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A Novel Photoelectrochemical Platform for Detection of Protease

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A novel photoelectrochemical (PEC) biosensing platform was constructed for protease detection. Firstly, a short designed peptide that can be hydrolyzed selectively by a target protease was synthesized and combined on indium tin oxide (ITO) electrode surface. Then, the negatively charged PEC material, such as CdTe QDs, was subsequently immobilized on the electrode surface by means of electrostatic attraction to form a PEC biosensor, which can produce strong photocurrent under irradiation. Once the platform is incubated with target protease, the photocurrent would decrease. The decrease on photocurrent is relation to the concentration of target protease and incubating time. Based on this, the concentration of target protease can be detected selectively under the given incubating time. Take trypsin for example, a sensitive and selective biosensor for trypsin had been constructed. The biosensor possesses a linear response range of 0.3-24 μg/mL trypsin with a correlation coefficient of 0.9979 and a detection limit of 0.07 μg/mL based on a signal-to-noise ratio of 3.

Keywords: Photoelectrochemistry, Biosensor, Protease, Trypsin, CdTe QDs.

1. INTRODUCTION

As one of the most significant physiological enzymes, proteases can catalyze the hydrolytic cleavage (proteolysis) of peptide bonds at specific sites along the amino acid sequence [1], and play critical roles in maintaining regular functions of biological systems, including cell proliferation, differentiation, signaling and apoptosis [2]. Up to now, proteases have been become a major target for the development of simple and sensitive analytical tools. The commonly employed methods for the determination of proteolytic enzyme activity include colorimetry [3], fluorescence [4, 5], gel electrophoresis, mass spectrometry [6] and ELISA [7, 8]. However, many of those methods have either the need of expensive reagents, specialized equipment or show a lack oflow- sensitivity. Therefore,

There it is, therefore, aan pressing needurgent requirement for a universal assay methods that are not expensive and yet highly sensitive for detection and quantitation of protease activities.

Photoelectrochemistry (PEC) is a newly appeared yet dynamically developing analytical method. Integrating photoirradiation with electrochemical detection, photoelectrochemical sensors have the characteristics of both optical methods and electrochemical sensors [9]. Owing to the separation of excitation source and detection signal, the sensitivity of PEC is potentially higher than conventional electrochemical methods because of the reduced background signals [10] . In addition, the photoelectrochemical method is well suited for the rapid and high-throughput biological assay, and especially has the more advantage of application in protein analysis of protein [11] . For these reasons, it is particularly meaningful to couple PEC with proteases analysis for the development of a set of universal and effective detection method.

In this study, trypsin was chosen as the model proteases to complete our proposals. Trypsin, one class of protease, is the most important digestive enzyme produced by the pancreas and plays a key role in controlling pancreatic exocrine function [12] . Both quantitative deficiency and mutation of trypsin can cause pancreatic diseases such as meconium ileus and hereditary pancreatitis [13]. In this study, trypsin was chosen as the model protease to construct a newly sensitive determination method for proteases. In order to detect the activity of trypsin, we designed a short peptide containing several arginines sequence that can be hydrolyzed selectively by trypsin. Firstly, Te nanoparticles (NPs) were electrodeposited onto the ITO electrode by potentiostatic method [14]. Then the designed peptide ended with cysteine residue was connected to the Te NPs subsequently. Because of the positively charged arginines, the negatively charged PEC material, mercaptopropionic acid (MPA) functioned CdTe QDs, could be anchored on the electrode surface by virtue of electrostatic attraction to form a PEC biosensing platform. The obtained biosensing can give rise to strong photocurrent under irradiation. But once incubated with trypsin, the photocurrent would decline. The decrease of photocurrent has correlation with the concentration of trypsin under the given incubating time. The constructed biosensing platform exhibited excellent selectivity, high sensitivity and low limits of detection. Importantly, the platform can be easily adapted to any other protease by using an appropriate peptide, which makes it an ideal and highly versatile tool for the early detection of any protease-related disease.

2. EXPERIMENTAL

2.1 Materials

Trypsin was purchased from biodee Co. Ltd (Beijing, China). The designed peptide, Cys-Ala-Pro-Gly-GLy-Ala-Arg-Arg-Arg-Arg-Arg was synthesized from by apeptide Co., Ltd (Shanghai, China). It contains five positively charged arginines that can be hydrolyzed selectively by trypsin, its purity (HPLC) is more than 99%. All other reagents were of analytical grade and used as received. All aqueous solutions were prepared with triply distilled water (18 MΩ/cm), Phosphate buffer (pH 7.4, 10mM) was triply distilled water (18 MΩ/cm). Phosphate buffer (pH 7.4, 10mM) was used for

preparation of the peptide and QDs solution, washing buffer solution,. and 1mM mercaptoethanol was employed as blocking reagent. Phosphate buffer (pH 8.5, 1M) was used for trypsin incubating. 0.1M phosphate Phosphate buffer (pH 8.5, 1M) was used for trypsin incubating. Phosphate buffer solution (pH 7.4, 0.1M) containing 0.1M AA was used employed as supporting electrolyte for photocurrent measurements.

CdTe QDs functionalized with MPA were prepared according to the previous protocol [15].

2.2 Apparatus

Photoelectrochemical measurements were performed with a home-built photoelectrochemical system. A 500W Xe lamp equipped with monochromator was used as the irradiation source to produce the monochromatic illuminating light. The light intensity was about 400 μW/cm^2. Photocurrent was measured on an electrochemical workstation (CHI 660b660B) with a three electrode system: a 0.25 cm^2 modified ITO, a Pt wire and a saturated Ag/AgCl electrode used as the working, counter and reference electrode, respectively, . Scanning electron microscopic (SEM) images were recorded by a Hitachi S4800 (Hitachi, Japan). Electrochemical impedance spectroscopy (EIS) was carried out with an electrochemical workstation (CHI 660B).

2.3 Fabrication of the PEC biosensor

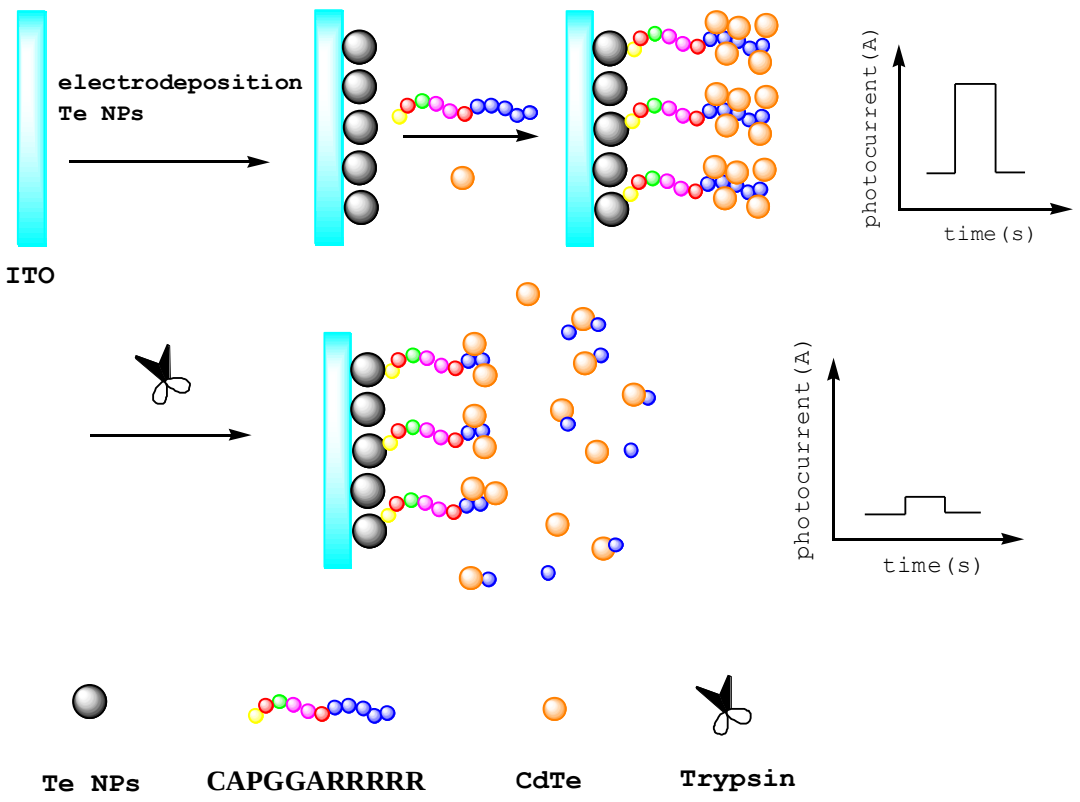

Figure 1. Schematic diagram of the biosensor construction process.

The ITO slices were was sonicated in diluted ammonia, water, ethanol and water respectively, for about 15 min each, respectively, . then The cleaned ITO slice was followed treated by a constant-potential deposition at -0.4 V for 5s to form Te NPs modified electrode [14]. Then To immobilize peptide, the electrode was incubated in 0.5 mL 0.01 M PBS (pH 7.4, 10 mM) at pH7.4 containing 0.02 mg/mL peptides for 1h at room temperature to immobilize peptide, followed by block non-specific sites with 1mM 2-Mercaptoethanol (ME), after which the as-obtained electrode was immersed into CdTe QDs solution diluted to 2.2×10^{-7} M with 10mM PBS (pH 7.4, 10 mM) for 1h beforeere Washedwashing with buffer solution and triply distilled water. The fabrication and with washing buffer solution and triply distilled water after each assembly step. The fabrication and sensing process of photoelectrochemical biosensor is presented on Fig.1.

3. RESULTS AND DISCUSSION

3.1 the The characterization of the PEC proformbiosensor

The scanning electron microscopy (SEM) images of the as-obtained modified electrodes were shown Fig. 2.

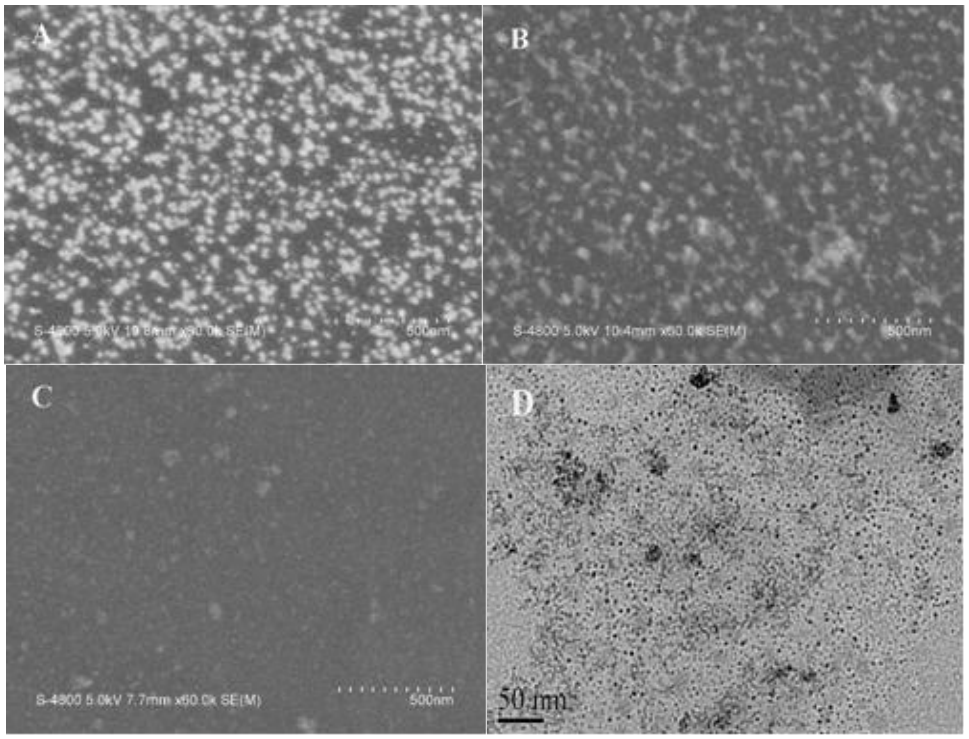

Figure 2. SEM micrographs of (A) Te NPs (B) Te NPs /peptide/CdTe and (C) Te NPs/peptide/CdTe/trypsin modified electrode; TEM image of (D) CdTe QDs.

Te NPs have been successfully deposited onto the ITO glass surface with a quasi-spherical shape whose average size was is in the range of 20–35 nm with a symmetric distribution (Fig. 2A), and

laid provided a solid foundation for anchoring peptides. The morphology of the prepared CdTe QDs displayedwere observed as shown in Fig. 2D (the average size of CdTe QDs is about 5 nm), which was consistent with the literature reports [15]. After the peptides and CdTe QDs were bound to sensor Te NPs surface(surface (Fig. 2B) ,), some flocculent substances forms emerged on the electrode surface which could be attributed toconsidered as the aggregation effect of peptides and CdTe QDs. While trypsin is introduced (Fig. 2C), it can cut off short peptides could be cut off, meanwhile, and CdTe QDs could be released from modified electrode surface. Through before and after morphology comparison of morphology before and after the introduction of trypsin, it can be seen that we can clearly see the effect of trypsin hydrolysis reaction is blindingly obvious, which and further verify the feasibility of the biosensing platform.

The construction process of the biosensor was also characterized by photoelectrochemical measurements and electrochemical impedance spectroscopy (EIS). Fig. 3A depicts the photocurrent responses of different modified electrodes. There are not found any photocurrent on ITO/Te NPs and ITO/Te NPs/peptide electrodes (Fig. 3A, curve a and b). After the anchoring of CdTe QDs on short peptides, the photocurrent increased prominently (Fig. 3A, curve c), indicating that CdTe has excellent light response. Under light irradiation, photoexcition of CdTe QDs would result in the electron transfer from VB to CB of QDs, thus yielding electron-hole pairs. Once the process happened, the e–h pairs would recombine or the charges would be transferred in order to avoid photodissolution of CdTe QDs. The holes could be scavenged by an electron donor and thus give rise to generation of stable anodic photocurrent. Na_2SO_3, triethanolamine (TEA) and ascorbic acid (AA) are usually used as efficient electron donors for CdTe. In our experiment system, the photocurrent intensities of the electrodes in 0.1M Na_2SO_3 and TEA solution were lower than that in 0.1M AA. Ascorbic acid, a widely used powerful antioxidants with the oxidation potential of −0.185V (vs.SCE) [16], can be easily oxidized by the holes (E° = 0.26V) generated by illuminated CdTe QDs [17, 18]. Therefore, AA was chosen as an electron donor in our experiments. The intensity of resulting anodic photocurrent can be controlled by the applied potentials [19] and furthermore the photocurrent was enhanced with the decrease of applied potential from -0.1 to -0.6 V, hence -0.6V was used as applied potential. The decline of photocurrent during irradiation might be due to ultrafast capture of the electron by the surface defects [20]. Fig. 3B shows the electrochemical impedance Nyquist plot of different modified electrodes using $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ as redox probe. Compared with ITO electrode (Fig. 3B, curve a), the diameter of the high frequency semicircle decreased after Te NPs was introduced to the electrode surface (Fig. 3B, curve b). This obvious change indicated that the Te NPs enhance the conductivity of the electrode interface and accelerate electron transfer of the electrochemical probe. After the electrode was conjugated with peptides (Fig. 3B, curve c), the diameter of the high frequency semicircle increased. The reason for the resistance increase was that non-conductive properties of peptides would act as an inert layer, obstructing the diffusion of the redox probe to the electrode [21, 22]. The result also showed that peptides were successfully immobilized on the electrode surface. Subsequently, CdTe QDs were combined on the Te NPs/peptides composite film and the R_{et} increased again (Fig. 3B, curve e), resulting from the further obstructive behavior of the film for the probe transport. After incubated with the corresponding trypsin (Fig. 3B, curve f), as expected, the resistance was decreased owing to the hydrolysis of the protease.

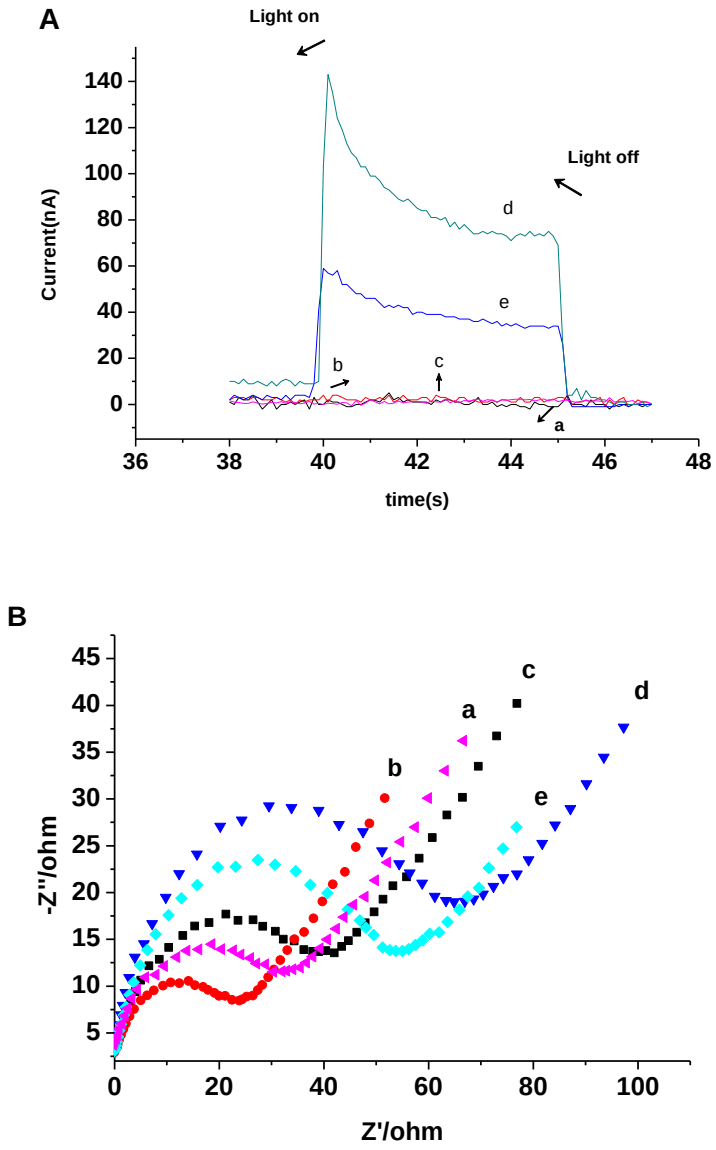

Figure 3. (A) Photocurrent response of the modified ITO electrodes: (a) ITO/Te NPs, (b) ITO/Te NPs/peptide, (c) ITO/Te NPs/peptide/QDs, (d) ITO/Te NPs/peptide/QDs/trypsin (4.8μg/mL). The photocurrent responses were measured in 0.1 M PBS (pH 7.4) containing 0.1 M AA with 400 nm light on and off and -0.6V applied potential. (B) EIS of the modified electrodes: (a) ITO, (b) ITO/Te NPs, (c) ITO/Te NPs/peptide, (d) ITO/Te NPs/peptide/ME, (e) ITO/Te NPs/peptide/ME/QDs, (f) ITO/Te NPs /peptide/ME/QDs/trypsin (4.8μg/mL). EIS were recorded from 0.01 Hz to 100 kHz with applied voltage of 5 mV in 0.1 M KCl containing 5 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ (1:1).

3.2 Photoelectrochemical response of the PEC biosensor to trypsin

In order to construct an effective PEC platform for protease detection, take trypsin as model, we investigated the effect of trypsin concentration on the photocurrent responses. Fig. 4 presents the photocurrent decrement after incubation in variable concentrations of trypsin for 30 min at 37℃. The photocurrent signal descends constantly as trypsin concentration increases up to 24 μg/mL. The inset

of Fig. 4 depicted the derived calibration curve, indicating that the percentage of the photocurrent decrement was proportional to the logarithmic value of trypsin concentration in a linear range from 0.3 μg/mL to 24 μg/mL (R^2=0.9979). The sensor was prone to saturation when the concentration of trypsin over 30μg/mL and the photocurrent did not show further decrease. The detection limit was estimated to be 0.07 μg/mL (S/N=3). The proposed PEC platform showed a wider linear range as well as lower detection limit. This demonstrates that the novel strategy would open a new insightful prospect in PEC determination of proteases and bring new opportunities for design of more novel sensing strategies and expansion of its application in different fields.

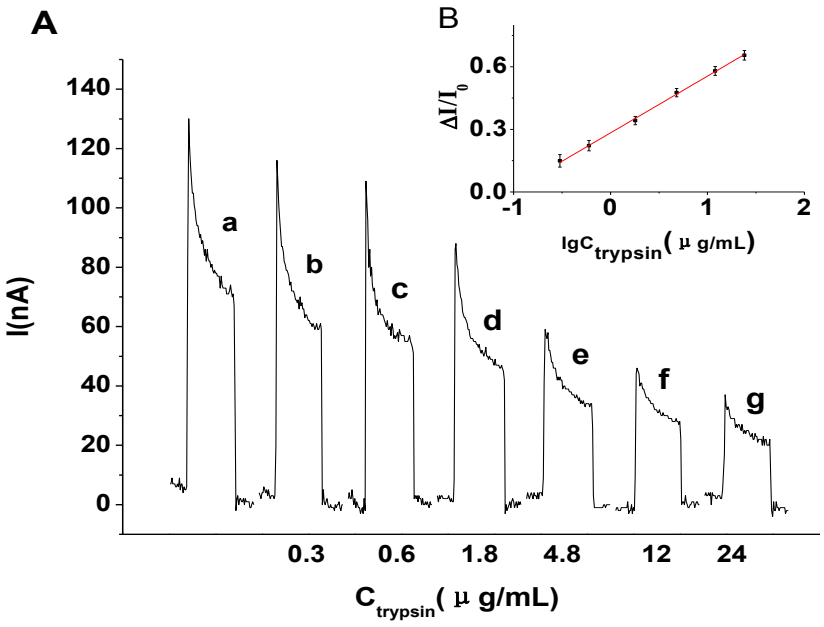

Figure 4. (A) Effect of different concentrations of trypsin on the differential photocurrent responses. (B) The corresponding calibration curve. $\Delta I = I_0 - I$, I_0 was the photocurrent of the CdTe QDs modified electrode and I was the final photocurrent of the electrode after incubation with trypsin of elevated concentrations corresponding to (a) 0, (b) 0.3, (c) 0.6, (d) 1.8, (e) 4.8, (f) 12, and (g) 24 μg/mL, respectively. The photocurrent responses were measured in 0.1 M PBS containing 0.1 M AA with an applied potential of -0.6 V and light wavelength of 400 nm.

As is well known, the trypsin inhibitors can inhibit the cleavage of arginines by inactivating trypsin. Accordingly, photocurrent would not decline when the corresponding inhibitors of trypsin are present in the solution. A Bowman–Birk inhibitor (BBI) from soybeans was selected as an example of the inhibitor to further illustrate the mechanism. The corresponding IC_{50} value (the concentration of the inhibitor that leads to 50% inhibition of the enzyme activity) of BBI toward trypsin was estimated to be 0.07 μg/mL (Fig. 5). This IC_{50} value of BBI is lower than previous report [23]. Therefore, all these results obviously indicate that the protease assay method is easily accessible and can be utilized not only for proteases activity assay but also for the corresponding inhibitor-screening.

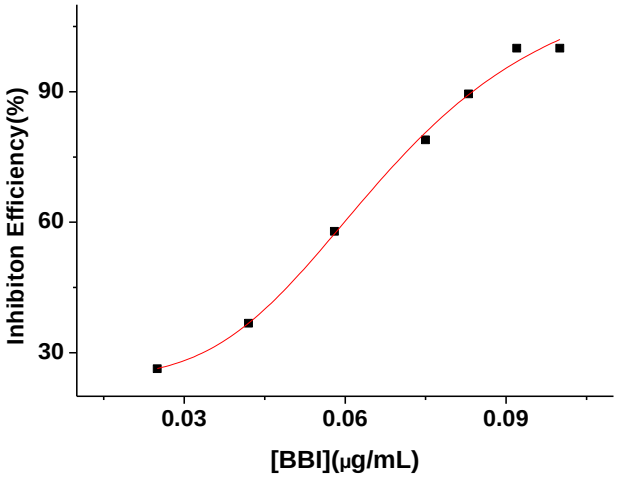

Figure 5. The plot of inhibiton efficiency vs. the concentration of BBI.

3.3 Stability, Specificity, Reproducibility, and Application

The selectivity of the proposed protocol was investigated by measuring the photocurrent response of 30 μg/mL thrombin, lysozyme (Fig. 6). No obvious photocurrent drop could be observed. It also clearly demonstrates that the observed photocurrent decrease is assigned to the specificity of the designed platform to trypsin. The relative standard derivation was obtained by measuring 4.8 μg/mL trypsin with five electrodes prepared independently under identical experimental conditions. The results are shown in Table 1, and indicated satisfactory reproducibility of the proposed protocol. The stability of the as-obtained electrode was also evaluated by performing 20 times repeated measurements. The photocurrent was very stable over time without any noticeable decrease. When the modified electrode was stored in dark under nitrogen environment one week at 4 °C, no apparent change in photocurrent response was found, thanks to its good storage stability.

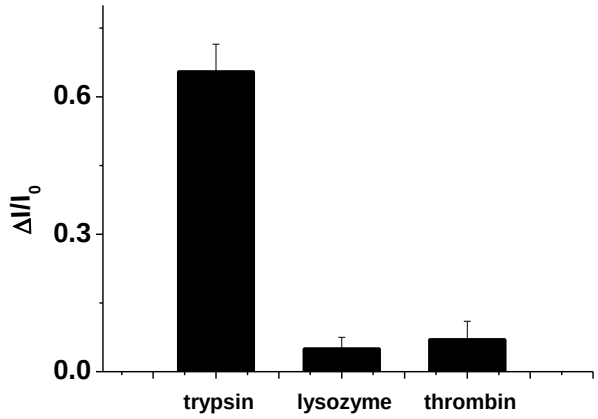

Figure 6. Selectivity of the proposed biosensor to trypsin by comparing it to the interfering enzyme at the 30 μg/mL level: lysozyme and thrombin. The error bars showed the standard deviation of four replicate determinations.

Table 1. Assay results of trypsin (4.8 μg/mL) determinations with five electrodes.

Electrode number	Current(nA)	RSD (%)
1	34	
2	35	
3	36	5.3
4	37	
5	39	

The feasibility of this strategy for clinical application was investigated on several real samples of human serum. Serum samples were diluted to a specific concentration with 10 mM phosphate buffer solution (pH 7.4). The acceptable recoveries of 1 μg/mL and 5 μg/mL of trypsin are 113% and 112%, respectively, indicating that it is feasible to utilize the developed photoelectrochemical biosensor for practical applications.

4. CONCLUSION

In conclusion, we have taken trypsin as a model protease for the development of PEC protease biosensor with satisfactory performance. The biosensoring platform with increased specificity by well-designed the sequence of the peptide substrate is capable of simultaneous/multiplex detecting other proteases or kinases. What is more, we believe that there is much broader prospects for creating photoelectrochemically addressable multifunctional architectures by combining biological networks and well-established methods.

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