Photoelectrochemical device based on modified by osmium polymer to detection of enzymatic activities

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Abstract

Development of sensitive electrochemical devices opens the possibility for the design of simple assays for a large number of enzymes based on photocatalytic activity of semiconductor nanoparticles (SNPs). We report an innovative photoelectrochemical (PEC) system modified by osmium complex via electrostatic adsorption method. The system is based on the in situ generation of CdS quantum dots (QDs) by an enzymatic reaction. Alkaline phosphatase (ALP) catalyzes the reduction of sodium thiophosphate (TP) with 1-thioglycerol (TG) to generate H_2S. The latter reacts with cadmium cations to form CdS QDs. Controlling potential (0.31 V vs. Ag/AgCl) and wavelength (λ_{ex} = 365 nm), photocurrent is generated. Combined with immune recognition and PEC methodology, immunoassay based on specific enzyme-linked immune-sorbent assay (ELISA) was developed, as it is known ALP-based ELISA. Selecting BSA as a model, anti-BSA antibody (AB) with ALP-labeled antibody (ALP-AB) was detected, displaying a linear range up to 20 ng mL^{-1} and a detection limit of 2 ng mL^{-1} (S/N = 3). The sensitivity of our PEC system was enhanced 5 and 2 times as regarding chromogenic and fluorogenic methods, respectively.

Keywords: Photoelectrochemistry, Quantum dots, ELISA, enzyme catalysis, immunoassay

1. Introduction

Nowadays, photoelectrochemical (PEC) measurements as a newly emerging technique are taking off with high sensitivity. PEC systems are based on the photocatalytic reduction or oxidation of biomolecules to carry out photogenerated electron transfer between the target analyte and electrode under light irradiation to amplify the signal (Zhao et al., 2015). The PEC detection, where photoactive species are excited by light and generated current is used as the detection signal, possesses potentially higher sensitivity than classic electrochemical methods. Furthermore, it makes the devices cheaper and easier to miniaturize than conventional optical methods. However, PEC systems exhibit several drawbacks due to the use of presynthesized semiconductors as signal source, generating high production times, low sensitivity, increased cost, etc.

For that reason, the technological advances in nanoscience have focused on searching manifold nanomaterials that improve the performance in PEC devices (Devadoss et. al., 2015). In particular, inorganic semiconductors such as SnO_2 (Wu et al., 2013), TiO_2 (Zhang and Guo, 2012; Zhao et al., 2012a) and quantum dots (QDs) have been used extensively in these biosensors. The latter have become a promising alternative based on the enzyme-catalyzed growth of QDs. Their size-tunable photonic properties attract great interest in both ultrasensitive optical and electrochemical bioassays. PEC biosensors generating in situ QDs as electrochemical labels are being developed for enzymatic sensing and immunoassay (Rzigalinski and Strobl, 2009; Tanne et al., 2011; Zeng et al., 2014, Zhao et al., 2012b).

Semiconductor nanoparticles (SNPs) QDs can generate anodic or cathodic photocurrent depending on the applied potential. The electron transfer might occur from the conduction band (CB) to the electrode, generating anodic photocurrent. But if the

electron transfer is produced from the electrode to the valence band (VB), cathodic photocurrent is achieved. However, the relatively low carrier mobility, high recombination rate of photogenerated electron–hole pairs of QDs attached to harsh operating conditions limit the overall response. To solve these problems, some effective approaches have been developed to further optimize the photoelectric performance, including conductive polymers immobilized on the electrode surface (Gazotti et al., 2000; Li et al., 2014; Wang et al., 2013) and redox active mediators in solution, such as ferri/ferrocyanide (Stoll et al., 2006) and ascorbic acid (Zhao et al., 2012b, 2013).

We report here a miniature PEC device based on two samples of the in situ generation of CdS QDs: i) enzymatic biosensing assay and, ii) immunoassay based on specific enzyme-linked immune-sorbent assay (ELISA). First, we demonstrate that the reaction product of alkaline phosphatase (ALP) enzyme is able to catalyze the production of CdS QDs as it is shown in Scheme 1. On the other hand, through a competitive immune recognition reaction (Scheme 2), Bovine serum albumin (BSA) antibody (AB) was specifically conjugated to its ALP-labeled antirabbit-IgG (ALP-AB). ALP then catalyzed de reduction of TP to generate H_2S and form CdS QDs, which are photoexcited under ultraviolet light to produce a photocurrent as signal. The specific interaction between Ab and ALP-Ab is detected by recording the enhanced photocurrent of CdS QDs sensitized by conductive osmium poly(4-vinylpiridine) polymer and thioglycerol (TG) redox mediator. In both applications, the model system operates with the efficient chemistry of ALP which is widespread in the human body and also acts as a crucial biomarker in clinical diagnosis (Millán, 2006a, 2006b). Furthermore, our system is based on BSA as the test object due to its abundance of blood plasma, being a carrier of many low-polar metabolites and drugs (Peters, 1996) and employed in multiply clinical probes (Kumaran et al., 2015).

2. Materials and Methods

2.1. Chemicals

Alkaline phosphatase (ALP) from bovine intestinal mucose, albumin from bovine serum (BSA), antibovine serum albumin (developed in rabbit, AB), ALP-labeled antirabbit-IgG (ALP-AB), magnesium chloride ($MgCl_2$), sodium thiophospahte (TP), 4-nitrophenyl phosphate disodium (PNPP), cadmium nitrate ($Cd(NO_3)_2$), 1-thioglycerol (TG), trizma base, clorhidric acid (HCl), diethanolamine and casein blocking buffer were obtained from Sigma-Aldrich. All water used was Mili-Q ultrapure grade (18.2 MΩ cm). The osmium complex $[Os^{II}(bpy)_2Cl(4\text{-vinylpyridine})Cl]$ was prepared according to the literature procedure (Valery Thesis, Popescu et al., 1999; Virel et al., 2009).

2.2. Preparation of graphite electrodes modified by osmium polymer

Spectrographic graphite rods of 2 mm in diameter (Bay Carbon, USA) were cut into pieces of 3 cm in longitude, introduced into heat shrinkable PVC plastic tubes, shrunk by heating, wet polished on fine emery paper (Akasel, Denmark) and sonicated in water/ethanol (1:1) for 10 min. Then it was dried in a stream of N_2 at room temperature (RT) for 10 min. The modified graphite electrodes were obtained by immersing the electrode surface onto 100 µL of 1.375 mg mL^{-1} osmium complex solution, depositing by cyclic voltammetry (CV) scanning.

2.3. PEC measurements

PEC measurements were performed with a homemade device equipped with a UVPL-25 Lamp. Photocurrent was measured on PGSTAT302N electrochemical workstation (Metrohm-Autolab) with a three-electrode microsystem: a modified osmium conductive polymer electrode with a geometrical area of 0.03 cm^2 as the

working electrode, a gold wire as the counter electrode and a saturated Ag/AgCl electrode (PINE Instruments) as the reference electrode. All PEC measurements were carried out under 365 nm of irradiation at a constant potential of 0.31 V (vs. Ag/AgCl) in Tris-HCl (50mM, pH 8.8).

2.4. Redox mediator concentration

Varying amounts of TG (from 0 to 500 mM) were added to the samples containing CdS QDs. The samples were composed of 100 µL Tris-HCl buffer (0.05 M, pH 8.8) containing, Na_2S (1 mM) and $Cd(NO_3)_2$ (10 mM). The mixtures were incubated for 5 min at RT. The photocurrent of the resulting suspensions was recorded at 0.31 V (vs. Ag/AgCl) with 365 nm irradiation. All measurements were carried out in triplicates; the error bars represent the standard deviation of three independent measurements.

2.5. Enzimatic ALP assay.

Various amounts of TP (from 0 to 500 µM) were incubated with different amounts of ALP (from 0 to 1600 mU mL^{-1}) in Tris-HCl buffer (0.05 M, pH 8.8) containing $MgCl_2$ (1mM) at 37ºC for 90 min. After that, $Cd(NO_3)_2$ (5 µL, 100 mM) and TG (10 µL, 2M) were added to the samples (97.5 µL). The PEC response was recorded at 0.31 V (vs. Ag/AgCl) with 365 nm irradiation. All measurements were carried out in triplicates.

2.6. Detection of anti-BSA antibody

BSA (2 µg mL^{-1}) in phosphate buffered saline (PBS) was coated onto 96-well plate (NUNC, Denmark) overnight at 4 ºC. After blocking with casein buffer for 2 hours at RT, different concentrations of AB (from 0 to 200 ng mL^{-1}) in PBS were incubated for 2

hours at RT. After that, the ALP-AB (2 µg mL^{-1}) in PBS was incubated for 1 hour at RT. The plate was then incubated with 85 µL of ALP substrate (500 µM TP in 1mM $MgCl_2$ and 50 mM Tris-HCl buffer, pH 8.8) at 37 ºC for 90 min. Finally, 5 µL of 100 mM $Cd(NO_3)_2$ and 10 µL of 2 M TG were added and the photocurrent of the resulting suspension were recorded at 0.31 V (vs. Ag/AgCl) with 365 nm irradiation. All measurements were carried out in triplicates.

3. Results and discussion

3.1. Characterization and Optimization of the detection conditions.

The detection conditions were optimized for PEC system. The photocurrent is related to potential and charge (number of cycles) which provide the best relationship between signal generated by CdS QDs and osmium complex. It was found the best potential (0.31 V vs. Ag/AgCl) and osmium amount (number of cycles = 2) as it is shown in Fig. 1. The efficient anchoring of osmium polymer onto electrode surface is due to electrostatic adsorption by means of CV. Reversible waves are achieved in the osmium complex, revealing that only the central osmium atom is involved in the redox process (Fig. S1 in the Supplementary Material). These characteristic curves were also observed in other similar osmium complex (Battaglini et al., 2000; Gao et al., 2002; Virel et al., 2009).

Furthermore, the content of TG was assessed to get the best PEC response (Fig. S2 in the Supplementary Material). The PEC response achieved a plateau in the presence of 200 mM concentration, selecting for enzyme-catalyzed reaction. TG has been used as capping agent of nanoparticles (Kim et al., 2003; Uchihara et al. 2006) but also TG can remarkably improve the electronic cascade (Yang, 2006).

To confirm that under our experimental conditions for different assays, all components (osmium complex, TP, 1-thioglycerol, $MgCl_2$, Tris-HCl buffer, casein, ALP, BSA, AB, ALP-AB) were studied individually, not providing enhancement in the photocurrent higher than 2 nA (data not shown). No obvious difference in the photocurrent was observed compared to the background signal. Noted that the influence of osmium complex in the photocurrent is minimal, never being greater than 1.5 nA. The control experiments conducted without cadmium cations do not provide any significant photocurrent signal.

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- The irradiation wavelength (only 354 nm)
- The surface coverage was calculated from the under-peak areas recorded during CV measurements (data not shown). We have found that the optimum concentration of osmium complex for PEC assays was X.

3.2. PEC biosensing

ALP is widely used in bionalysis, monitoring its activity by colorimetric and fluorometric assays (Malashikhina et al., 2013; Saa et al., 2010). However, several drawbacks limit their application, making less sensitive than PEC system. We developed two photoenzymatic systems in which generation of CdS QDs was linked to the presence of ALP.

One alternative is based on the growth of CdS QDs in the presence of ALP, TP, $Cd(NO_3)_2$ and TG. The mechanism of the system is depicted in Scheme 1, according to which TP is hydrolyzed by ALP to orthophosphate (PO_4^{3-}) and H_2S. The latter reacts quickly with Cd^{2+} cations, generating CdS QDs. The reaction is supported by TG,

favouring the hidoxilation process. When generated nanoparticles are excited at 354 nm and 0.31 V (vs. Ag/AgCl) a photocurrent signal is observed.

The influence of the different amounts of TP in enhanced photocurrent is shown in Fig. 2A and B. Higher concentrations of TP higher increase in the photocurrent response. It clearly indicates that generated CdS QDs are related to hydrolysis of TP by ALP. We also investigated the influence of different ALP concentrations on the photocurrent signal using 500 µM as the optimal concentration TP as it is depicted in Fig. 3A and B. In absence of enzymatic substrate, the reaction was performed containing ALP at maximum concentration (1800 mU mL^{-1}). No significant photocurrent response was observed (Fig. 3A). In the same way, no signal was observed without ALP as it is shown in Fig 2A. From the calibration plot, it is observed a linear response up to 50 mU mL^{-1} and detection limit for ALP of 0.7 mU mL^{-1} at a signal-to-noise ratio of 3 (S/N = 3). Comparing with fluorogenic method (Saa et al., 2010), the PEC system is ten-times more sensitive.

On the other hand, we offer a second alternative to validate the applicability of our PEC enzymatic assay in ELISA assays. BSA was selected as a model of clinical heterogeneous immunoassays for the detection of different antibodies in human serum. Scheme 2 outlines the model assay for detection of AB by generation of CdS QDs. First, we have to take into account the best concentration of TP where the maximum increased photocurrent is reached (Fig. 2B). Therefore, we selected saturating 500 µM TP in ELISA to achieve the highest enzymatic reaction rate.

The immune assay consists of coating plates with BSA and casein to block unspecific binding followed by successive incubation with AB and ALP-AB. The photocurrent response corresponding to variable AB (from 0 to 200 ng mL^{-1}) is depicted in Fig. 4A. Higher adsorbed AB leads to increase in the photocurrent response. Prior to

the assay, graphite electrode modified by the osmium complex was directly inserted into the incubation solution without AB (Fig. 4A) and ALP-AB (data no shown) as control experiments. No obvious variation in photocurrent was observed compared to the background signal. Fig. 4B shows the calibration plot of different AB concentrations, showing a linear response up to 20 ng mL^{-1}. The lowest amount of AB that could be detected by this system was found to be 2 ng mL^{-1} (S/N = 3).

Chromogenic and fluorogenic assays were measured, using PNPP and TP substrates respectively with the same protocol of PEC method. Both methods are described in Supplementary Material. The fluorescence emission spectra of CdS QDs corresponding to different amounts of AB at λ_{ex} = 290 nm and the absorbance data are shown in Fig. S3 and Fig. S4, respectively. Afterwards, the lowest AB concentration detected by the fluorogenic and standard chromogenic method was found to be 4 ng mL^{-1} and 10 ng mL^{-1}, respectively. The sensitivity of PEC system was found to be 2 and 5 times higher than conventional methods. In previous work (Malashikhina et al.), the generation of CdS QDs was limited due to the possible inhibition of ALP by TP, restricting the application of generation of CdS QDs in ALP-based ELISAs. For that reason, the detection limit of fluorogenic method is not relatively true.

Our PEC systems can find broad application for measurements of different enzymes in a large number of bioanalytical assays due to time and costs are significantly reduced with PEC devices. Our future works will be concentrated on detection different tumor marker from real serum samples, supporting ABs and enzyme-ABs on electrode for achieving biosensors.

4. Conclusions

In summary, a new opportunity for the development of numerous rapid and cheaper analytical techniques using the PEC method is opened. The present work describes for the first time a device based on assembling of osmium complex onto the graphite electrode. The in situ growth of CdS QDs by an ALP enzymatic reaction leads to detection of photocurrent via nanoparticles (CdS QDs) or antibodies (ABs). This strategy was successfully applied to BSA determination, via AB. Better detection limit was achieved than classic methods. For that reason, we believe that PEC immunoanalytical may be extended for probing multiple biological interactions of the interest. Also, it may support a large number of applications in clinical diagnosis, such as biomarkers for cancer detection. Development of biosensors based on osmium complex is the next step of our investigations.

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