



CdS nanocrystals/graphene oxide-AuNPs based electrochemiluminescence immunosensor in sensitive quantification of a cancer biomarker: p53

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ABSTRACT

An ultrahigh sensitive, simple and reliable Electrochemiluminescence (ECL) immunosensor for selective quantification of p53 protein was designed according to the enhancement effects of AuNPs on ECL emission of CdS nanocrystals (CdS NCs). CdS NCs were immobilized on the glassy carbon electrode and AuNPs introduced to the process through formation of a sandwich-type immunocomplex between first anti-p53/p53/ secondary anti-p53. ECL of CdS NCs firstly evoked the SPR of AuNPs which in return amplified the CdS NCs ECL intensity. By using graphene oxide in immunosensor fabrication procedure, and attaching more AuNPs on the surface of the electrode, the ECL intensity was further increased resulting in much higher sensitivity. After applying the optimum conditions, the linear range of the developed immunosensor was found between 20 and 1000 fg/ml with a calculated limit of detection of 4 fg/ml. Moreover, the interference, reproducibility and storage stability studies of the immunosensor were investigated. Finally, immunosensor's authenticity was evaluated by detecting the p53 protein in human spikes which offers it as a potential in early detection of cancer, monitoring the cancer progress and clinical prognosis.

1. Introduction

Since protein biomarkers have been on the focus of the researchers in recent decades, there is an increasing demand for highly sensitive detection of these biomarkers. Development of new drugs, diagnosis of different kinds of diseases which could help monitoring cancer treatments (Doustvandi et al., 2017; Navaeipour et al., 2016), Immunology and other medical applications are greatly dependent on quantification of protein biomarkers (Afsharan et al., 2016a; Hasanzadeh et al., 2017). In particular, early cancer prognosis and sickness monitoring showed indisputable dependence to cancer biomarkers. p53 protein, a widely known tumor suppressor gene and a prominent cancer biomarker, plays inevitable role in cell proliferation and specially in apoptosis. It prevents genome mutation and thus, a failure in its functionality leads to highly risks of cancer (Afsharan et al., 2016c). From this point of view, finding sensitive, reliable and low-cost methods for detection of p53

protein seems very desirable and has drawn a lot of attention in recent years (Hasanzadeh et al.).

Between different procedures which have been used to detect p53 protein such as electrochemical (Afsharan et al., 2016b), Surface Plasmon resonance (SPR) (Wang et al., 2009), colorimetric (Li et al., 2013), chemiluminescence (Chen et al., 2013) and field-effect transistor (FET) (Han et al., 2010),

Electrochemiluminescence (ECL) method is one of the non-invasive optical methods (Ramanaviciene et al., 2012) which has been on the focus according to its superb characteristics including simplicity, low-cost, low response time, great selectivity and more importantly ultra-high sensitivity (Afsharan et al., 2016c; Khalilzadeh et al., 2016) thanks to compatibility with utilizing nanoparticles (Balal et al., 2009; Khalilzadeh et al., 2011; Saghatforoush et al., 2009).

It should be noted here that; concurrent employment of different methods together could be taken into consideration. For instance, the

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authors in (Ramanaviciene et al., 2012), was developed and introduced the first method for simultaneous application of SPR, electrochemical and ECL techniques for the detection of the human growth hormone immobilized on the SPR-chip surface. Although these kinds of works are more reliable and sensitive compared to other methods, but still, the works focused on one method are of high importance and value. ECL is one of those methods.

Moreover, in some ECL studies, luminophores and gold nanoparticles (AuNPs) can be used as quenchers or ECL emission enhancers (Khalilzadeh et al., 2018). Quenching effects happen due to the Forster resonance energy transfer (FRET) when AuNPs and luminophore are adjacent to each other, while enhancing phenomena takes place when AuNPs and luminophore are at certain distance in account of AuNPs-enhanced Raman scattering, or SPR (Bagdeli et al., 2017; Shan et al., 2009; Zhang et al., 2014). In here, Nano Crystals (NCs) are used as luminophores and thus with placing AuNPs at a close proximity to these NCs, we can quench the photons emission of NCs by the non-radiative energy dispersion into the metal core of the AuNPs (Zhang et al., 2014). On the other hand, when they are at a certain distance from each other, ECL of the NCs would induce surface Plasmon resonance (SPR) of AuNPs, and in return, this induced SPR would intensify the ECL response of NCs (Hao et al., 2014; Qian et al., 2010).

In this work, an ultra-sensitive ECL immunosensor for selective and sensitive quantification of p53 protein via sandwich method was designed and fabricated through enhancing the CdS NCs as ECL donors utilizing the AuNPs as acceptors. Huge applications in analytical experiments, easy and fast preparation in aqueous solution and other impressively splendid features such as size/surface-trap controlled luminescence and good stability against photo bleaching are made CdS NCs a great candidate for using as an ECL luminophore (Zhang et al., 2014). The immunosensor was prepared by immobilizing first capture antibody via glassy carbon electrode (GCE)/CdS NCs, forming of the immunocomplex between first antibody and p53 protein, and adding the tGO-AuNPs conjugated secondary antibody, respectively. Afterwards, different conditions influencing the performance of the engineered immunosensor was optimized, selectivity and interference studies were done and finally the p53 immunosensor was used to evaluate the human spike sera samples.

2. Materials and methods

2.1. Materials and reagents

Monoclonal anti-human p53 from mouse (first antibody), monoclonal biotinylated anti-human p53 from goat (secondary antibody) and p53 human protein in an ELISA pack were purchased from Boster Biological Technology Co., Ltd (Pleasanton, CA, USA). trisodium citrate and citric acid were purchased from Merck (Hohenbrunn, Germany). $\text{Na}_2\text{S}\cdot x\text{H}_2\text{O}$ (32–38%) and Graphene oxide (GO) were received from Fluka (Switzerland). Gold (III) chloride trihydrate ($\text{HAuCl}_4\cdot 3\text{H}_2\text{O}$), tri (2-carboxyethyl) phosphine hydrochloride (TCEP), Cadmium nitrate tetrahydrate ($\text{Cd}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$), bovine serum albumin (BSA, purity $\geq 98\%$) and hydrogen peroxide (H_2O_2) were bought from Sigma-Aldrich (St. Louis, MO, USA). Streptavidin was provided from Abcam (Cambridge, USA). Double-distilled water (DW) was used thoroughly in all the experiments carried out in this study. In addition, 0.1 M Na_2HPO_4 , 0.1 M KH_2PO_4 and 0.1 M KCl were added to DW to prepare phosphate buffer solutions (PBS). pH of the PBS was adjusted using NaOH and then stored in 4 °C for further usage.

2.2. Apparatus and procedures

Centrifuging and sonication were performed by Sartorius 1–15P (SIGMA, Göttingen, Germany) SONOPLUS GM3100 (Bandelin

electronic, Berlin, Germany), respectively. Auto-Lab equipped with three electrode setup (a glassy carbon -GCE, with a surface of 0.03 cm^2 -, an Ag/AgCl and a platinum rode) was used in both ECL and electrochemical (including square wave voltammetry; SWV and differential pulse voltammetry; DPV) investigations. Furthermore, to detect the ECL emitting photons, a R12829 photomultiplier-tube (Hamamatsu, Japan) was coupled with PGSTA T302 N Auto-Lab (ECO Chemie, Utrecht, Netherlands). The experiments were carried out in complete darkness and room temperature.

2.3. Synthesizing the CdS

According to (Hao et al., 2014), CdS was synthesized by dissolving 0.16 g $\text{Cd}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$ in 60 ml preheated (to 70 °C) of DW and stirring for 30 min. Next, 0.59 g Na_2S was added to the mixture and the obtained yellowish solution was kept stirring for another 3 h under reflux condition. Then, the mixture was centrifuged and rinsed with water and ethanol 3 times to remove any unreacted reactants. Finally, after re-dispersing the orange remaining sediment in DW and centrifuging again, the upper supernatant solution was collected. Fourier transforms infrared (FTIR) spectra were carried out with a Shimadzu 8101M FT-IR spectrophotometer (Kyoto, Japan) over the range of $4000\text{--}400\text{ cm}^{-1}$. UV-Visible spectra were measured on a Shimadzu UV-1650PC (Kyoto, Japan). A photoluminescence Shimadzu RF-5301PC was used for recording the PL spectra. A scanning electron microscope (SEM, S-360 Cambridge, UK) was utilized to record the SEM images.

2.4. Preparation of t-GO, AuNPs and streptavidin modified AuNPs

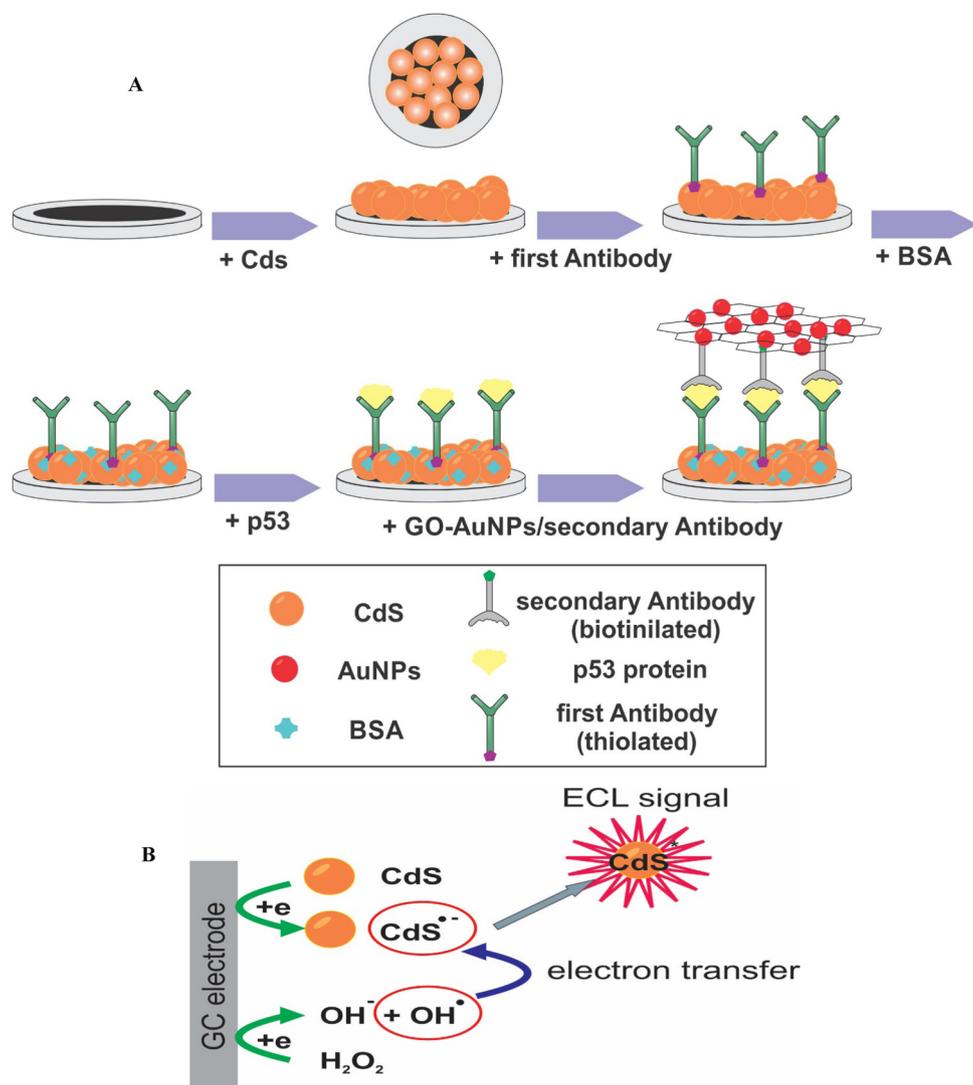
Graphene oxide thiolation (t-GO) process, gold nanoparticles (AuNPs) synthesis and preparation of streptavidin modified AuNPs were done according to our previous publication (Rashidiani et al., 2018) and used here without further manipulations. Also the characterization of AuNPs, tGO, tGO decorated with AuNPs and CdS NCs was available in the [Supplementary information](#).

2.4.1. tGO-AuNPs conjugated p53 secondary antibody formation

thiol-functionalized GO (t-GO) was decorated with AuNPs in ethanol with vigorous stirring for 8 h at reflux condition and 60 °C (with the optimized ratio of 10: 15 μl tGO: AuNPs). The solution was then washed, centrifuged and dried naturally. The remnant sediment (tGO-AuNPs) was finally obtained (Khalilzadeh et al., 2016). Afterwards, tGO-AuNPs was then re-dispersed in PBS (pH = 7.5) and blended with biotinylated secondary p53-antibody at 4 °C for 5 h to form the Biotin-Avidin reaction. This bio-conjugate is one of the most durable reactions that cannot be broken easily. Subsequently, a mixture of BSA (1% w/v) was added to block any possible active sites.

2.5. Fabrication of p53 ECL immunosensor and immunosensing procedure

As illustrated in [Scheme 1A](#), the immunosensor fabrication was done as follow: first, GC electrode was polished on a pad carefully to obtain a mirror-like surface and then rinsed with DW. This step repeated for three time to make sure no unintended materials were interfering the experiments. Next, CdS modified on GCE by drop-casting 20 μl of CdS the GCE and drying at room temperature. In a previously published article, Yan-Yan Zhang et al. demonstrated that by activating GCE/CdS NCs in an activating buffer, containing both H_2O_2 and citric acid, resulted ECL emission was 58 times bigger than that of conventional method. Therefore, we did the same and activated GCE/CdS before performing further modification as it has been done in (Zhang et al., 2014). After that, the GCE/CdS electrode was immersed in first p53-antibody and incubated in humid conditions for 24 h at 4 °C. It must be noted that first antibody was treated with 25 μl TCEP prior to



Scheme 1. Schematic illustration of (A) the designed ECL immunosensor using CdS NCs and tGO-AuNPs and (B) ECL signal generation of CdS in the presence of H_2O_2 .

use to activate the $-\text{SH}$ (thiol) functionalization of antibody. Afterwards, prepared GCE/CdS/p53-Ab1 was incubated (1 h, 4°C) in BSA (1% w/v) solution to avoid any nonspecific bindings. Thereafter, different concentrations of p53 protein was drop-cast on the modified electrode and incubated for about half an hour in room temperature (25°C). As the final step for formation of sandwich method, secondary antibody was introduced to the electrode surface by immersing the electrode in $25\ \mu\text{l}$ of as-prepared tGO-AuNPs conjugated secondary antibody. This step was also performed in room temperature (Afsharan et al., 2016c). Thus, the desired immunosensing electrode including CdS as photon source, p53 as analyte, Au as CdS enhancer and tGO as amplification element was obtained. To authenticate the preparations steps, DPV, SWV and ECL characterizations were separately carried out. As a comment, two points must be mentioned; first, antibodies and p53 protein were kept in room temperature for 15 min prior use and second, after each step of modification, the electrode was rinsed by immersing in PBS (pH = 7.5) for 10 min in order to wash off the unreacted materials.

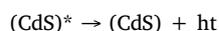
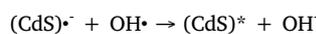
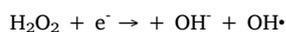
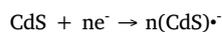
To detect and quantify p53 with the engineered electrode, it was used as working electrode and placed in electrochemical glass cell. The ECL measurements were performed in PBS (0.1 M, pH = 7.5) in the

presence of 20 mM H_2O_2 . The ECL intensity was detected by a photomultiplier tube (PMT) which was set on 800 V. Moreover, the electrochemical potential between -1.5 and -0.8 V was applied to ignite and excite the CdS distance dependent FRET mechanism.

3. Results and discussion

3.1. Electrochemiluminescence behavior of the proposed sensing method

CdS and H_2O_2 group is one of the most predominant and used ECL luminophores and coreactants. According to the following equations, after applying negative potential (around -1.25 V), CdS and H_2O_2 at the electrode surface were reduced to $(\text{CdS})^\bullet-$, and OH^\bullet , respectively. Next, OH^\bullet reacted with $(\text{CdS})^\bullet-$ led to the formation of the $(\text{CdS})^*$ excited state which can emit photons.



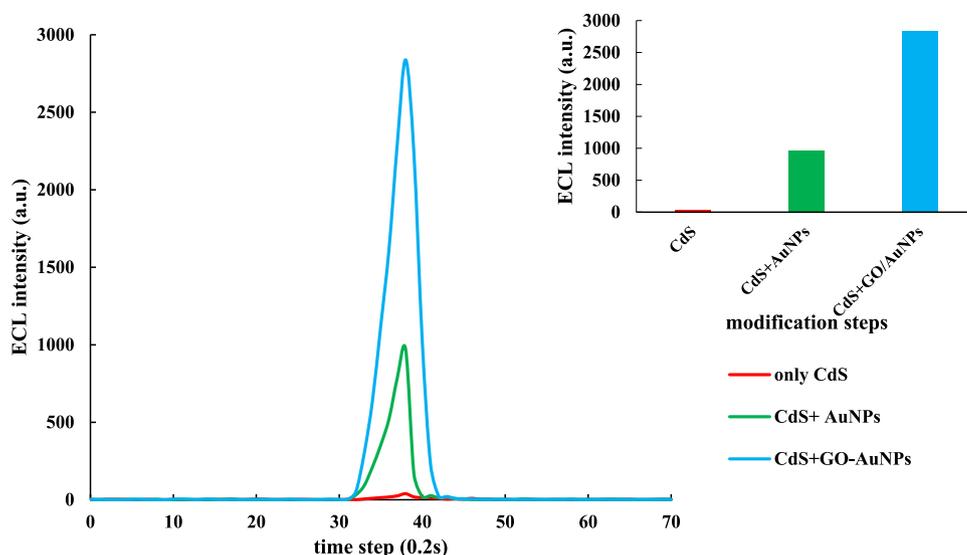


Fig. 1. ECL responses of modified GCE/CdS/p53-Ab1/p53/p53-Ab2 electrode in the absence of tGO and AuNPs (red), in the presence of only AuNPs (green) and in the presence of tGO-AuNPs (blue) in PBS (0.1 M, pH = 7.5) containing 20 mM H_2O_2 and the scan rate of 0.1 V/s (inset: ECL peak variations vs. different modification steps). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In this cycle, H_2O_2 could form freely by electrochemical reduction of oxygen in aqueous solution (Zhang et al., 2014). This procedure is illustrated in Scheme 1B.

Besides, AuNPs can play both quencher or enhancer roles when it comes to semiconductor nanocrystals. It is all a matter of distance which determines either AuNPs quench or amplify the ECL of CdS in the presence of H_2O_2 as co-reactant (Shan et al., 2009). In the current study, sandwich method was employed to create the desired distance between CdS (as ECL donors) and AuNPs (as ECL acceptors). Accordingly, as it can be seen in Fig. 1, amplification of engineered biosensor (GCE/CdS/p53-Ab1/p53/p53-Ab2-tGO-AuNPs) was investigated in PBS (pH = 7.5) containing 20 mM H_2O_2 at the scan rate of 0.1 V/s. Presence of AuNPs was greatly enhanced the ECL intensity of the synthesized CdS. This behavior is due to the induction of AuNPs surface Plasmon resonance (SPR) influenced by ECL of CdS, that in return amplifies the ECL emission of CdS through FRET. Furthermore, use of tGO as a platform to immobilize more AuNPs on the electrode surface had an extraordinary impact on the ECL output. This led to the introduction of more AuNPs to the CdS and as a result, create more SPR activities.

Moreover, in order to eradicate the baseline error which is created in account of background emitted photons from CdS even in the absence of AuNPs, the chosen drop-cast value of CdS was optimized perfectly and carefully, so that no “always on ECL intensity” occurs (Fig. S3A).

Beside all of this, the thermodynamical and kinetic characteristics of the proposed immunosensor could be calculated according to the Klotz, Langmuir or Langmuir-Freundlich equations (Viter et al., 2018). Through calculating these parameters (The analysis of the adsorption isotherms) such as binding affinity or affinity dissociation constant (K_D), we can evaluate how antigen-antibody react with each other. For example, K_D is the concentration at which 50% of binding sites (antibody) are occupied by antigen (p53). Similarly, the p53 affinity is equal to $1/K_D$. Higher K_D (lower binding affinity) means that it is difficult for antibody and antigen to bind. Here, and in this article, we do not calculate these data and will do these kind of experiments in another article.

3.2. Electrochemical and electrochemiluminescence characterization of engineered immunosensor

Characterizations are recommended to verify the authenticity of

immunosensor preparations. Thus, electrochemical and ECL characterization were conducted to meet these requirements.

DPV and SWV are simple and at the same time eligible ways used to check the electrochemical behavior of modified electrode. DPV and SWV of the modified electrode in each step has been shown in Fig. S1 in the PBS (0.1 M, pH = 7.5) containing 5 mM $K_4[Fe(CN)_6]$, $K_3[Fe(CN)_6]$ and 0.1 M KCl at scan rate of 100 mV/s. From Fig. S1A, it can be concluded that, after CdS was drop-cast on GCE, the DPV current decreased due to the semiconductor effects of CdS. Somehow it reduced the electron transference rate. Immersing the GCE/CdS electrode in p53-Ab1 resulted in more decline in output DPV current because antibody could shield the electron transfer process. This indicates that the antibody is successfully immobilized on the electrode. This decrement in peak current was further continued after introduction of p53 protein to GCE/CdS/p53-Ab1 prepared electrode. This reduction is clearly attributed to the hindered impacts of immunocomplex formation between antibody and p53. At last and after incubating p53-Ab2-tGO-AuNPs, a huge ascent and amplification was observed in DPV of the immunosensor. This behavior was not only due to the conductivity effects of AuNPs itself ending in acceleration of electron transfer rate, but also because of the increment of effective surface area and conductivity of tGO. In addition, SWV measurements of immunosensor were also showed almost the same variations in working electrode current after each immobilization step (Fig. S1B). Another interesting point which can be derived from Fig. S1B is attributed to this fact that by only using AuNPs without utilizing tGO, the peak current of both DPV and SWV was although experienced an increment, but not even close to the use of latter one (the gray bar in comparison with the yellow bar).

For further evaluation of the validity of the electrode modification, ECL measurements of engineered electrode after each step was investigated. Fig. S2 shows the ECL behavior of the electrode after adding CdS, p53-Ab1, p53 and p53-Ab2-tGO-AuNPs in PBS (0.1 M, pH = 7.5) with 20 mM H_2O_2 . It demonstrated that the ECL intensity of bare GC electrode increased after adding CdS which validate that the nanocrystals are perfectly coated on electrode. It was obvious that the coverage of first antibody and p53 on GCE, formed a barricade in electron transfer route, made the transfer rate decrease and so led to more reduction in ECL output. Subsequently, incubation of p53-Ab2-tGO-AuNPs was resulted in a dramatic rise in emitted ECL photons. The reason behind the amplification could be attributed to the high surface area of tGO enabling the ability to hold more AuNPs in distance from CdS to gain more SPR and so more ECL output intensity.

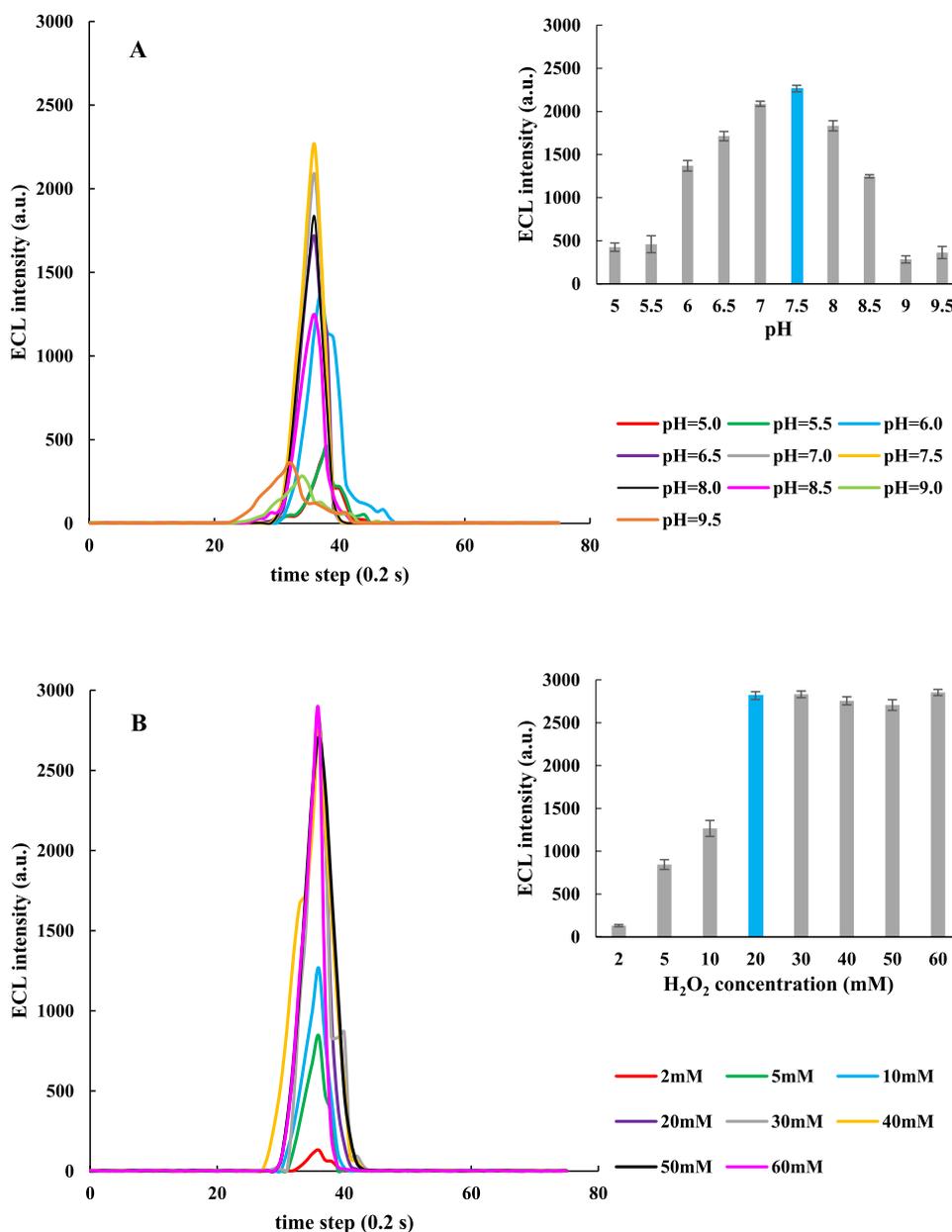


Fig. 2. (A) The effects of buffer pH on the proposed immunosensor ECL response in PBS (0.1 M, pH = 7.5) in the presence of 10 mM H₂O₂ and (B) data gathered from GCE/CdS/p53-Ab1/p53/p53-Ab2-tGO-AuNPs electrode in PBS (0.1 M, pH = 7.5) containing different concentrations of H₂O₂ (Insets: variation of the ECL intensities versus (A) pH of buffer solutions and (B) different concentrations of H₂O₂).

Changes in both electrochemical and ECL responses not only did prove the claim the authors made about using tGO-AuNPs concurrently, which can yield to huge enhancement in ECL behavior of CdS nanocrystals, but also show that the preparation of the electrode has not affected the CdS properties and thus synthesized CdS has retained its characteristics and could emit photons. Eventually, the resulted data indicate that the electrode was correctly modified and can be used for detection and quantification of p53 protein.

3.3. Optimizing the prepared p53 immunosensing conditions

Achieving higher performance in the detection of p53 using the proposed procedure, requires further endeavors to optimize the main parameters and conditions influencing the sensitivity.

3.3.1. pH optimization

pH of the solution is one of the predominant factors in obtaining higher p53 quantification sensitivity. So, the ECL data of the modified electrode with 1 pg/ml p53 in PBS (0.1 M) with different pH values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5) and in the presence of 10 mM H₂O₂ were recorded. The resulted curves are displayed in Fig. 2A. As seen, ECL intensity experienced an increment by reaching higher values from 5.0 to 7.5 and then decreased. Acidic environment disrupts both CdS functionality and p53 sandwich formation. Moreover, since higher pH values make H₂O₂ unstable and antibodies and proteins are working properly in physiological conditions (pH = 7.5), so further rise in pH of the buffer (alkaline environment) ended in lower ECL responses. Thus, pH = 7.5 was selected and utilized in sensitive detection of p53.

3.3.2. Finding the optimum concentration of H_2O_2 in PBS

ECL response of the biosensor is directly pertinent to the concentrations of H_2O_2 . Experiments were carried out in PBS (0.1 M, pH = 7.5) to acquire an optimum concentration for H_2O_2 and the ECL signals of the designed immunosensor (with 1 pg/ml p53) were investigated (Fig. 2B). In the absence of H_2O_2 , barely no ECL signal was observed. Furthermore, by increasing the concentrations, ECL intensity escalated and then reached a plateau and the variation stopped. The reason might be contributed to the saturation of H_2O_2 reactions that caused excited state CdS molecules and as a result ECL emission to stay steady. Consequently, to minimize the reagents consumption, 20 mM was selected as optimized concentration of H_2O_2 .

3.3.3. Optimization of CdS and tGO: AuNPs

Another important factor dealing with the sensitivity is CdS NCs. It is obvious that value of CdS can influence the emission of photons. Optimizing CdS was done as follows: first different immunosensors with 1, 2, 5, 10, 20, 30, 40, 50 and 100 μ l of CdS NCs were prepared and activated by a mixture of H_2O_2 and citric acid. Then the rest of the electrode modifications were immobilized on GCE/CdS and ECL measurements in PBS (0.1 M, pH = 7.5) with 10 mM H_2O_2 were recorded (Fig. S3A). increment in ECL response via drop-casting more CdS on GCE was observed in the range of 1–40 μ l. After adding more CdS to GCE surface (50 and 100 μ l), the variations slowed down. This behavior was expected because after a particular CdS value (here 40 μ l), the surface of GCE was all covered with CdS NCs and no active sites remained untouched to react with CdS molecules. Consequently, drop-casting more CdS NCs on GCE was considered a disadvantage rather than being an advantage due to the more loss of CdS. Besides, and as said before, although the highest ECL intensity was happened in 40 μ l of CdS, but to eliminate intensities of ECL photons from CdS when neither p53 protein nor AuNPs are present (CdS has a background ECL) and so to minimize the baseband errors, 20 μ l was chosen as optimum value of CdS and drop-cast on the GCE surface for further modifications.

As the final optimization condition, tGO: AuNPs was investigated by obtaining ECL response of modified electrode with various ratios of tGO: AuNPs. For this reason, 10 μ l tGO was separately decorated with 5–35 μ l o AuNPs and used as enhancer to amplify the ECL of CdS NCs. The resulted curves are shown in Fig. S3B indicates that 10: 15 μ l is the optimal ratio for tGO: AuNPs which presents the maximum amplification toward CdS.

3.4. Performance of the proposed ECL method in detection and quantification of p53 protein

Considering all of the optimizations obtained in the experiments, the performance of the ECL immunosensor was evaluated using designed immunosensor to detect and measure the p53 protein. The electrode was prepared employing different concentrations of p53. 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1 pg/ml of p53 were sandwiched between two antibodies following the ECL immunosensing quantification. The results are gathered in Fig. 3. It can be concluded that by incubation more concentration of p53 on the electrode, the ECL response increased linearly (Fig. 3B). limit of detection (LOD) and limit of quantification (LOQ) are two important factors which can be calculated to indicate the performance and efficiency of the biosensor. For the engineered p53 ECL immunosensor, these factors are calculated and obtained 4.66 and 15.56 fg/ml based on the signal to noise ratio (S/N) of 3 and 10, respectively.

Obtained results have been compared to those of other studies (Table S1). The represented LODs clearly represents that the analytical performance of the designed immunosensor is better than the other p53 immunoassays reported so far. Firstly, applying the method discussed in (Zhang et al., 2014), caused CdS NCs to become ECL activated and emit

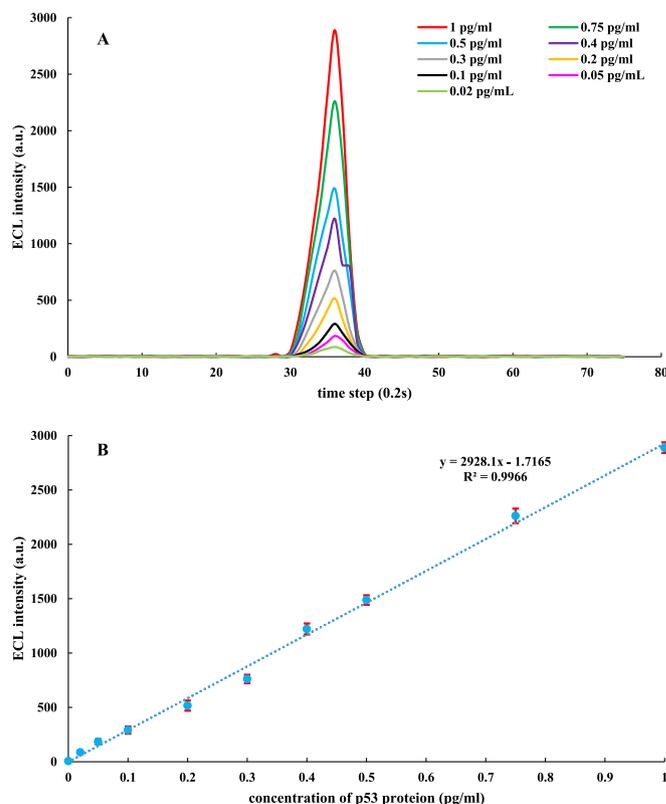


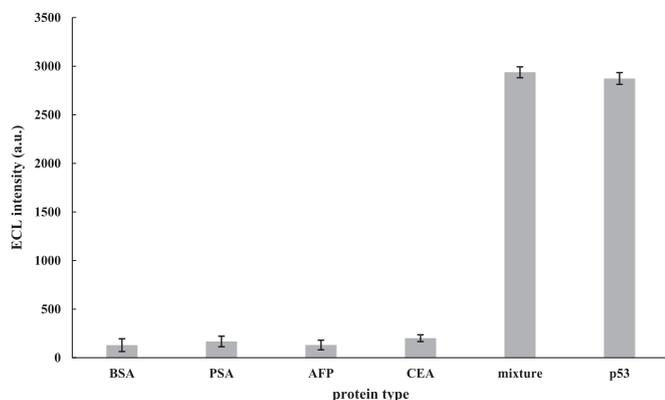
Fig. 3. (A) ECL intensity of the proposed immunosensor with 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1 pg/ml of p53 in PBS (0.1 M, pH = 7.5) containing 20 mM H_2O_2 and (B) the ECL response variations vs. concentrations of p53 protein.

more light. Secondly, role of tGO in the fabrication of the immunosensor and the huge impact it has on the sensitivity cannot be ruled out (Jiang et al., 2014). Use of tGO not only did provide extended active sites for loading more AuNPs and subsequently, more secondary antibody on the electrode, but also resulted in facile electron interference. In addition, AuNPs was also the other significant modification which rather than increasing the electron transfer rate, dramatically amplified the ECL intensity of the CdS NCs by providing possible surface Plasmon resonance, which in return, could enhance the ECL performance. Utilizing the streptavidin–biotin conjugate in the modification of the immunosensor was another reason ending in such a good LOD. Streptavidin–biotin was resulted in further increase in the amount of secondary antibody introduced to the electrode. Increment in number of secondary antibodies, finally led to more AuNPs and more ECL intensity. Last but not least, other reasons such as simplicity, low cost and short response time paved the way and helped the immunosensing process to obtain this remarkable data.

3.5. Feasibility of the immunosensor

3.5.1. Selectivity and interference evaluation

To evaluate the authenticity of the immunosensor, other investigations were done. As the first of them, specificity and selectivity was examined. Response of the electrode in the presence of different interferences (proteins) was assessed and the obtained data are presented in Fig. 4. Samples including 10 ng/ml BSA, 50 ng/ml PSA, 50 ng/ml CEA, 100 ng/ml AFP, the mixture of all proteins and 1 pg/ml p53 were incubated separately with the modified immunosensor. The results were promising. Investigating the obtained data revealed that the ECL peak intensities showed barely no response toward other proteins. In



BSA: Bovine serum albumin

PSA: Prostate specific antigen

AFP: Alpha-fetoprotein

CEA: Carcinoembryonic antigen

Fig. 4. The interference study: The ECL curves obtained from the modified electrode incubated in 10 ng/ml BSA, 50 ng/ml PSA, 50 ng/ml CEA, 100 ng/ml AFP, the mixture of all proteins and 1 pg/ml of p53 protein carried out in PBS (0.1 M, pH = 7.5) containing 20 mM H₂O₂ with scan rate of 100 mV/s.

Table 1

Recovery of the modified biosensor for detection of p53 protein in human serum samples.

Sample	Added (fg/ml)	ECL intensity (a.u.)	Found (fg/ml)	Recovery (%)
1	850.0	2431.5	831.6	96.6
2	450.0	1333.5	456.1	103.5
3	225.0	636.6	218.5	93.5
4	105.0	308.6	106.9	101.8
5	52.0	150.5	52.7	107.1
6	12.0	30.4	11.3	94.1

other words, immunosensor's response was imperceptible and negligible. However, ECL of the sample containing mixture of all samples is almost the same as sample of 1 pg/ml p53 protein. In conclusion, other proteins cannot interrupt the quantification of p53 and the designed immunosensor is surely reliable. This level of specificity might be due to the use of monoclonal anti-p53 which only conjugates with p53 proteins

3.5.2. Reproducibility study and stability investigation of the modified immunosensor

The proposed assay's reproducibility as the second feasibility investigations was evaluated during measurements of the prepared electrode under 15 consecutive cycles in (0.1 M, pH = 7.5). The associated relative standard deviations (RSD) were then calculated less than 4.82% (illustrated in Fig. S4A).

Furthermore, storage stability as the third possibility of immunosensor from the practical application point of view was examined by finding ECL intensities of designed electrode including 1 pg/ml p53 protein in different time intervals (including immediately after preparation and after 2, 4, 8, 24, 48 and 168 h of modification). After each measurement, the electrode was washed with PBS and then kept in 4 °C in PBS (pH = 7.5) until next experiment. The resulted ECL curves are displayed in Fig. S4B for the fabricated immunosensor. According to the data, almost no noticeable changes were observed after one week. 10.8% was calculated for the ECL peak differences almost a week after first preparation of the immunosensor as follow:

$$\frac{ECL \text{ peak intensity (immediately - after 168 h)}}{ECL \text{ peak intensity (immediately)}} \times 100 = 10.8 \%$$

3.5.3. Application: detection of p53 protein in human real samples

To further evaluate the feasibility of the proposed immunosensor, recovery experiments were performed. Electrode was prepared with human spiked samples. These human samples were prepared by standard addition method prior to use. The collected data are listed in Table 1 which were calculated by the regression equation. According to the data, the recovery was obtained between 93.5% and 107.1%. Consequently, recovery data indicate that the developed immunosensor can be used for detection and quantification of p53 in human serums in clinical diagnosis.

4. Conclusion

In this article, p53, a major cancer biomarker, was detected using an innovative method with ultrahigh sensitivity. This method was to sandwich p53 protein between a capture anti-p53, immobilized on the GCE through CdS nanocrystals, and a secondary anti-p53 attached to tGO decorated with AuNPs as enhancing agents. After finding optimum conditions and taking them into consideration, applying potential led to an intensified ECL intensity of CdS. In other words, the CdS/H₂O₂ ECL was dramatically amplified due to the presence of AuNPs/tGO in a close distance from CdS. It is anticipated that excited SPR of AuNPs amplified the ECL of CdS. Furthermore, employing tGO in the immunosensor fabrication, give rise to more AuNPs load on the secondary anti-p53 and played huge role in amplification procedure. Using this method, limit of detection for p53 measurements was calculated as low as 4 fg/ml. Also, experiments showed that the engineered immunosensor had good biocompatibility, low response time, reliable selectivity, extra-ordinary sensitivity. We believe that, these gathered data is indicative of authenticity of CdS/H₂O₂ plus AuNPs system in immunosensors and so, could pave the way for future endeavors toward using this method in clinical trials or diagnosis of cancer related illnesses.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2018.10.031.

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