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RESEARCH PAPER

An Aptasensor Based on Electrosynthesized Conducting Polymers, Cu_2O–Carbon Dots and Biosynthesized Gold Nanoparticles, for Monitoring Carcinoembryonic Antigen

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ABSTRACT

Current work proposes an inimitable composite, with great electrical conductivity and quite enhanced surface area, (including conducting polymers (poly (cathechol)), Cu_2O–carbon dots and green synthesized gold nanoparticles) for detecting acute carcinoembryonic antigen. At current work, the electropolymerization was offered instead of enzyme-catalyzed polymerization of poly (catechol). Four cost-effective electrochemical techniques (Differential Pulse Voltammetry, Electrochemical Impedance Spectroscopy, Cyclic Voltammetry and Crono amperometry) were engaged to investigate aptasensors construction, immobilization, hybridization, sensitivity, selectivity, repeatability, long-term stability and real PCR sample detections. According to the data, the values of I_{peak} is linearly related to the logarithm of the concentrations of carcinoembryonic antigen in the range from 1.0 pg mL^{-1} to 0.001 g mL^{-1}, with a detection limit of 0.19 pg mL^{-1} for the target antigen (carcinoembryonic antigen).

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INTRODUCTION

Cancer, which is among the deadliest disease, can start almost anywhere in the human body. When the cells grow old or become damaged, other cells grow and divide to form new ones. Cancer breaks down this normal procedure, old cells survive and other cells grow and divide without stopping until they make cancerous tumors. The worst part of this, is metastasis and it is when cancerous cells spread (through the blood or the lymph system) into the other parts of the body, to make new tumors far from the original tumor. Cancer is hard-to-treat after metastasis, hence it had to be diagnosed before it has the chance to spread to other parts of the body.

There are a bunch of diagnostic device and methods, like biopsy and imaging procedures; but these methods are not always safe and sometimes causes unknown harmful effects on the human body, besides these equipment are not available everywhere, and some of costly diagnostic methods are not covered by all insurance plans.

Since, there is an urgent need for the cost cutting diagnostic devices, which involve tests and procedures to confirm the presence of the cancer at an early stage (before the tumor get metastatic), without any side effects. Electrochemical biosensors, as a new-wave diagnostic devices, are fulfilling all of the above mentioned conditions. An electrochemical biosensor is an analytical equipment, which is used for the detection of a biological analyte, with the aid of an electrochemical detector [1, 2].

There are various types of electrochemical biosensors, but one of the most promising biosensors, is electrochemical aptasensor due to their unique selectivity, sensitivity, chemical stability and cost effectiveness. The electrochemical aptasensor

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is a robust class of sensors for the detection of biomolecules, which consist of two main components: an aptamer as a bioreceptor and an electrochemical detector.

In this article, we used an electrochemical aptasensor for the detection of carcinoembryonic antigen (CEA); CEA level remains at record low unless different forms of cancers are present, like pancreas, stomach, breast, lung, colon, blood and ovarian cancers [3]. Hence, CEA aptsensor has a vast potential for the detection of different kinds of cancers. Conductive polymers and nanoparticles are two promising new classes of materials, which can elevate the privileges of the electrochemical biosensors and specially aptasensors. Polymers are among the most used materials in the modern world; conductive polymers are polymers with unique features, such as air stability, mechanical strength and very good electrical conductivity. These features make them good candidates to improve the structure of an electrochemical biosensor [4]. Poly (catechol), as a conductive polymer, draw a lot of interest in the electrical equipment field because of its high conductivity. Enzyme-catalyzed polymerization of catechol was developed as a useful, yet expensive and multistep method; which was employed horseradish peroxidase (HRP) as a catalyst. Horseradish peroxidase is a quite expensive enzyme which may decay during the time [5].

At current work the electropolymerization was offered instead of enzyme-catalyzed polymerization of catechol. Electropolymerization is a one-step, fast, easy operated, cost-effective, well-controlled method. At this work, electropolymerization was performed via controlled potential coulometry (CPC), which is a well-known electrochemical method. The electrosynthesized poly (catechol), was applied to modify the electrical properties of an aptasensors surface area.

Nanomaterials revolutionize lots of electrical equipment due to their small size and unique electrical properties. The small size of nanoparticles (which is between 1 to 100 nanometers) leads to increased surface area-to-volume ratio. Larger surface area-to-volume ratio allows more available space and more of the particles are exposed to the other reactants [6].

At current work, modified carbon quantum dots have been used for modification of the aptasensors surface area. Carbon quantum dots (CQDs, C-dots or CDs) are a new class of nanomaterials with the size less than 10 nanometers and a quite large surface area-to-volume ratio. The tiny nature of carbon quantum dots results in perfect features, like the enhanced surface area to which other materials can bond, this means that CDs can broaden the surface area. In 2014 Jianhui Deng and his coworkers proposed a one-step, easy operated procedure for the electrochemical synthesis of CDs directly from alcohols [7]. In 2015 Qitong Huang and his coworkers improved the electrical properties of CDs, with Cu $(NO_3)_2$ modifications, to form Cu_2O–CDs [8].

At current work, Cu_2O–CDs have been used for modification of the aptasensors. Using CDs for modification of the electrode surface area, would lead to a larger surface area-to-volume ratio; and using Cu_2O–CDs would lead to the larger surface area-to-volume ratio besides improving the electrical properties of the electrode.

Immobilization of the aptamer on the surface of an electrode is the critical, challenging step, in aptasensors construction. Among some of different immobilization methods, immobilization by forming a strong covalent bond between gold nanoparticles (AuNP) and thiol-modified aptamers, provides the most promising strategy in aptasensors fabrication.

There are various methods to synthesis AuNP, yet biological synthesis got many advantages, such as, cost-effectiveness, easy operating, great biocompatibility and being ecofriendly.

At current work, *Saccharomyces cerevisiae* yeast strains were applied to biosynthesize gold nanoparticles (BioAuNP). These BioAuNPs were used for the immobilization of the thiol-modified aptamers, on the surface of the electrode [9].

The present work aims to introduce an electrochemical aptasensor, with unique electrical properties, based on both conducting polymers and nanomaterials (modified carbon quantum dots and biosynthesized gold nanoparticles) for accurate detection of carcinoembryonic antigen (CEA) (scheme S1). As above- mentioned, pancreas, stomach, breast, lung, colon, blood and ovarian cancers cause an increased CEA level. Hence, the mentioned modified aptasensor has a vast potential for the detection of different kinds of cancers.

MATERIALS AND METHODS

Apparatus and reagents

Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 6-Mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich. Graphite powder, catechol, trisodium citrate, hydrochloric acid,

hydrogen peroxide, sulfuric acid, sodium hydroxide, phosphoric acid, disodium hydrogen phosphate, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), ethanol, sodium azide, $Na[AuCl_4]$, Cu $(NO_3)_2$ and bovine serum albumin were obtained from Merck. Other chemicals and stock solutions were of the analytical grade and were used without any more treatments.

For biosensors investigations screen-printed electrodes, were employed. Screen-printed electrodes (SPEs), which are applied as inexpensive electrodes, have gone through considerable progresses over the last years, with respect to both their materials and formats. Due to their beneficial material features, such as uniformity, and rapid responses (which leads to a better electrical conductivity) SPEs have been successfully employed for the accurate, rapid in situ studies of analytical samples. The SPEs electrochemical cell consists of:
Working electrode: Carbon (4 mm diameter)
Auxiliary electrode: Carbon
Reference electrode: Silver
All SPEs were purchased from Dropsens Company (www.dropsens.com).

Another three-electrode system of a Pt (as the counter electrode), a graphite electrode (as working electrode) and an Ag/AgCl/KCl (3.0 M) (as the reference electrode) employed to synthesize C-dots and the poly (catechol).

Saccharomyces cerevisiae yeast was growth in microbial collection of Yazd University, Iran.

The functionalization steps involved in the assembly of the aptasensor were performed using 8 µL of the appropriate solution deposited on the modified SPEs working electrode surface.
The aptamer sequences is as follows:
Aptamer sequence (APTA):
5'-Thiol-TTT TTT TTT TTT ATA CCA GCT TAT TCA ATT-3'

The stock solutions of aptamer (100.0 µM) were prepared in Tris-EDTA buffer solution and kept frozen at −20 °C, Carcinoembryonic antigen (CEA) were prepared in 0 in Tris-EDTA buffer and 0.2% sodium azide and kept frozen at −20 °C. The double distilled water and buffers were sterilized using an autoclave.

The electrochemical measurements were performed with an Autolab potentiostat/galvanostat (PGSTAT-302 N, Eco Chemie, Netherlands). The experimental conditions were controlled by General Purpose Electrochemical System (GPES) and Frequency Response Analyzer (FRA) software.

A Metrohm 691 pH/ion Meter was used for pH evaluations. A GFL1101 shaker was used to speed up the yeast growth.

Preparation of modified electrodes
Electrochemical synthesis of conductive polymer

According to our previous work [10], electropolymerization of catechol was offered through a one-step, fast, easy operated, cost-effective, well-controlled method. As far as we know, there are no former reports, using controlled potential coulometry (CPC) for the poly (catechol) synthesizing. In detail, the reaction took place in a traditional three electrode cell: a Pt electrode as the counter electrode, a graphite electrode as working electrode, and a calomel electrode was engaged as the reference.

The procedure of electropolymerization of catechol is as follow: 0.0264 g of catechol were added to 60 mL 0.01 M sodium phosphate buffer solution (pH 6), the reaction proceeded for about 1 h at the potential of 0.5 V until the stock solution turned reddish-brown. The mentioned solution was dialyzed for 24 hours to remove unreacted monomers.

Preparation of Cu_2O–CDs nanoparticles

Electrochemical synthesized of carbon dots (CDs) was done through well-controlled electrochemical carbonizations of ethanol. In detail, the reaction took place a traditional three electrode cell: a Pt electrode was engaged as the counter electrode, a graphite electrode was employed as working electrode, and a calomel electrode was engaged as the reference. 70 mL of ethanol solution was mixed with 5 mL of water, and then under strong magnetic stirring, 0.5 g of NaOH was subjoined. The reaction proceeded through, for about 24 h at the potential of 3 V until the stock solution turned brown. Then excess ethanol (150 mL) was subjoined for salting out the excess NaOH, and the solution was then remains, during the night. The mixture was heated to 80 °C and the temperature was remained constant for about 24 h to yield a stable yellow powder.

The synthesis of Cu_2O–CDs, is as follows: 100 mL of 1.0 M aqueous solutions of Cu $(NO_3)_2$ was added to the 200 mL aqueous solution of NaOH (1.0 M). After 20 min stirring at room temperature, 100 mL of CDs solution (8.0 mg mL^{-1}) was added to the mentioned solution. Then the mixture

was heated to 90 °C and the temperature was remained constant for about 30 min until a dark purple powder was procured [8, 9].

Eco-friendly biosynthesis of gold nanoparticles

The *Saccharomyces cerevisiae* yeast strains were grown in YNBG broth liquid medium at 27°C under shaking at 80 rpm for 72 h, after that the biomass was separated from the YNBG broth by 5000 rpm, for 10 min centrifuging. Then the supernatant of the mentioned solution was subjected to the 0.6 mM $HAuCl_4$ solutions and placed in a shaker at 27°C (80 rpm) for 48 h. The mentioned aqueous solution should be centrifuged (10000 rpm, for 10 min) to get eco-friendly biosynthesis gold nanoparticles [9].

Modification, Immobilization and hybridization steps

The biosensor modification was as follows:

First step: 8 µL of 0.007 g mL^{-1} of Cu_2O–CDs solution (pH 7.4) were dropped on the SPEs working surface and let it dry at room temperature (Cu_2O–CDs /SPE).

Second step: 8 µL of 0.01 gr mL^{-1} of poly (catechol) (Pol) solution was dropped on the Cu_2O–CDs / SPE, then let it dry at room temperature (Pol/ Cu_2O–CDs/SPE).

Third step: 8 µL of 0.004 M green synthesized gold nanoparticles was dropped on the surface of the Pol/Cu_2O–CDs/SPE to form Bio AuNP/Pol/ Cu_2O–CDs/SPE.

Forth step (immobilization process): 8 µL of 10^{-5} M 5′-SH aptamer (Apta) was incubated on the surface of the Bio AuNP/Pol/Cu_2O–CDs/SPE and remained stable for the optimal immobilization time. The immobilization took place due to the strong bonded between thiol modified aptamer and green synthesized gold nanoparticles (Apta/ Bio AuNP/Pol/Cu_2O–CDs/ SPE).

Fifth step: To avoid nonspecifically adsorption of aptamers, the Apta/ Bio AuNP/Pol/Cu_2O–CDs/ SPE was rinsed with 0.1 M pH 7.4 phosphate buffer saline (PBS buffer) solution; then 8 µL of 1mM MCH solution was dropped on the surface of the Apta/Bio AuNP/Pol/Cu_2O–CDs/SPE, and remained stable for 1h.

Last step (hybridization process): 8 µL of CEA solution (Anti) was dropped on Apta/Bio AuNP/ Pol/ Cu_2O–CDs/SPE, and remained stable for the optimum hybridization time (scheme S1).

Electrochemical measurements

Four well known electrochemical methods (DPV, EIS, CV and Crono amperometry) was applied to analysis the construction, immobilization and hybridization of the aptasensor.

EIS analysis was measured at a bias potential of 200 mV with an alternating voltage of 10 mV in the frequency ranging from 0. 1 Hz to 10^5 Hz.

DPV investigations were measured in the potential range from −0.5 to −0.1 V at modulation amplitude of 70 mVand potentials step of 5 mV. To investigate the chronoamperometric measurements the working electrode potential was set at 500 mV.

RESULTS AND DISCUSSION

Scanning electron microscopy (SEM), NMR and FT-IR and UV-Visible spectroscopy Characterization

The morphology of CDs, Cu_2O–CDs and biosynthesized Bio AuNPs were investigated by SEM. The typical SEM images of CDs, Cu_2O–CDs and Bio AuNPs were shown in Fig. 1a, Fig. 2a, Fig. 2, respectively. CDs, Cu_2O–CDs and Bio AuNPs SEMs exhibited a uniform spheres structure morphology.

According to our previous work [10], FT-IR spectroscopy of poly (catechol) shows a peak series

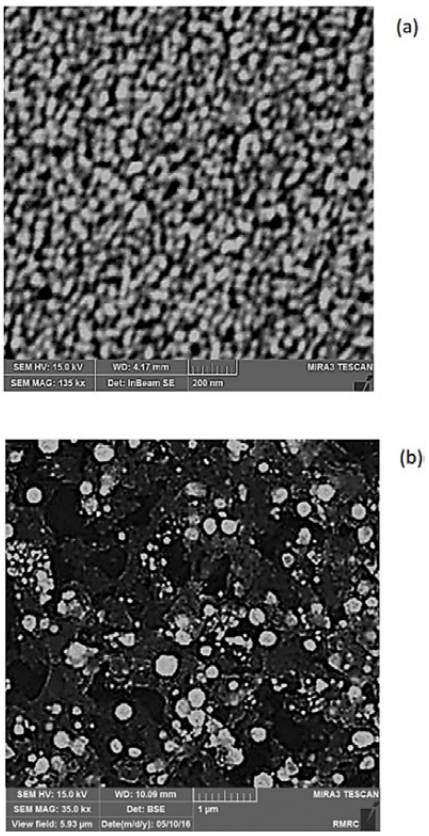

Fig. 1. A SEM micrograph of the carbon dot (CD) (a) and Cu_2O_CD (b).

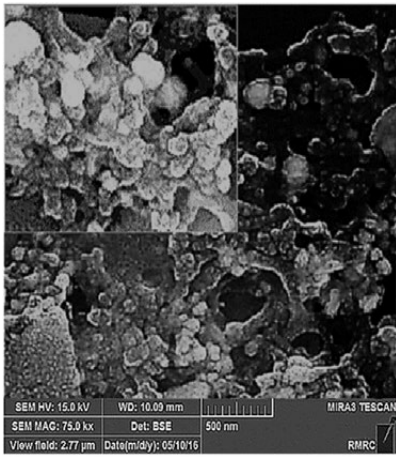

Fig. 2. A SEM micrograph of the biosynthesized gold nanoparticles.

in 3437, 3000, 1500, 1210 and 1120 which belongs to the Ar-OH-H bound, Ar-H stretch, aromatic C-C stretch, overlapping peaks of asymmetric vibration of C–O–C linkage and C–OH vibrations, respectively [11, 12]. The NMR spectroscopy of poly (catechol), confirms the FT-IR spectroscopy results.

UV-Visible spectroscopy of Bio AuNPs, showed a peak at 550 nm, which is due to the electron transitions between gold nanoparticles.

Optimization conditions

At present paper, four well-known electrochemical techniques (Differential Pulse Voltammetry (DPV), Electrochemical Impedance Spectroscopy (EIS), Cyclic Voltammetry (CV) and Chrono amperometry) were engaged to investigate different optimized parameters from aptamer concentration, probe solution types to the best immobilization and hybridization time [12, 13].

All investigations took place in 2 mM of catechols solution in 0.1 M pH 7.4 phosphate buffer saline (PBS buffer), which was the best choice as a probe solution (Fig. S1).

To optimize the aptamer concentrations, which has a major effect on signal strength; different concentrations of aptamer (10^{-9} to 10^{-4} M) were dropped on surface of Bio AuNP/Pol/Cu_2O-CD/SPE. According to the data, the best value for the aptamer concentration is 10^{-5} M (Fig. S2).

Electrochemical investigations of construction, immobilization and hybridization

Four different analyzing methods (DPV, EIS, CV and Crono amperometry) was applied simultaneously, to enhance the quality of investigations. Cyclic voltammetry (CV) as a potentiodynamic electrochemical measurement, was employed to study the construction, immobilization and hybridization of the aptasensor. The CV investigations were done in 2 mM catechol solution, with a scan rate of 100 mV/s.

As can be seen in Fig. 3, Pol/ SPE surface modification was shown an obvious increase in the peak current, and it's because of unique electrical properties of conductive polymers

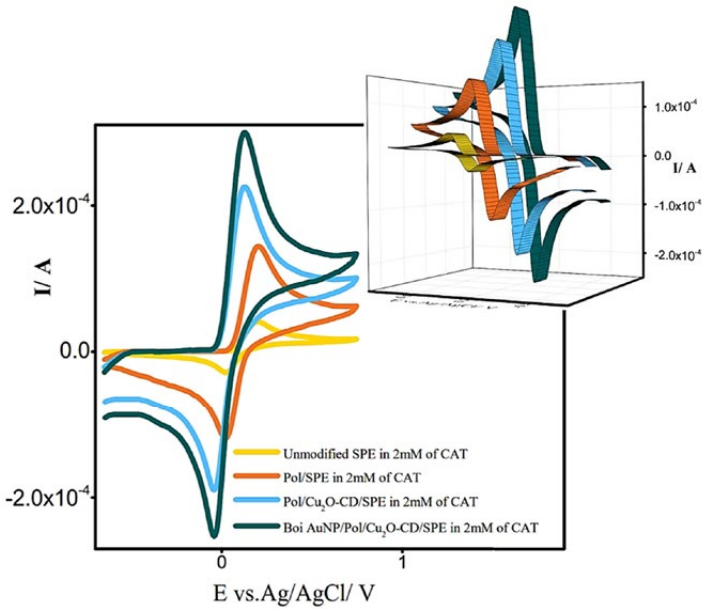

Fig. 3. Detecting of the SPE, Pol/ SPE, Pol /Cu_2O_CD/SPE, Bio AuNP/ Pol/ Cu_2O_CD/SPE in 2 mM of catechol in PBS.

which impressively improve the sensitivity of the aptasensor.

According to the Fig. 3, Pol/Cu_2O-CD/SPE surface modification compare to the Pol/SPE surface modification shows an obvious peak current increased, besides there is a clear peak shift to less positive potentials due to the CDs quantum size effect and enhancing the surface unit to volume ratio.

Bio AuNP/Pol/Cu_2O-CD/SPE surface modification was shown an increase in the peak magnitude and a slight shift of the peak maximum towards less positive potentials (Fig. 3).

In the next step, 5'-SH aptamer immobilized on the surface of Bio AuNP/ Pol/Cu_2O-CD/SPE which leads to a decrease in peak current and it's due to the covalent coupling of thiol modified aptamer to the gold nanoparticles. The peak current amplitude was most decreased due to the hybridization of the aptamer and antigen (CEA) (Fig. 4).

Electrochemical impedance spectroscopy (EIS) is an experimental method which characterizes

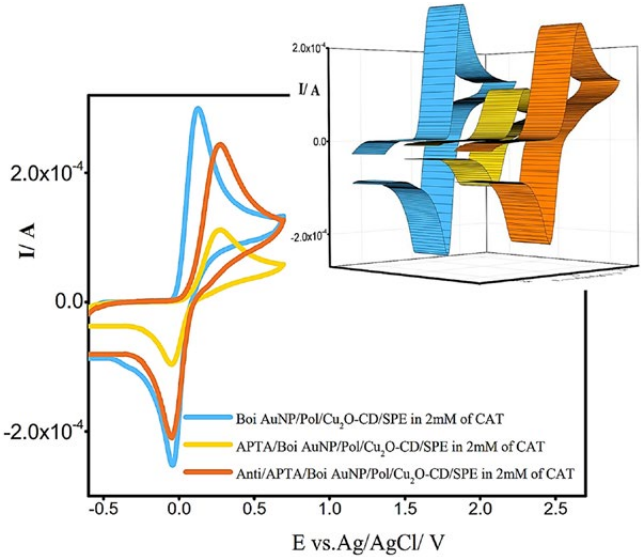

Fig. 4. Detecting of the immobilization and hybridization process at Bio AuNP/ Pol/ Cu_2O_CD / SPE and APTA/Bio AuNP/Pol/Cu_2O_CD/SPE, respectively, in 2 mM of catechol in PBS.

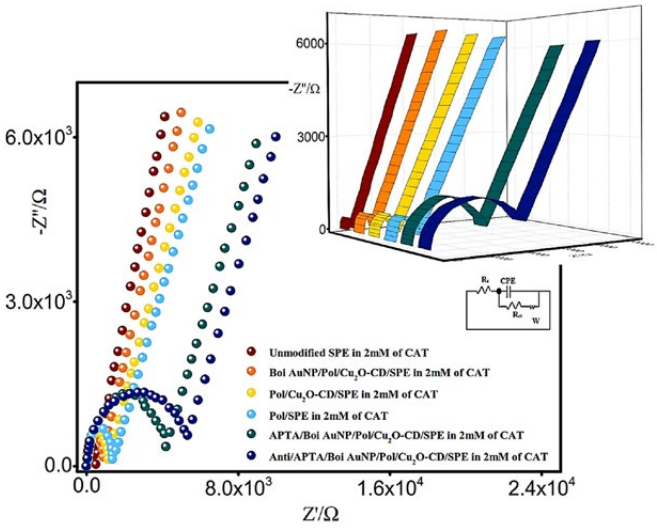

Fig. 5. Nyquist plots of SPE, Pol/ SPE, Pol /Cu_2O_CD / SPE, Bio AuNP/ Pol/ Cu_2O_CD / SPE APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE, Anti/ APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE in 1 mM of $[Fe(CN)_6]^{3-/4-}$ in PBS. Inset: Equivalent circuit for fitting the plots.

the impedance of a medium over a range of frequencies. $[Fe(CN)_6]^{3-/4-}$ solution was applied as a probe solution to investigate EIS studies.

According to the results, unmodified SPE showed the minimum value for $R_{ct.}$ As can be seen, after modification of SPE with Pol/Cu_2O-CD the $R_{ct,}$ increased and it is due to the formation of the Pol/Cu_2O-CD on the electrode surface. After modification of electrode with Bio AuNP/ Pol/Cu_2O-CD the R_{ct} decreased due to the conductive nature of conductive Bio AuNP/ Pol/Cu_2O-CD composite. After immobilization step the R_{ct}, increased which means the prospering immobilization of SH-modified aptamer on the surface of Bio AuNP/Pol/ Cu_2O-CD. After hybridization step, R_{ct} was most enhanced which means the aptamer matches with the CEA. As can be seen, the electrochemical impedance spectroscopy results confirmed cyclic voltammetry studies (Fig. 5).

Differential pulse voltammetry (DPV) investigations were done to confirm CV and EIS result, and according to the DPV studies lead to similar results (Fig. S3).

Chronoamperometry technique is an electrochemical method which can be used for quantitative analysis. At this work, chronoamperometry was used to study the electrode behaviors. According to the data, chronoamperometry results proved cyclic voltammetry, differential pulse voltammetry and electrochemical impedance spectroscopy studies (Fig. 6).

Cyclic voltammetry technique was applied to investigate the optimized immobilization and hybridization time. According to the results, the optimum aptamers immobilization time was 2h (Fig. 7a), which is a reasonably good result, and the optimum hybridization time was 3h (Fig. 7b).

Repeatability, reproducibility and long-term stability detection

The repeatability and storage stability of the biosensor were also investigated. CV evaluations were applied to study repeatability and reproducibility of the mentioned biosensor [14]. The relative standard deviation (RSD), for four parallel Anti/APTA/Bio AuNP/Pol/Cu_2O-CD/ SPEs which was made individually in four various days, was about 0.007 which is a very good result, all investigations were done in 2mM of catechol solution (pH 7.4) (Fig. S4).

The storage stability of the APTA/Bio AuNP/ Pol/Cu_2O-CD / SPEs was evaluated. The mentioned biosensors were stored at 4°C in 0.1 M Tris-EDTA buffer solution for whole a week, the outcomes suggested a good long-term storage stability and that might be attributed to the biocompatible nature of biosynthesis gold nanoparticles.

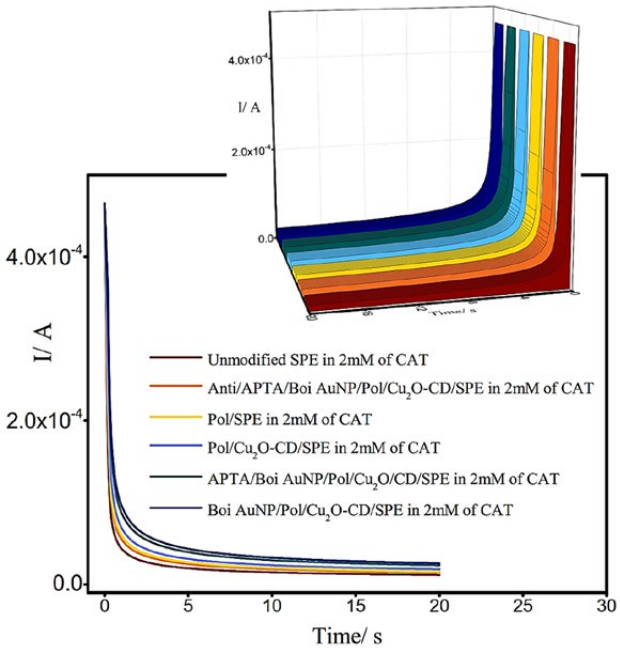

Fig. 6. Chronoamperometricb results, obtained at SPE, Pol/ SPE, Pol /Cu_2O_CD / SPE, Bio AuNP/ Pol/ Cu_2O_CD / SPE, APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE, Anti/ APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE in 2 mM of catechol in PBS.

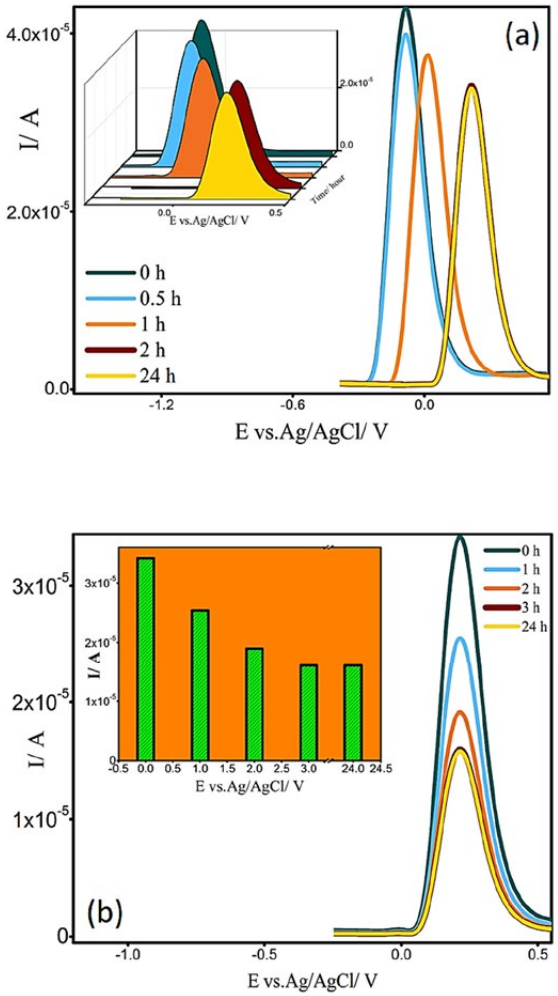

Fig. 7. Optimizing the immobilization time of APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE (a) and the hybridization time of Anti/ APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE (b) in 2 mM of catechol in PBS.

Evaluating the selectivity of the aptasensor

The C.V studies were done to investigate selectivity of APTA/Bio AuNP/ Pol/Cu_2O-CD/ SPE biosensor. The hybridization studies were performed with match antigen (CEA) and mismatch target (bovine serum albumin). According to data (Fig. 8) the match antigen (CEA) showed a certain decrease in the voltammetric signal, which means that the hybridization occurred. The same did not happened to the mismatch target (bovine serum albumin) which means that no hybridization occurred [15].

Sensitive detection of CEA

The sensitivity of the mentioned biosensor was investigated using DPV analysis. The APTA/ Bio AuNP/Pol/Cu_2O-CD/SPEs were hybridized with different concentrations of target antigen (CEA). As expected, DPV signals decreased with increasing target CEA concentrations.

As shown in Fig. 9, the values of I_{peak} is linearly related to the logarithm of the concentrations of CEA in the range from 1.0 pg mL^{-1} to 0.001 g mL^{-1}, with a detection limit of 0.19 pg mL^{-1} for the target antigen (CEA).

Real PCR Samples screening

The real samples (which was extracted from patients with CEA-positive samples), were hybridized onto the Bio AuNP/Pol/Cu_2O-CD/SPE. The electrochemical investigations were done through DPV analysis [16].

According to the data, the DPV peak current decrease due to the hybridization between the aptamer and CEA-positive real blood sample (Fig. S5).

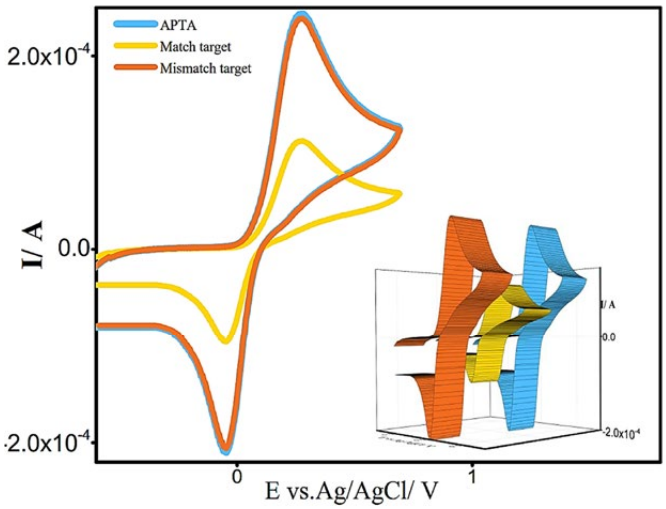

Fig. 8. Detecting of the selectivity of APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE in 2 mM of catechol in PBS.

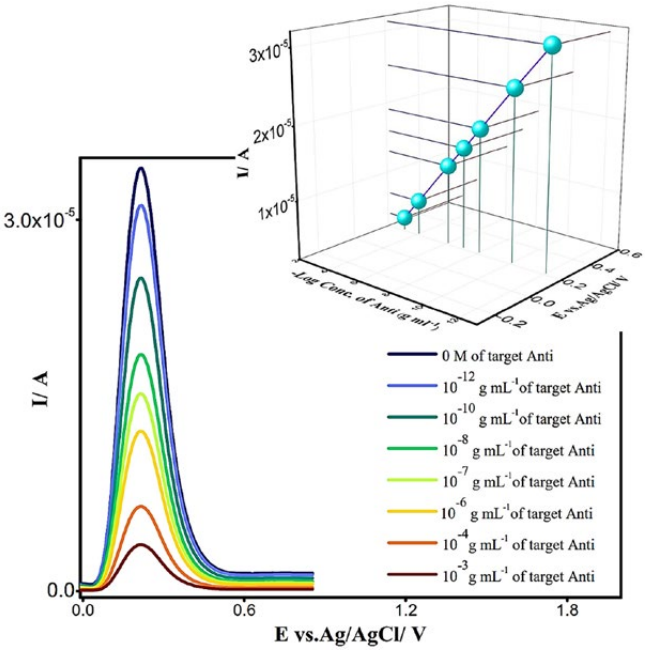

Fig. 9. DPVs of Anti/ APTA/Bio AuNP/ Pol/ Cu_2O_CD / SPE in 2 mM of catechol in PBS, at different concentrations of target antigen (CEA).

CONCLUSION

In this research, a screen-printed apatsensor, modified with an inimitable composite (based on conducting polymers (poly(cathechol)), Cu_2O–carbon dots and biosynthesized gold nanoparticles) with unique electrical conductivity and quite enhanced surface area, was investigated for the detection of carcinoembryonic antigen.

The values of I_{peak} is linearly related to the logarithm of the concentrations of carcinoembryonic antigen in the range from 1.0 pg mL^{-1} to 0.001 g mL^{-1}, with a detection limit of 0.19 pg mL^{-1} for the target antigen (carcinoembryonic antigen).

Four different electrochemical techniques (Differential Pulse Voltammetry, Electrochemical Impedance Spectroscopy, Cyclic Voltammetry and Chrono amperometry) were engaged to investigate aptasensors construction, immobilization, hybridization, sensitivity, selectivity, repeatability, long-term stability and real PCR samples detections.

It was anticipated, that the mentioned modified aptasensor has a vast potential for the detection of different kinds of cancers.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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