Received: 13 July 2021 | Revised: 8 August 2021 | Accepted: 10 August 2021

DOI: 10.1002/jmr.2932

RESEARCH ARTICLE

Journal of Molecular Recognition WILEY

Electrochemical immunoplatform to assist in the diagnosis of oral cancer through the determination of CYFRA 21.1 biomarker in human saliva samples: Preparation of a novel portable biosensor toward non-invasive diagnosis of oral cancer

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Funding information
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Abstract

In this study, a novel, low-cost, and flexible paper-based electrochemical immunosensor was developed for the bioanalysis of Cyfra 21.1 biomarker in human saliva samples by using stabilization of synthesis Ag nano-ink on the surface of paper using pen-on-paper technology. The employed electrochemical techniques for the evaluation of immunoplatform performance were differential pulse voltammetry and chronoamperometry. Also, the prepared immunosensor showed great ability in the determination of Cyfra21.1 in human saliva specimens. Under the optimized conditions, the obtained linear range was from 0.0025 to 10 ng/mL, and the obtained LLOQ was 0.0025 ng/mL. The developed immunosensor is easy to prepare, sensitive, cost-effective, portable, and simple. So proposed immunoplatform can be an accomplished biodevice in clinical laboratories. The proposed paper-based immunosensor could be a hopefully new and cheap tool for the diagnosis of other biomarkers. Also, the prepared immunosensor showed great ability in the determination of Cyfra21.1 biomarker in human saliva specimens.

KEYWORDS
advanced nanomaterial, biomedical analysis, biotechnology, Cyfra 21.1 biomarker, immunosensor, oral cancer, protein

1 | INTRODUCTION

Nowadays, cancer as the second threat to human health has caused human death around the world.[1] Each year, the number of new cancer cases and deaths is increasing due to enhancing rate of smoking,[2] air pollution,[3] high alcohol consumption,[4] and high-risk diet.[5] Among different type of cancer, oral cancer (OC) as the sixth most common cancer in the world have attracted considered attention to involves all malignancies in the oral cavity, lips, nasopharynx, and pharynx.[6] The most common and severe type of the disease is oral squamous cell carcinoma and also happen in the lateral edge of the tongue, the mouth floor, buccal mucosa, and gingiva.[7] It is worth considering that this type of cancer accounts for 90% of OC diagnostic cases.[8,9] The stage of OC is an important factor for recognizing the level of malignancy. In details, if malignancy is measured in stage T1, the survival rate is 90%, while in stages of high-grade (T3 and T4) stages, it drops to 20%.[10] At the last stages (metastatic stage), OC cells quickly and easily expand to other organs of the body, mostly through the lymphatic system. The lymph nodes in the neck are the first and main organ to be targeted for OC metastasis. Furthermore, the second

organ to be targeted for OC metastasis is lung cells.[11,12] Hence, early-stage detection or prevention of cancer is a highly difficult task that plays a significant role in the prevention of disease in the initial grade (T1) or advances the treatment efficiency of OC.[13-15] A biomarker (reliable indicator) of OC with the chemical nature of protein, DNA, and RNA can provide valuable information about the state of disease of the human body.[16] Various body fluids (including saliva, blood, serum, and plasma) are high potential candidate for measuring of these compounds. Serum cytokeratin fragment 21.1 (Cyfra 21.1) is also establish as a high potential biomarker in many epithelial cell cancers.[17] This biomarker is a soluble fragment of cytokeratin-19 that is released during cell apoptosis. In 2015, Cyfra 21.1 have exploited as an OC biomarker by Kurokawa and colleagues for the first time.[18] Afterward, several types of clinical studies have developed for recognition of Cyfra 21.1 salivary biomarkers. Therefore, this biomarker is reliable for early detection of OC.[19-21]

Although several conventional methods including biopsy,[22] toluidine blue staining,[23] oral cytology/brush biopsy,[24] fluorescence spectroscopy,[25] imaging,[26] and chemiluminescent illumination[27] have developed for early detection of OC, there is a lack of comprehensive method, which does not suffer from rapid, sensitive, accurate, and inexpensive screening. Thus, various type of sensors including electrochemical, optical, and quartz crystal microbalance (QCM) sensor as novel and next generation of the analytical method have received great attention for determination of OC.[28] Among different type of sensors, electrochemical sensors have widely used due to significant advantages such as being practical, sensitive, fast, selective, and having high detection capabilities and low detection limits.[29-31] Despite the development of the various type of electrochemical sensor based on several substrates, there is a shortage in the application of low-cost and portable substrate.[32] Therefore, we attempted to design a novel and unique electrochemical biodevice for the detection of OC.

Over the decades, various type of bioreceptor including aptamer, antibody, DNA, RNA, peptide, and enzyme have exploited for improving properties of the electrochemical sensor and creating a different type of electrochemical biosensors such as an immunosensor, aptasensor, genosensor, and also enzyme-based electrochemical biosensor.[33,34] Among different type of biosensors, the immunosensor has attracted particular attention in the sensing field. This type of biosensor has operated based on antibody–antigen interaction for providing appropriate biodevice. Besides, application of paper with high conductive ink have been provided excellent portable and cost-effective substrate for creating paper based electrochemical biosensor for detection of Cyfra 21.1.[35,36] The three electrode system have designed with a different type of inks for creating paper-based electrochemical immunosensor by hand-drawing as most popular due to low cost and simplicity.[37] Specific thermal and optical features and also high electrical conductivity have caused attracted considerable attention for designing conductive inks containing AgNPs, which widely exploited for indirect printing.[38] Therefore, we synthesized and used Ag ink for all of the electrodes preparation and stabilization on the surface of photographic paper.

In this study, we developed a novel, low-cost, and flexible three-electrode paper-based electrochemical immunosensor based on the interaction of specific antibody with Cyfra 21.1 on the sensing zone of working electrode for the selective and sensitive monitoring of OC. For the first time, these types of flexible electrodes were used for the screening of Cyfra 21.1 in human saliva samples. So, a noninvasive method was proposed for the early-stage diagnosis of OC. For this purpose, the counter, reference, and working electrodes were drawn on the surface of the photographic paper by pen-on-paper method and using synthesized conductive Ag ink. Then, the sensing zone was decorated with specific antibody for efficient capture of Cyfra 21.1 antigen. Afterward, the BSA was used for blocking free functional group of Ag ink on the surface of sensing zone. Finally, quantitative application of engineered immunoplatform was applied for the measurement of Cyfra 21.1 antigen in human saliva samples.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

All utilized reagent and Chemical in this research are in analytical grade. Glutaraldehyde (GA), bovine serum albumin (BSA), cysteamine (CysA), and silver nitrate ($AgNO_3$) were supplied from Sigma Aldrich (Ontario, Canada). Sodium dihydrogen phosphate monohydrate ($H_2NaPO_4.H_2O$), sodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCL), potassium ferricyanide ($K_3Fe(CN)_6$), and potassium ferrocyanide ($K_4Fe(CN)_6$) were obtained from Merck (Germany). Human Cytokeratin Fragment Antigen 21-1 (CYFRA21-1) ELISA Kit was provided from MyBiosource (San Diego, California, USA). Purity of chemical was 99.9%.

2.2 | Apparatus

PalmSens 4c system and paper-based three-electrode system drawn with Ag nano-ink were employed for electrochemical studies. Morphology, crystallographic structure, and size of synthesized Ag nano-ink were evaluated by using transmission electron microscopy (TEM), Philips-CM30, Adelaide, Australia, with an operating voltage of 200 kV. Superficies morphology of bulk Ag nano-ink and different steps of immunosensor modification was analyzed by high-resolution field emission scanning electron microscope (FE-SEM), Hitachi-SU8020, Czech operated at 3 kV. EDS (energy-dispersive spectroscopy) coupled with the FE-SEM was utilized for investigation of chemical composition. Ohmmeter (XIOLE, XL830L, China, multimeter) was also to assess the resistance of synthesized Ag nano-ink.

2.3 | Collection of human saliva specimen

Unprocessed human saliva specimens were used as a real sample to detect early-stage OC. Fasting saliva specimens were collected in the

full fasting state (at least 8 hours have passed since the last eating and drinking). For this purpose, to ensure the cleanliness of the oral cavity before sampling, the oral cavity was rinsed using PBS buffer (0.1 M, pH = 7.4) for 30 seconds. After discarding PBS, the oral cavity was rinsed with deionized water for 20 seconds. Finally, saliva specimens were collected, and after ensuring its transparency and the absence of suspended particles, it was used.

2.4 | Synthesis of silver nano-ink

For this purpose, first 1.915 g of PAA was mixed with 40 g of DEA and 50 g of water and stirred in a water bath for about 2 hours, and silver nitrate solution was added. After stirring for 22 hours, it was kept in an ultrasonic water bath for about 1.5 hours at 65°C. It was then mixed with ethanol and centrifuged (20 minutes, 9000 rpm). The resulting precipitate was mixed with water and centrifuged again. The washing step was repeated three times. The final precipitate was dissolved in a 2 wt% solution of hydroxypropyl cellulose and homogenized for 3 minutes at 2000 rpm.

2.5 | Conductivity analysis of silver nano-ink

To investigate the resistance of the synthesized ink and its ability to conduct electrical current, conductive patterns were drawn using it on a photographic paper. Electrical resistance was measured using an ohmmeter. To test the electrical conductivity, the cathode leg of the 1.5 V LED lamp was connected to the battery anode and the cathode surface of the battery was connected to one side of the conductive pattern. By connecting the LED anode leg to the other side of the conductive pattern, the electrical circuit was turned on. The mechanical strength of the ink was also investigated on the paper surface (Figure S1).

2.6 | Construction of immunosensor

The three-electrode system (2.5 × 1.5 mm^2) was created by handwriting of Ag ink on the superficies of photographic paper using a sampler. About 15 μL ink was used for every sensor. After drying at room temperature, the prepared paper electrode was used for the fabrication of immunosensor. For this aim, firstly carboxyl group (—COOH) of Fc (fragment crystallizable) region of anti-Cyfra21.1 antibody was activated through N-hydroxysuccinimide (NHS)/1-ethyl-3-[3-dimethylami-nopropyl]-carbodiimide hydrochloride (EDC) and immobilized (5 μL) on the surface of the sensing zone. This is carried out by combining the antibody with the 0.4 M of NHS and 0.1 M of EDC solution and keeping it at room temperature (25°C) for 30 minutes, which leads to the covalent attachment of the antibody to the Ag ink-based paper electrode superficies.

After 12 hours of incubation at 4°C, 50 μg/mL BSA (5 μL) was employed as a blocking agent for the sites of the surface that were not bound to the antibody. After 1 hour, the sensing zone of paper electrode washed with distilled water (DW) to remove unbound species. Finally, Cyfra21.1 antigen (5 μL) was incubated at 4°C for 4 hours and rinsed with DW to remove unbound species. For further use, it was stored at 4°C. The construction process of the engineered immunosensor was summarized in Scheme 1.

2.7 | Characterization

2.7.1 | Characterization of Ag ink

To evaluate the morphology of the synthesized Ag nano-ink, FE-SEM and TEM imaging were employed. As shown in Figure S2, AgNPs have a uniform and spherical structure, and there is no mass of AgNPs. The average size of the nanoparticles is about 50 nm. Figure S3 shows the TEM images proves FE-SEM imaging, and spherical and uniform nanoparticles are also seen in this imaging. The particle size is excellent and is in the nanometer range. EDS spectra also utilized to study the elements and analyze the organized obligation. As shown in Figure S4, the presence of AgNPs in large quantities indicates the successful synthesis of conductive Ag-ink.

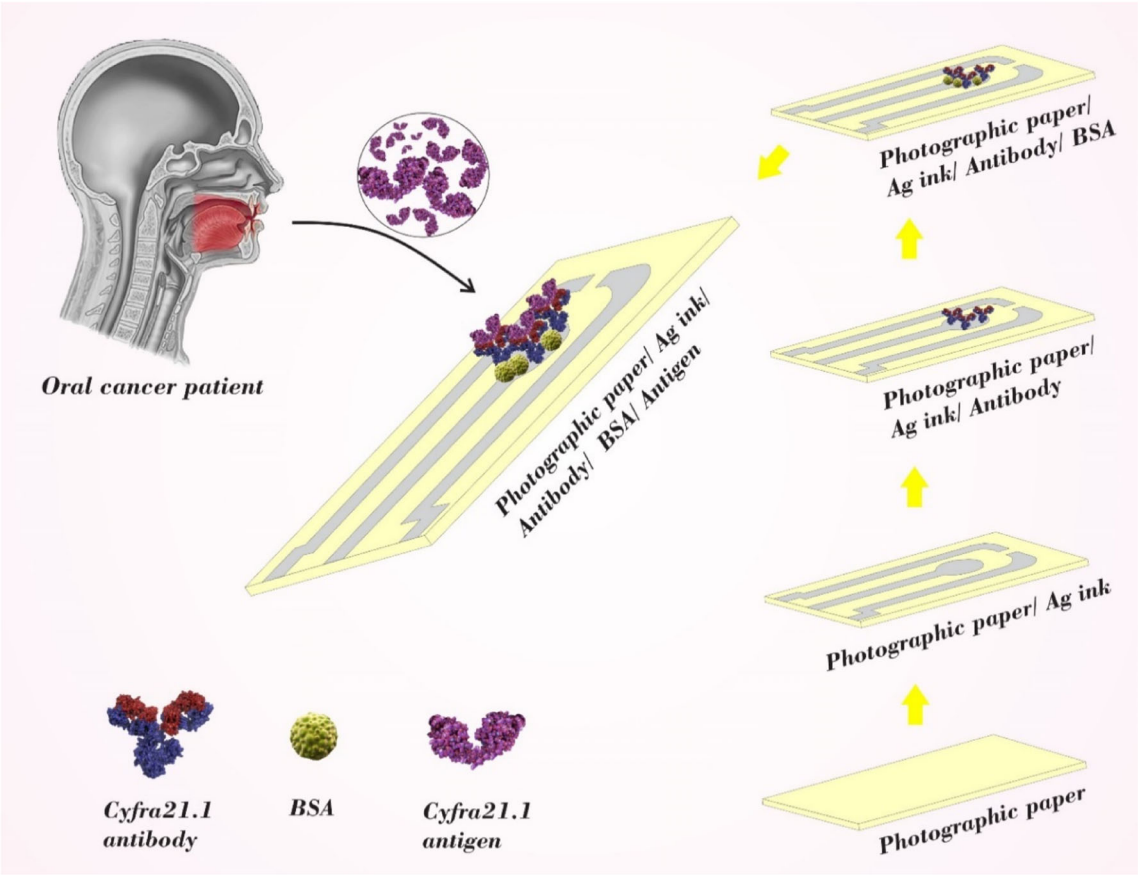
2.7.2 | Characterization of fabricated immunosensor

Investigation of morphological changes in the different steps of prepared immunosensor was recorded by FE-SEM imaging. Figure S5A,B displays the spherical and uneven structure of Ag-ink on the surface of photographic paper. After antibody and antigen incubation, the morphology completely changes, which shows successful consolidation of biological materials on the surface of paper. According to EDC elemental analysis (Figure S6), AgNPs is the most abundant due to Ag nano-ink. In the later stages after incubation of biological materials, the amount of phosphate and sulfur elements increases, which indicates their successful stabilization on the surface.

3 | RESULTS AND DISCUSSION

3.1 | Electrochemical behavior of engineered immunosensor

Electrochemical behavior of the engineered immunosensor in diverse steps was analyzed by using ChA technique in 0.005 M $[Fe(CN)_6]^{3-/4-}$/KCl. According to Figure 1, the peak current intensity of the synthesized Ag nano-ink is equal to 26 μA, and it can be concluded that Ag nano-ink has good current intensity and can provide a great surface for immobilization of antibody. After incubation of the antibody, the electron transfer rate is increased by $[Fe(CN)_6]^{3-/4-}$/KCl solution, which indicates a proper substrate for early detection of OC. Also, after immobilization of BSA and Cyfra 21.1 antigen, the current intensity increases to around 157 μA, which indicates the successful hybridization of the antigen–antibody complex.



SCHEME 1 The schematic illustration of paper-based immunosensor for the detection of Cyfra21.1 biomarker

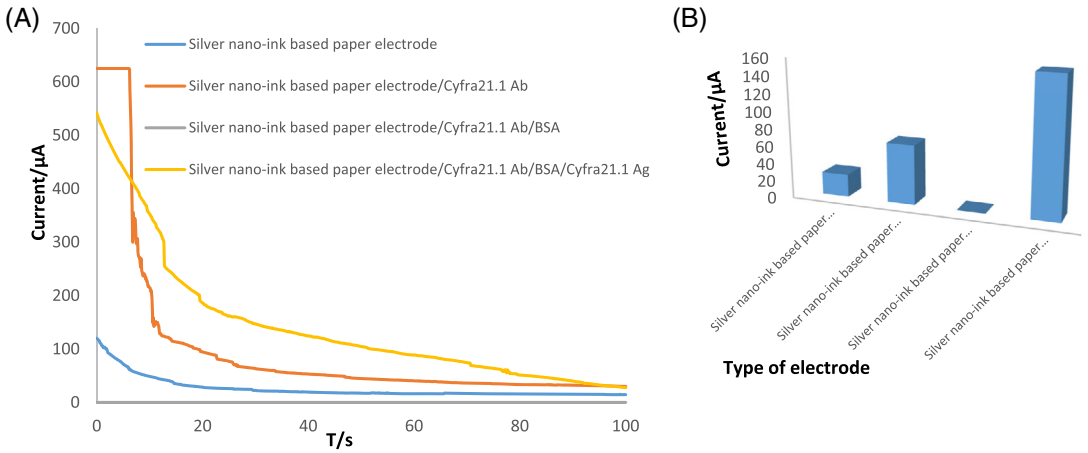


FIGURE 1 A, Chlorohydroxyanilines (ChAs) of diverse steps of the prepared electrochemical immunosensor on the surface of photographic paper in 0.01 M $[Fe(CN)_6]^{3-/4-}$/KCl solution as supporting electrolyte. E = 0.6 V, duration time = 100 s. B, Histogram of peak current vs type of electrode

3.2 | Analytical performance

3.2.1 | Sensitivity study in standard specimens

By using the optimal circumstances evaluated, the differential pulse voltammetric and chronoamperometric assessments of the engineered immunosensor for diverse concentration of Cyfra21.1 antigen were revealed in Figure 2. Also, Figure 3 displayed the calibration curve of current vs Cyfra21.1 Ag various concentration. The results exhibited excellent linearity in the concentration range of 0.0025-12.5 ng/mL and 0.0025-30 ng/mL by ChA and differential pulse voltammetry (DPV) technique, respectively. The lower limit of quantification (LLOQ) obtained 0.0025 ng/mL. The linear regression equation obtained with each technique is as follows:

$I\ (\mu A) = 16.87\ C_{(Cyfra21.1)} + 75.293,\ R^2 = 0.9136$ (DPV)

$I\ (\mu A) = 21.031\ C_{(Cyfra21.1)} + 18.104,\ R^2 = 0.9603$ (ChA)

One of the most important factors in the development of high-performance biosensors is the excellent electrical conductivity of the matrix, which can lead to high-speed electron transfer. In addition, the structural properties of the matrix are of particular importance for creating a suitable and wide surface for the stabilization of biological agents and the possibility of biological activity. Cellulose papers, due to their very fine porous structures, can provide a good substrate for antibody stabilization and can provide portable analytical tools with high detection sensitivity.

3.2.2 | Sensitivity study in human saliva specimens

As presented by Figures 4 and 5, chlorohydroxyanilines (ChAs) and DPVs were recorded for investigation of prepared immunosensor ability to determine the diverse concentration of Cyfra21.1 Ag in human saliva specimen. Examination of biomarkers in human saliva specimens exhibited a great linear dependence in the range of 0.00025 to 10 ng/mL using chronoamperometry and DPV technique. The linear regression equation obtained with each technique is as follows:

$I\ (\mu A) = 23.734\ C_{(Cyfra21.1)} + 137.7,\ R^2 = 0.9528$ (DPV)

$I\ (\mu A) = 4.2452\ C_{(Cyfra21.1)} + 22.827,\ R^2 = 0.909$ (ChA)

LLOQ was 0.025 ng/mL for DPV and ChA. Table 1 compares the sensitivity of the immunosensors used to detect biomarkers Cyfra21.1 in human saliva specimens with the present study.[6,29,39-41]

In addition to biosensing techniques, various conventional methods for identifying Cyfra21.1 biomarker in a variety of biological specimens for the diagnosis of OC have been reported in several studies. Examination of Cyfra21.1 biomarker in patients with OC using immunoradiometry method showed a significant difference between patient and control group ($n = 42$, 2.25 $ng.ml^{-1}$).[42] In another study performed using this technique, the concentration of Cyfra21.1 in the serum specimen was reported to be 2.9 $ng.ml^{-1}$.[43] Zhong et al,[44,45] also used the enzyme-linked immunosorbent assay (ELISA) method to detect S in serum and salivary specimens. Serum concentrations were reported to be 0.65 μg/L, and specificity and sensitivity were 0.964 and 0.57, respectively. Specificity and sensitivity were reported in salivary samples 0.86 and 0.5, respectively, with measured concentrations of 85.95 μg/L.[18] In another study using ELISA, Cyfra21.1 levels in patients' saliva and serum specimens were reported to be 17.46 $ng.ml^{-1}$ and 5.14 $ng.ml^{-1}$, respectively. Electrochemiluminescence immunoassay (ECLIA) is another method used to evaluate OC, with concentrations of 13.68 $ng.ml^{-1}$ and 5.01 $ng.ml^{-1}$ in saliva and serum samples, respectively.[46]

3.3 | Selectivity of engineered immunosensor

Selectivity is a significant feature of biosensors that demonstrates the ability of the bio-receptor to detect a particular analyte in the target specimen and is a starting point for sensor design. Therefore, the ability to accurately detect the Cyfra21.1 biomarker was evaluated by ChA and DPV techniques using a number of biological molecules (such as 2-Ag, CA-125, SNCA, p53, CA 15-3, CEA, PSA) that can interfere with the function of the biosensor. The results are presented in Figure S7. Comparison of Cyfra21.1 current with interfering species showed that 2-Ag alone has not interfered in the detection of Cyfra21., while other used biomolecules can interfere with the detection of Cyfra21.1 by changing the peak current.

3.4 | Reproducibility, precision, and stability of the immunosensor

Reproducibility and stability are two among the most critical factors affecting the performance of a biosensor. Reproducibility is the ability

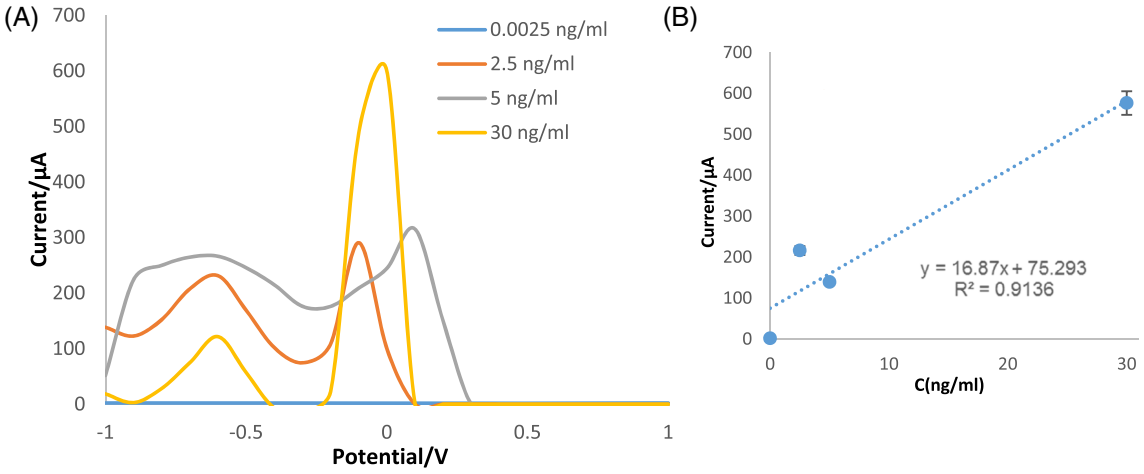


FIGURE 2 A, Differential pulse voltammetries (DPVs) of paper-based immunosensor in various concentrations of Cyfra21.1 Ag include: 0.000025, 0.0025, 2.5, 5, 30 ng/mL in 0.01 M $[Fe(CN)_6]^{3-/4-}$/KCl solution as supporting electrolyte, potential range of −1 to +1 V. B, Calibration curve

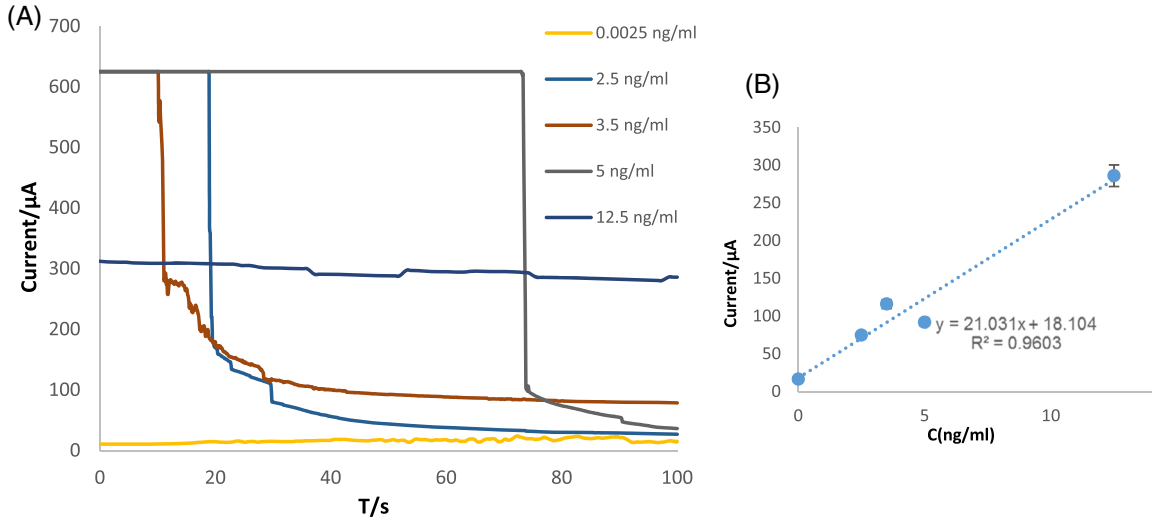


FIGURE 3 A, Chlorohydroxyanilines (ChAs) of paper-based immunosensor in various concentrations of Cyfra21.1 Ag include: 0.0025, 2.5, 3.5, 5, 5, 12.5 ng/mL in 0.01 M $[Fe(CN)_6]^{3-/4-}$/KCl solution as supporting electrolyte. E = 0.6 V, duration time = 100 s. B, Calibration curve

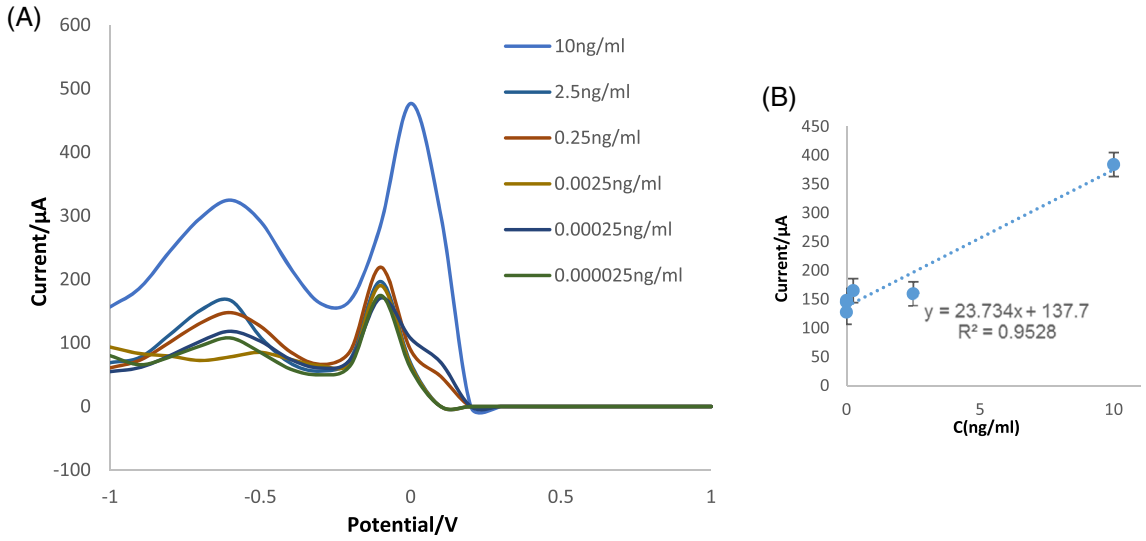


FIGURE 4 A, Differential pulse voltammetries (DPVs) of paper-based immunosensor in various concentrations of Cyfra21.1 Ag include: 0.000025, 0.00025, 0.0025, 0.25, 2.5, 10 ng/mL in 0.01 M $[Fe(CN)_6]^{3-/4-}$/KCl solution as supporting electrolyte, potential range of −1 to +1 V. B, Calibration curve

of a biosensor to produce identical responses in a duplicate experimental condition. Reproducibility in a biosensor is determined by the accuracy of the converter and electronics. To evaluate the reproducibility, current intensity was recorded using ChA technique by two separate electrodes (Figure S8). The resulting flows were not significantly different. Hence, it can be concluded that the designed three-electrode system can be reproduced. To investigate the effect of number of cycles on the performance of the three-electrode system, changes in current intensity with increasing number of cycles were recorded. The results presented in Figure S9 show a significant reduction in current intensity after 5 cycles. Therefore, it can be said that the sensor has the best performance in the initial cycles. The long-term stability of the immunosensor was also evaluated by keeping it at 4°C using the DPV technique. The results showed a significant decrease in the performance of the immunosensor after 24 hours (Figure S10). Therefore, the best performance of the immunosensor is on the same day of preparation.

3.5 | Regeneration of the immunosensor

Regeneration is an important factor in the production of practical biosensors. With the possibility of biosensor regeneration, an accessible method for multiple sampling is developed and the sensor-to-sensor variance is eliminated. This can be useful when measuring over a period of time or interrogating similar levels of analyte. For

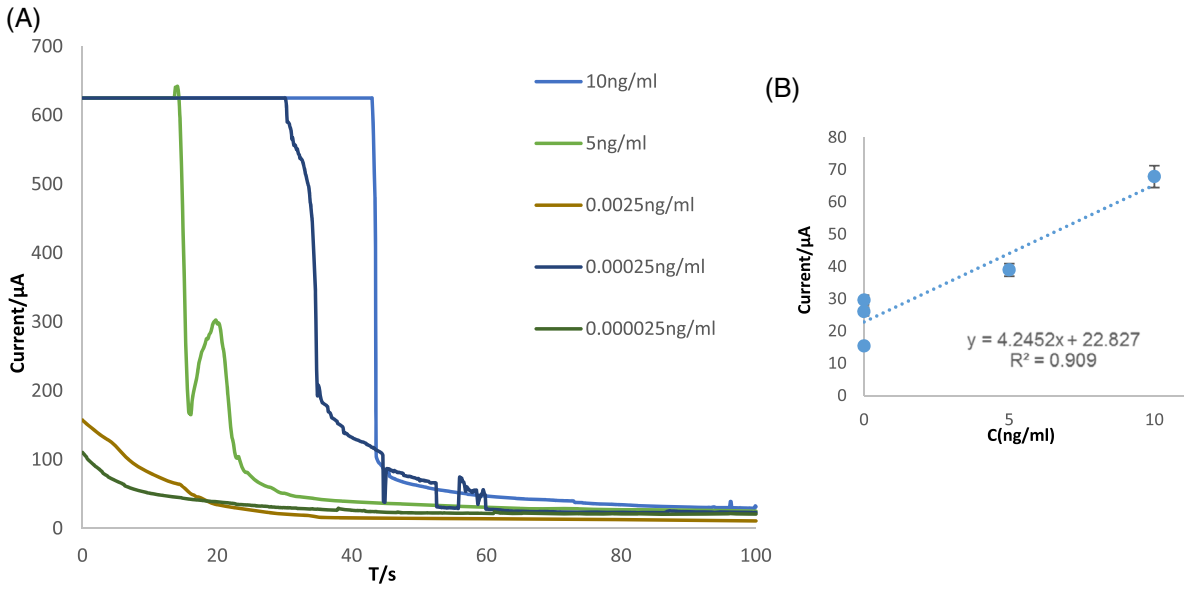


FIGURE 5 A, Chlorohydroxyanilines (ChAs) of paper-based immunosensor in various concentrations of Cyfra21.1 include 0.000025, 0.00025, 0.0025, 5, 10 ng/mL in 0.01 M $[Fe(CN)_6]^{3-/4-}$/KCl solution as supporting electrolyte. E = 0.6 V, duration time = 100 s. B, Calibration curve

TABLE 1 Comparison of the performance and sensitivity of the designed paper-based immunosensor for detection of Cyfra21.1

Substrate	Bioassay strategy	Method	Specimen	Linear range	LOD/LLOQ	Refs.
CysA-GA/AuE	Immunosensor	SWV	Saliva	2.5-50 ng/mL	2.5 ng/mL	[6]
Cys-La$(OH)_3$/ITO		DPV	Saliva	0.001-10.2 ng/mL	0.001 ng/mL	[39]
APTES/Zro_2 RGO/ITO			Saliva	2-22 ng/mL	0.122 ng/mL	[40]
APTES-ZrO_2 /ITO		CV	Saliva	2-16 ng/mL	0.08 ng/mL	[29]
APTES/nHfo_{O2}/ITO			Saliva	2-18 ng/mL	0.21 ng/mL	[41]
Ag ink-based paper electrode		DPV-ChA	Saliva	0.025-10 ng/mL	0.025 ng/mL (LLOQ)	This work

Abbreviations: CV, cyclic voltammetry; DPV, differential pulse voltammetry; LLOQ, low limit of quantification; LOD, limit of detection; SWV, square wave voltammetry.

regeneration, DPV was first recorded using an immunosensor. The electrode was then treated with urea solution for 30 minutes to separate the antigen from the antibody. After washing with water, the regenerated immunosensor was used to detect the same concentration of Cyfra21.1 antigen. According to the results of Figure S11, the performance of the regenerated sensor has changed slightly compared to the original sensor.

4 | CONCLUSION

In this study, a novel and low-cost electrochemical immunosensor based on the interaction of a specific antibody with Cyfra 21.1 antigen was developed. A three-electrode system was designed by using synthesized Ag ink with pen-on-paper technology on the surface of photographic paper. The engineered substrate was suitable for antibody and antigen immobilization. One of the unique features of the prepared immunosensor is the synthesized Ag-nano ink, which has excellent physicochemical properties that can increase the stability, selectivity, and sensitivity of the immunosensor. The engineered immunosensor was label-free, but it has a suitable analytical performance. This immunosensor also tested in human plasma samples, which had good analytical power. The developed immunosensor is easy to prepare, sensitive, cost-effective, portable, and simple. So proposed immunoplatform can be an accomplished biodevice in clinical laboratories. The proposed paper-based immunosensor could be a hopefully new and cheap tool for the diagnosis of other biomarkers. Also, the prepared immunosensor showed great ability in the determination of Cyfra21.1 biomarker in human saliva specimens.

ACKNOWLEDGEMENT

This work was supported by TBZMED (Tabriz University of Medical Sciences).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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How to cite this article: Tofighi FB, Saadati A, Kholafazad-kordasht H, Farshchi F, Hasanzadeh M, Samiei M. Electrochemical immunoplatform to assist in the diagnosis of oral cancer through the determination of CYFRA 21.1 biomarker in human saliva samples: Preparation of a novel portable biosensor toward non-invasive diagnosis of oral cancer. *J Mol Recognit*. 2021;e2932. doi:10.1002/jmr.2932