021;336:129748. doi:10.1016/j.snb.2021.129748

18. Milligan C, Ghindilis A. Laccase Based Sandwich Scheme Immunosensor Employing Mediatorless Electrocatalysis. Electroanalysis. 2002;14(6):415–9. doi:10.1002/1521-4109(200203)14:6<415::AID-ELAN415>3.0.CO;2-8

19. Hassanpour S, Hasanzadeh M, Saadati A, Shadjou N, Soleymani J, Jouyban A. A novel paper based immunoassay of breast cancer specific carbohydrate (CA 15.3) using silver nanoparticles-reduced graphene oxide nano-ink technology: A new platform to construction of microfluidic paper-based analytical devices (μPADs) towards biomedica. Microchem J. 2019;146:345–58. doi:10.1016/j.microc.2019.01.018

20. Feng X, Gan N, Zhou J, Li T, Cao Y, Hu F, et al. A novel dual-template molecularly imprinted electrochemiluminescence immunosensor array using Ru(bpy)32+-Silica@Poly-L-lysine-Au composite nanoparticles as labels for near-simultaneous detection of tumor markers. Electrochim Acta. 2014;139:127–36. doi:10.1016/j.electacta.2014.07.008

21. Farshchi F, Saadati A, Hasanzadeh M. A novel immunosensor for the monitoring of PSA using binding of biotinylated antibody to the prostate specific antigen based on nano-ink modified flexible paper substrate: efficient method for diagnosis of cancer using biosensing technology. Heliyon. 2020;6(7):e04327. doi:10.1016/j.heliyon.2020.e04327

22. Thévenot DR, Toth K, Durst RA, Wilson GS. Electrochemical biosensors: recommended definitions and classification1International Union of Pure and Applied Chemistry: Physical Chemistry Division, Commission I.7 (Biophysical Chemistry); Analytical Chemistry Division, Commission V.5 (Electroanalytical. Biosens Bioelectron. 2001;16(1–2):121–31. doi:10.1016/S0956-5663(01)00115-4

23. Paleček E. Electrochemical behaviour of biological macromolecules. Bioelectrochemistry Bioenerg. 1986;15(2):275–95. doi:10.1016/0302-4598(86)80033-2

24. Wang Y, Qu Y, Liu G, Hou X, Huang Y, Wu W, et al. Electrochemical immunoassay for the prostate specific antigen using a reduced graphene oxide functionalized with a high molecular-weight silk peptide. Microchim Acta. 2015;182(11–12):2061–7. doi:10.1007/s00604-015-1552-2

25. He Y, Xie S, Yang X, Yuan R, Chai Y. Electrochemical Peptide Biosensor Based on in Situ Silver Deposition for Detection of Prostate Specific Antigen. ACS Appl Mater Interfaces. 2015;7(24):13360–6. doi:10.1021/acsami.5b01827

26. Farshchi F, Hasanzadeh M, Mokhtarzadeh A. A novel electroconductive interface based on Fe 3 O 4 magnetic nanoparticle and cysteamine functionalized AuNPs: Preparation and application as signal amplification element to minoring of antigen-antibody immunocomplex and biosensing of prostate cancer. J Mol Recognit. 2020;33(4). doi:10.1002/jmr.2825

27. Farshchi F, Hasanzadeh M, Solhi E. Immunosensing of prostate cancer in human plasma samples using immobilization of antibody on the surface of mesoporous silica-modified silver nanoparticles and its immunocomplex with prostate-specific antigen. Anal Methods. 2019;11(48):6159–67. doi:10.1039/C9AY02058F

28. Oliveira N, Costa-Rama E, Viswanathan S, Delerue-Matos C, Pereira L, Morais S. Label-free Voltammetric Immunosensor for Prostate Specific Antigen Detection. Electroanalysis. 2018;30(11):2604–11. doi:10.1002/elan.201800417

29. Deng H, Li J, Zhang Y, Pan H, Xu G. A new strategy for label-free electrochemical immunoassay based on "gate-effect" of β-cyclodextrin modified electrode. Anal Chim Acta. 2016;926:48–54. doi:10.1016/j.aca.2016.04.035

30. Abbasy L, Mohammadzadeh A, Hasanzadeh M, Ehsani M, Mokhtarzadeh A. Biosensing of prostate specific antigen (PSA) in human plasma samples using biomacromolecule encapsulation into KCC-1-npr-NH2: A new platform for prostate cancer detection. Int J Biol Macromol. 2020;154:584–95. doi:10.1016/j.ijbiomac.2020.03.093