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A novel protein binding strategy for energy-transfer-based photoelectrochemical detection of enzymatic activity of botulinum neurotoxin A

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ABSTRACT

In this work, we propose a novel energy-transfer-based photoelectrochemical (PEC) platform for probing of protein-protein interaction, which associates intimately with zinc-dependent cleavage and substrate specificities in the enzymatic activities of botulinum neurotoxin (BoNT). Specifically, by using substrate protein SNAP-25 as the energy-transfer nanoprobe, an exciton-plasmon interaction (EPI) based strategy between CdS quantum dots (QDs) and Au nanoparticles (NPs) in a PEC system is constructed with the photocurrent declining. Interestingly, the EPI effect is then interrupted by the target botulinum neurotoxin serotype A light chain (BoNT-LCA) special cleavage of the probe SNAP-25, leading to the photocurrent recovery. Therefore, the enzymatic activity of BoNT-LCA could be sensitively detected with a detection limit of 1 pg/mL. Unlike conventional DNA-programable assembly, a protein probe is used to bridge the excitons and plasmons in this work, which provides a new route for the investigation of the EPI-based bioassay.

1. Introduction

Botulinum neurotoxin (BoNT) is a protein produced by bacterium *Clostridium botulinum*, which is considered as the most toxic substance for human beings [1]. It has been found that four serotypes of BoNT labelled as A, B, E, and F may result in human diseases. Normally, BoNT serotype A (BoNT/A), is the most important cause of the human botulism [2]. BoNT/A consists of a light chain (LC) and a heavy chain (HC). Its neurotoxicity is mainly due to BoNT/A light chain (BoNT-LCA) specific cleavage of synaptosome-associated protein of 25 KDa (SNAP-25), at the site between 197 and 198 [3]. As a main target for toxin detection, BoNT-LCA has gained more and more attention. Thus, there is an urgent demand for the rapid and sensitive detection of enzymatic activity of BoNT-LCA for pre-diagnosis and pre-treatment. Although the established mouse bioassay method is commonly used for the detection of BoNT-LCA with high sensitivity (LOD of 7–15 pg/mL), it suffers a few drawbacks such as laborious, expensive and time-consuming processes [4]. Alternative methods, including enzyme-linked immunosorbent assays (ELISA) [5] and immunological strategies [6,7], seem to realize good performance with faster and more sensitive detection, in which the LOD of 0.1–100 pg/mL can be obtained. However, it should be noted that these methods are susceptible to the equipment and assay performance, and they just detect the presence of toxin, while not the activity of the toxin. Therefore, accurate, rapid, low-cost, sensitive and specific detection of enzymatic activity of BoNT-LCA is still of critical urgency.

Recently, photoelectrochemical (PEC) detection, a newly emerged but rapid developing analysis technology, has attracted considerable scientific interest in bioanalysis due to its rapid response, low cost, and high sensitivity [8–10]. Many researchers have devoted to seek delicate and effective sensing strategy to promote advanced PEC sensing [11–13]. Among them, exciton–plasmon interaction (EPI) between the quantum dots (QDs) and metal nanoparticles (NPs), a unique phenomenon of interparticle energy transfer, is promising with the advantages of simplicity and direct response [14,15]. Numerous efforts have been devoted to utilization of this effect in PEC system, and

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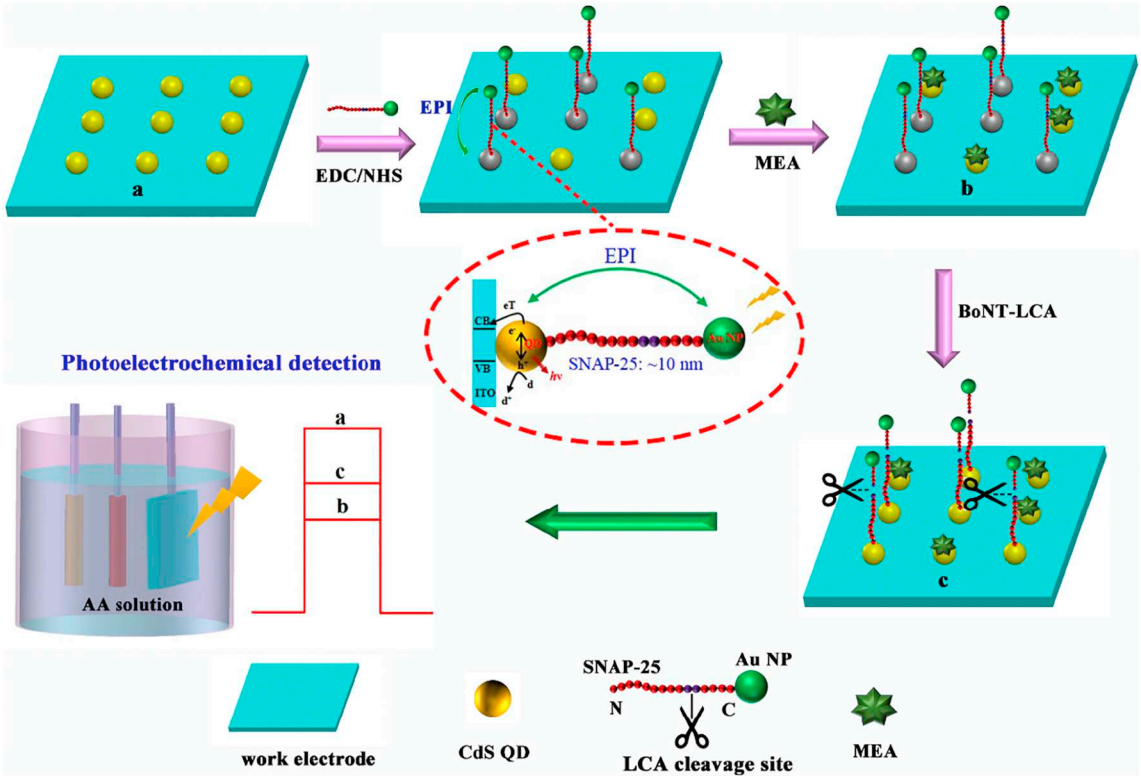

Scheme 1. Schematic diagram of PEC platform based on EPI effect for sensitive and specific detection of enzymatic activity of BoNT-LCA.

unfavorable signal disturbances between the photoactivated sensing surface and ambient environment could be efficiently avoided, thus the sensitivity of PEC biosensor is improved [16]. As an analytical basis for PEC detection, the EPI-effect based strategy has fueled many studies in sensing applications, such as heavy metal ions [15,17], DNA [18,19], thrombin [20,21], protein [22,23], and so on. Combining with the EPI effect between NPs and QDs, a novel and sensitive PEC strategy is expected to be constructed for BoNT detection.

Normally, the EPI-based PEC detection strategy relies on DNA binding events. Particular interest here is to explore new probes for advanced EPI-based bioanalysis of protein. Furthermore, the enzymatic activity of BoNT-LCA would induce a cleavage of the substrate protein SNAP-25, which is also very interesting to be utilized for the design of new PEC bioanalysis. Thus, in this work, we propose a novel protein binding strategy for EPI-based PEC detection of enzymatic activity of BoNT-LCA. Specifically, as shown in Scheme 1, under 420-nm light illumination, a stable anodic photocurrent is obtained due to the electron transfer from CdS QDs to ITO electrode, as shown in Step a. After the immobilization of the energy-transfer nanoprobes by using substrate protein SNAP-25 as the unique distance mediators, the labelled Au NPs are placed in close proximity with CdS QDs, inducing an EPI phenomenon of interparticle energy transfer and thus the photocurrent declining, as depicted in Step b. During the exposure to the target BoNT-LCA, the nanoprobe of SNAP-25 is specifically cut into two fragments at the cleavage sites by BoNT-LCA, resulting in the release of Au NPs. Interestingly, the EPI effect between CdS QDs and Au NPs is then interrupted, leading to the photocurrent recovery, as shown in Step c. By measuring the photocurrent signal change, the enzymatic activity of BoNT-LCA is successfully detected with a detection limit of 1 pg/mL. This platform provides a new route for the investigation of EPI-based biosensing.

2. Materials and methods

2.1. Materials and reagents

Indium tin oxide (ITO) was purchased from China Southern Glass Holding Co., LTD, China. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-Hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich. Ascorbic acid (AA) and monoethanolamine (MEA) were purchased from Aladdin (China). All other reagents were of analytical grade and were used as received. The washing buffer was 100 mM phosphate-buffered saline (PBS, pH 7.4).

2.2. Labelling of substrate protein SNAP-25 with Au NPs

Au NPs with average diameter 5 ± 1 nm was synthesized referred to the previous report [24]. Briefly, TCEP was firstly used to reduce the disulfide bonds of protein SNAP-25. Then, 100 μL of 0.8 mg/mL SNAP-25 was mixed with 1 mL of Au NPs colloid solution (6×10^{-8} M) containing 0.1 M NaCl and the mixed solution was kept shaking gently for 2 h at room temperature. Finally, the mixed solution was centrifuged for 30 min at 15000 rpm to collect the bioconjugates. The supernatant was removed, the sediment was redispersed in PBS and recentrifuged to wash for three times.

2.3. Immobilization of SNAP-25 and detection of BoNT-LCA activity

The CdS QDs modified ITO electrode was prepared using the previous technology [24]. Then the immobilization of SNAP-25 probe to the CdS QDs/ITO electrodes was realized via the classic coupling reactions between the NH_2 groups on SNAP-25 and COOH groups on CdS QDs. Firstly, the prepared CdS QDs/ITO electrodes were dipped in a solution containing 20 mg/mL EDC and 10 mg/mL NHS for 60 min at

room temperature. After washing with PBS solution carefully, 25 μL of SNAP-25 (1 mg/mL, Au NPs-labelled or label-free) was dripped onto the surface of the electrodes and incubated at room temperature for 2 h. After that, the electrodes were washed with PBS solution to remove the non-immobilized SNAP-25. The excess COOH groups of CdS QDs on the electrodes were blocked with 1 mM MEA at 4 °C for 2 h. Finally, the electrodes were rinsed with PBS solution carefully.

For the detection of BoNT-LCA, 25 μL of the BoNT-LCA with different concentrations were dropped on the electrodes (SNAP-25 immobilized) for 20 min incubation at 37 °C. Then, the electrodes were washed carefully for three times with PBS solution.

2.4. Electrical measurements

The PBS solution containing 0.1 M AA was used as electrolyte. A 500 W Xe lamp equipped with monochromator was served as the irradiation source. Photocurrent was measured by an electrochemical workstation (CHI 660E, China) with a three-electrode system (a modified ITO electrode as the working electrode, a Pt slice as the counter electrode, and a saturated Ag/AgCl as the reference electrode) at a constant potential of 0 V (vs saturated Ag/AgCl).

3. Results and discussion

The typical photocurrent responses for each step were recorded in Fig. 1a. At first, CdS QDs synthesized with uniform diameter (5 ± 1 nm) were modified onto the ITO work electrode [24]. A time-based photocurrent response is obtained and the fast rise reveals the efficient photoexcited carriers (electron-hole pairs) in CdS QDs and the superior electron transfer from CdS QDs to ITO electrode, as shown in curve a (Fig. 1a).

After SNAP-25/Au NPs is grafted to CdS QDs, the photocurrent decreases significantly as depicted in curve b (Fig. 1a), when compared to the initial intensity in curve a (Fig. 1a). This decrease could be mostly attributed to the photocurrent declining by the EPI effect between CdS QDs and Au NPs [24]. During the charge transfer between CdS QDs and ITO electrode, the recombination of electron-hole pairs occurs at the same time. By using the substrate SNAP-25 as the distance controller, Au NPs are in close proximity with CdS QDs. The spontaneous emission of CdS QDs by the radiative decay excites the surface plasmon resonance (SPR) of Au NPs, which enhances the radiative decay rate conversely and thus the recombination of electron-hole pairs [25,26]. Along with the SPR effect, the exciton energy transfer (EET) between Au NPs and CdS QDs would cause a non-radiative decay, which induces another route for the recombination of electron-hole pairs [27,28]. Therefore, the photocurrent would be declined.

For efficient excitation of the EPI effect, two factors are critical. First, an adequate spectral overlap between the absorption spectra of Au NPs and the emission spectra of CdS QDs is needed [19], which could be verified in our previous report [24]. Second, the appropriate interparticle distance between Au NPs and CdS QDs is essential. It has been reported that the interparticle distance of 8–12 nm is most efficient for EPI effect in PEC bioanalysis [18,29]. As the length of SNAP-25 constructed with residues of 141 to 206 is about 10 nm [29]. It is beneficial for the efficient excitation of EPI effect in our system.

After that, the novel EPI-based PEC system was used for the detection of enzymatic activity of BoNT-LCA. The platform was firstly exposed to the target BoNT-LCA with a concentration of 10^{-6} g/mL. We can find that the photocurrent recovers partly, as shown in curve c (Fig. 1a). The recovery of photocurrent is mainly due to the specific cleavage of substrate SNAP-25 by BoNT-LCA. During the exposure to

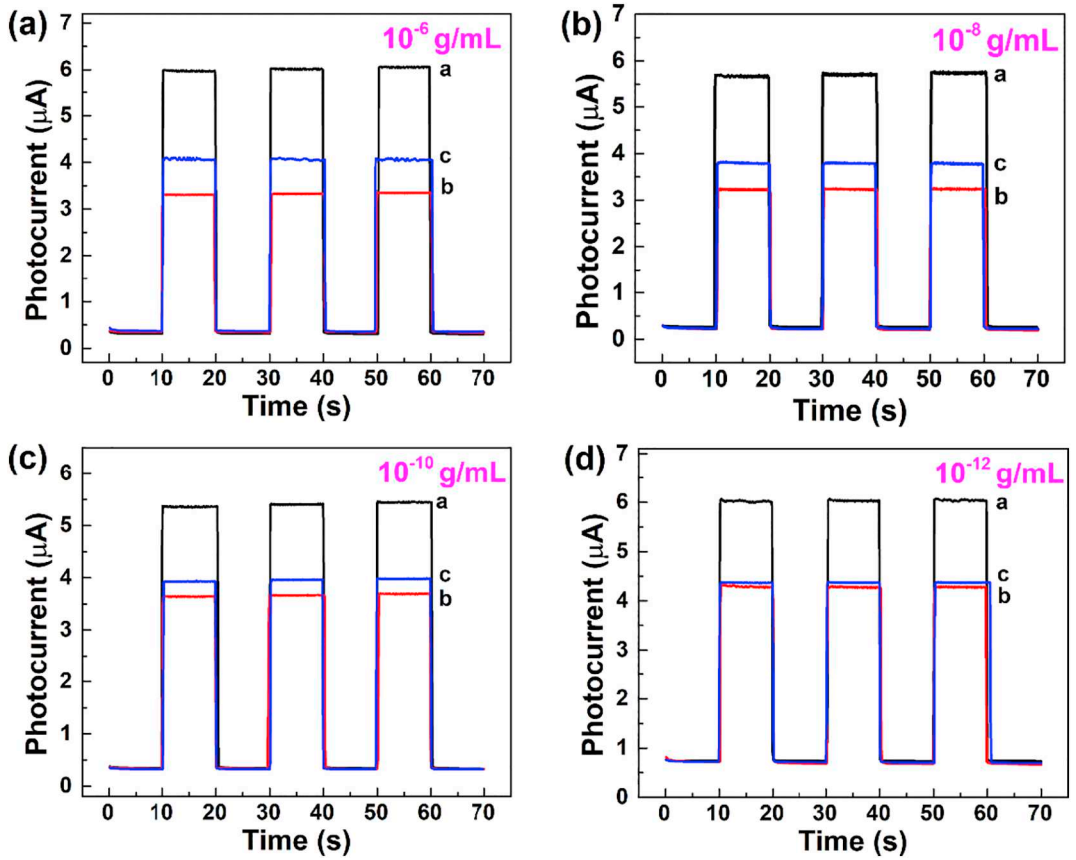

Fig. 1. Photocurrent responses measured in 0.1 M AA solution: (curve a) ITO electrode modified with CdS QDs, (curve b) grafted with SNAP-25/Au NPs with EPI effect, (curve c) exposed to the target BoNT-LCA. The irradiation light wavelength is 420 nm, intensity is 0.2 mW/cm^2. The concentration of BoNT-LCA is: (a) 10^{-6} g/mL, (b) 10^{-8} g/mL, (c) 10^{-10} g/mL, (d) 10^{-12} g/mL.

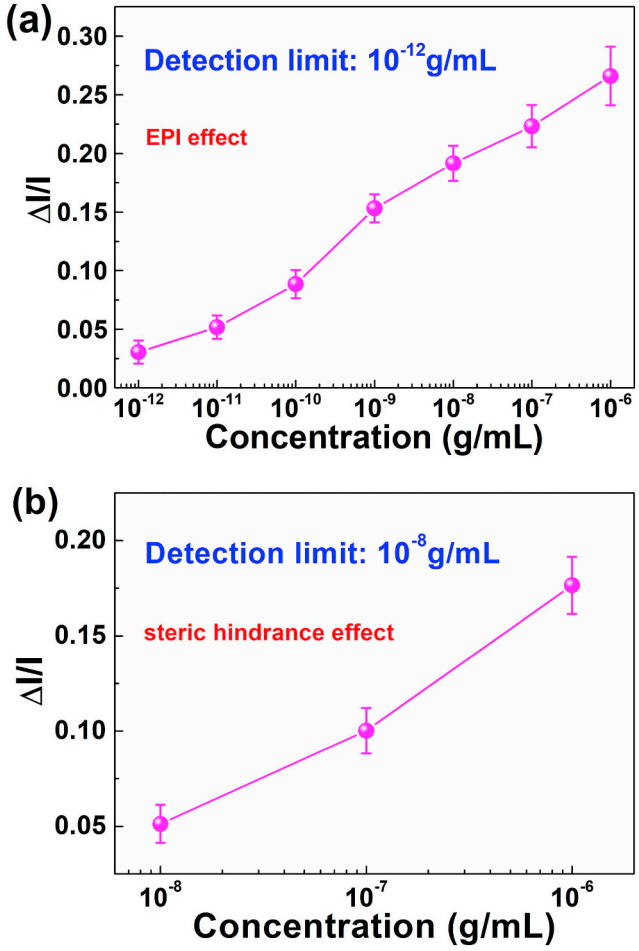

Fig. 2. The percentage of photocurrent change ($\Delta I/I = (I_c - I_b)/I_b$) induced during the detection of BoNT-LCA with different concentrations in 0.1 M AA solution: (a) mainly based on the EPI effect, (b) only based on the steric hindrance effect.

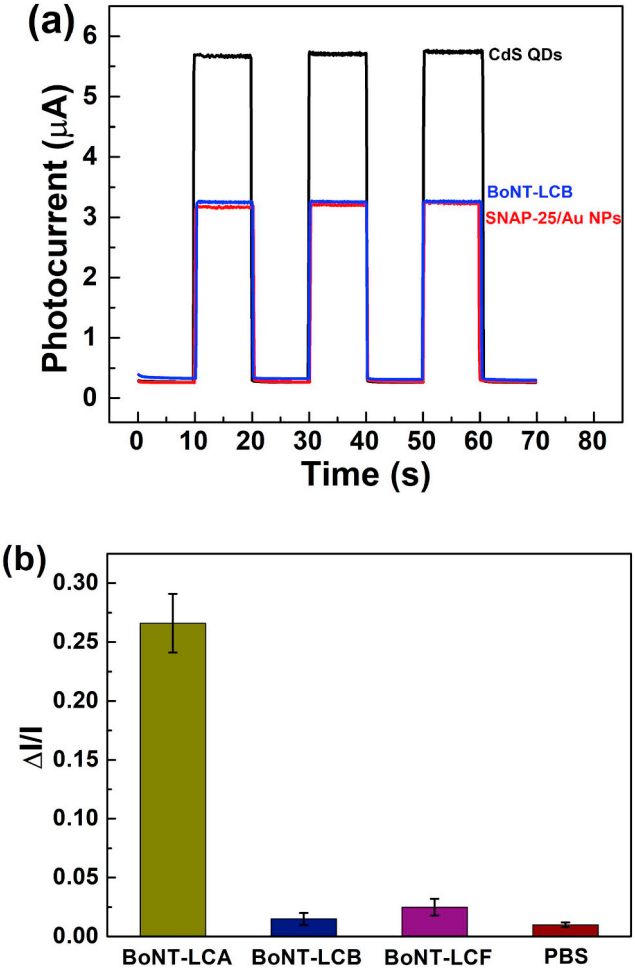

Fig. 3. (a) Photocurrent responses measured in 0.1 M AA solution: (black) ITO electrode modified with CdS QDs, (red) grafted with SNAP-25/Au NPs with EPI effect, (blue) exposed to BoNT-LCB with a concentration of 10^{-6} g/mL. (b) The selectivity of the PEC-based biosensor, which shows $\Delta I/I$ for the detection of BoNT-LCB (10^{-6} g/mL), BoNT-LCF (10^{-6} g/mL), and pure PBS solution (0.1 M, pH 7.4), compared to BoNT-LCA (10^{-6} g/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

BoNT-LCA, the nanoprobe of SNAP-25/Au NPs could be cleaved into two fragments and then Au NPs would be released, leading to the interruption of EPI effect and thus the recovery of photocurrent. Therefore, the enzymatic activity of BoNT-LCA could be detected significantly. The zinc-dependent cleavage and substrate specificities in the enzymatic activities of BoNT provides a new process with a photocurrent recovery, which is different from other PEC sensors with only photocurrent declining based on the EPI effect [18,19].

Using the same method, the detection limit of BoNT-LCA was also studied by varying its concentration from 1×10^{-12} to 1×10^{-6} g/mL, while the concentration of SNAP-25/Au NPs was fixed at 0.8 mg/mL, as shown in Fig. 2. Under each condition, at least three samples were tested. Here we use the percentage of photocurrent change ($\Delta I/I$) to represent the signal response caused by different concentrations of BoNT-LCA, which is given by the following equation:

$$\frac{\Delta I}{I} = \frac{I_c - I_b}{I_b} \quad (1)$$

where I_b and I_c correspond to the photocurrent responses after the immobilization of SNAP-25/Au NPs and the detection of BoNT-LCA, respectively. Fig. 2a shows the dependence of $\Delta I/I$ on the concentration of BoNT-LCA. It can be found that $\Delta I/I$ decreases from 26.6% to 3.1% when the concentration of BoNT-LCA decreases from 1×10^{-6} to 1×10^{-12} g/mL. The control experiment measured by pure PBS solution shows that the baseline noise of $\Delta I/I$ is only 1%, so more than 3% of the current signal response can be regarded as the real signal caused by the analyte. Hence, the detection limit of BoNT-LCA by our PEC-based biosensor can be considered as 1×10^{-12} g/mL, which is comparable to or even better than those of some other methods (LOD in pg/mL level).

In fact, the substrate protein and toxin protein have a certain volume which will impede the electron transfer in PEC system because of the steric hindrance effect. This suggests that the steric hindrance effect may also influence on the sensing performance during the detection of BoNT-LCA. Nevertheless, when the nanoprobes of substrate SNAP-25 without Au NPs labelling were used for comparison, the detection limit of the sensor is only 1×10^{-8} g/mL (Fig. 2b), much worse than that of EPI-based biosensor. Hence, we can conclude that the steric hindrance effect exhibits very little influence on the sensing performance of EPI-based biosensors. Therefore, the sensing mechanism for the detection of BoNT-LCA by PEC-based biosensor is mainly attributed to the EPI effect between CdS QDs and Au NPs.

To investigate the specificity of PEC-based biosensor, the other two serotypes of BoNT, BoNT-LCB and BoNT-LCF with concentration of 1×10^{-6} g/mL, and the background solution (0.1 M PBS, pH 7.4) were tested as control experiments following the same measuring procedure. As shown in Fig. 3a, no obvious photocurrent change occurs due to the non-specific effect of BoNT-LCB to SNAP-25. Similar results can be

observed for BoNT-LCF and PBS solution. Compared with 26.6% of photocurrent change for BoNT-LCA (1×10^{-6} g/mL), the signal response changes of the three non-specific analytes are all below 2.5%, as shown in Fig. 3b, which indicates the excellent specificity of EPI-based biosensors.

4. Conclusions

In summary, this study presents a novel protein binding event for EPI-based PEC detection of enzymatic activity of BoNT-LCA. By using substrate protein SNAP-25 as a unique distance controller, the labelled Au NPs are placed in close proximity with CdS QDs, inducing the photocurrent declining due to the EPI effect between the two particles. After specific cleavage of SNAP-25 by the target BoNT-LCA, the EPI phenomenon is interrupted with a photocurrent recovery. By measuring the photocurrent change, the enzymatic activity of BoNT-LCA could be detected in a simple, rapid, specific and sensitive way with a detection limit of 1 pg/mL. This work provides a feasible method for BoNT detection with high sensitivity and selectivity. More significantly, by using protein probe as the bridge, this strategy provides a new rout for energy-transfer based photoelectrochemical bioanalysis.

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