RESEARCH PAPER

Facile incorporation of DNA-templated quantum dots for sensitive electrochemical detection of the oral cancer biomarker interleukin-8

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Abstract

Recent studies reveal a great value of interleukin-8 (IL-8), a pro-inflammatory cytokine, as a potent biomarker for early diagnosis of oral cancer. Herein, a new electrochemical method is proposed to detect IL-8 by facilely incorporating DNA-templated quantum dots (QDs). In principle, target IL-8 is first treated with the reducing agent tris(2-carboxyethyl)phosphine (TCEP) to yield active thiols and then captured by antibody-functionalized magnetic beads (MBs). Thereafter, via the Michael addition reaction between the active thiol and maleimide group, a maleimide-modified DNA probe is linked to the surface of MBs, which can initiate a process of rolling circle amplification. In this way, long-range DNA strands are generated on the MB surface, subsequently recruiting DNA-templated CdTe/CdS QDs (DNA-QDs) to act as electrochemical reporters. By tracing the responses of DNA-QDs, the method allows IL-8 detection in a linear range from 5 to 5000 fg/mL with a detection limit of 3.36 fg/mL. The selectivity, reproducibility, and applicability in complex serum samples are also demonstrated to be favorable, indicating that the method may have a great potential in the future. More importantly, the use of TCEP treatment in the method not only provides a facile way to incorporate DNA-QDs, avoiding the complicated and time-consuming preparation process of antibody-DNA conjugates or functional nanomaterials; but also makes the method capable of being extended to detect other protein biomarkers in view of widespread presence of disulfides, which may hold a broad potential to facilitate efficient biosensing designs.

Keywords Oral cancer · Interleukin-8 · DNA-templated CdTe/CdS quantum dots · Tris(2-carboxyethyl)phosphine · Michael addition reaction

Introduction

Oral cancer is one of the most common cancers worldwide and its occurrence has increased in most counties [1]. The survival rate of oral cancer patients is around 60~80% when they are diagnosed at early stage, but this value sharply decreases if they reach the advanced stage [2]. So, early diagnosis is of great value for the successful treatment of oral cancer.

Detection of a certain biomarker in body fluids (e.g., blood and saliva) is considered to be a useful and non-invasive way for early diagnosis of oral cancer [3, 4]. Interleukin-8 (IL-8), a pro-inflammatory cytokine that is reported to be detected at higher concentrations in oral cancer patients, is one of representative oral cancer biomarkers [5]. Up to now, many efforts have been made to detect IL-8 [6–9]. Among them, electrochemical methods have attracted considerable attention in view of their fast response, low cost, and portability [10]. For example, Verma et al. functionalized indium tin oxide electrode with antibodies and gold nanoparticle/reduced graphene oxide composite and applied it as an immunosensing platform for IL-8 detection [6]. Aydın et al. proposed an electrochemical method to detect IL-8 by modification of electrode surface with conductive carbon black and star PGMA polymer composite material [8]. Although these methods display acceptable sensitivity and selectivity, they are typically hampered by complicated nanomaterial synthesis and electrode modification processes. In this sense,

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developing new IL-8 electrochemical detection methods with high sensitivity, selectivity, and simplicity is highly desired.

In past few years, much process has been made with respect to DNA-templated nanomaterials, such as DNA-templated silver nanoclusters, copper nanoparticles, and quantum dots (QDs) [11]. Accumulating studies reveal that these nanomaterials can be prepared in a fast, facile, and straightforward route and easily coupled with DNA-based molecular recognition or signal amplification owing to multiple functions of DNA molecules [12–15]. More interestingly, DNA-templated nanomaterials can serve as electrochemical signal reporters based on their elemental compositions, which avoids complicated electrode modification process [16–18]. Hence, incorporating DNA-templated nanomaterials with electrochemical technique may provide a promising solution to construct a sensitive, selective, and simple IL-8 detection method.

How to incorporate DNA-templated nanomaterials into electrochemical method for IL-8 detection? A routine strategy is to prepare antibody/DNA-modified nanomaterials or antibody-DNA conjugates through certain chemical means, such as hydrazone chemistry, the Diels-Alder reaction, and biotin-avidin recognition [19–21]. However, this strategy is usually high-cost and time-consuming and often results in unideal conjugating efficiency. A recent advance in cell engineering may provide us with a superior choice. It was found that treatment of cells with the reducing agent tris(2-carboxyethyl)phosphine (TCEP) reduced disulfides of cell surface proteins to active thiols, which could further coupled with thiol-reactive molecules for cell coating and labeling and had no effect on immunoreaction activity [22–24]. Inspired by these works and considering the presence of disulfides within IL-8 molecule, we anticipate that we can realize facile incorporation of DNA-templated nanomaterials for IL-8 electrochemical detection by making use of TCEP treatment. Specifically, TCEP treatment is employed to yield active thiols, and antibody-functionalized magnetic beads (MBs) are employed to recognize and capture IL-8, while a thiol-reactive maleimide-modified DNA probe is employed to incorporate a typical kind of DNA-templated nanomaterials (CdTe/CdS QDs) to serve as electrochemical reporters. To achieve high sensitivity, rolling circle amplification (RCA) is also integrated into the detection method. Experimental results demonstrate the feasibility, sensitivity, and selectivity of the method, indicating a great potential for non-invasive diagnosis of oral cancer.

Materials and methods

Materials and reagents

Recombinant human IL-8, anti-IL-8 antibody, and interleukin-6 (IL-6) were purchased from Abcam (Shanghai,

China). Tellurium powder, sodium borohydride (NaBH4), cadmium chloride ($CdCl_2$), glutathione (GSH), thiourea, TCEP, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Shanghai, China). Phi29 DNA polymerase, T4 DNA ligase, and deoxynucleotide set (dNTPs) were purchased from New England Biolabs (Beijing, China). Carboxylated MBs and Ellman's reagent were ordered from Invitrogen (Shanghai, China). Carbohydrate antigen 125 (CA-125), cytokeratin 19 fragment antigen 21 (CYFRA-21), and lactoperoxidase (LPO) were obtained from LMAI Bio (Shanghai, China). DNA probes (Mal-Primer, Padlock, Template, and so on) were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Their sequences are listed in Table S1 in the Electronic Supplementary Material (ESM). Other chemicals were of analytical grade and used as received. Buffer solutions were prepared with double-distilled water (18 MΩ cm), which was from a Direct-8 Millipore purification system.

Preparation of DNA-templated CdTe/CdS QDs

DNA-templated CdTe/CdS QDs (DNA-QDs) were prepared according to the method reported previously [25]. Firstly, 20 mg tellurium powder and 12.5 mg $NaBH_4$ were dissolved in 0.5 mL double-distilled water and incubated at 60 °C for 40 min to make up sodium hydrogen telluride (NaHTe) solution. In the meantime, 1.25 mM $CdCl_2$ and 1.05 mM GSH were mixed to make up $CdCl_2$-GSH stock solution, the pH of which was adjusted to 9.0 with 1.0 M NaOH. After that, 1 μL of the freshly made NaHTe solution and 1 μL of 100 μM Template (or Mal-Template) were added into 500 μL of $CdCl_2$-GSH stock solution and the mixture was heated to 100 °C for 5 min. After being cooled to room temperature, the mixture was purified with an ultrafiltration centrifuge tube (MWCO 30 K) at 12,500 rpm for 2 min and then resuspended in 500 μL of NaOH (pH 10.5). Thereafter, 7 μL of $CdCl_2$-GSH-thiourea stock solution (30 mM $CdCl_2$, 75 mM GSH, 25 mM thiourea, pH 11.0) was added into the resuspension and heated to 95 °C for 30 min. Then, other aliquots of $CdCl_2$-GSH-thiourea stock solution were added into the mixture. After an additional reaction for 30 min, the resulting solution was gradually cooled to room temperature and the obtained DNA-QDs were kept at 4 °C for further use.

TCEP treatment

A typical experiment for TCEP treatment was performed as follows. Firstly, 100 μL of IL-8-contained sample solution was treated with 400 μL of 1 mM TCEP at 37 °C for 20 min. Then, the resulting mixture was centrifuged in an ultrafiltration centrifuge tube (MWCO 30 K) at 14,000 rpm

for 10 min to remove TCEP. Afterward, the amount of active thiols on IL-8 was determined by using Ellman's reagent.

Electrochemical detection of IL-8 based on facile incorporation of DNA-QDs

Prior to detection, carboxylated MBs were first activated with 0.22 M EDC and NHS for 30 min and then incubated with 50 μL of anti-IL-8 antibody under gentle rotation for 60 min to produce antibody-functionalized MBs (anti-IL-8@MBs). The remaining active sites on the MBs were blocked by incubating with 25 μL of 1 M ethanolamine for 30 min. Thereafter, 50 μL of TCEP-treated IL-8-contained sample solution was mixed with 250 μL of as-prepared anti-IL-8@MBs at 37 °C for 1 h to progress the specific immune-recognition. Afterward, the MBs were separated and further incubated with 100 μL of 1 μM Mal-Primer@Padlock that was prepared by incubating maleimide-modified DNA probe (Mal-Primer) and Padlock at 90 °C for 5 min. In this way, Mal-Primer was linked to the surface of MBs via the Michael addition reaction and subsequently used to trigger the process of RCA. In details, the Mal-Primer@Padlock was reacted with 10 U T4 DNA ligase in ligation buffer (50 mM Tris-HCl with 5 mM $MgCl_2$, 10 mM DTT, and 0.1 mM ATP, pH 7.5) to form circular DNA. After that, RCA was performed by exposing the MBs to 100 μL of amplification mixture containing 20 U Phi29 DNA polymerase, 0.5 mM dNTPs, and RCA buffer (50 mM Tris-HCl with 10 mM $MgCl_2$, 4 mM DTT, and 10 mM $(NH_4)_2SO_4$, pH 7.5). After 1-h duration, the resulting MBs were further incubated with 100 μL of DNA-QDs to create DNA-QDs@MBs, which were used for the following electrochemical measurements.

Electrochemical measurements

To perform electrochemical readout of DNA-QDs, 50 μL of DNA-QDs@MBs was incubated with 250 μL of 0.5 M HNO_3 at room temperature for 1 h to release Cd^{2+}. The resulting solution was then mixed with 4.7 mL of 0.2 M HAc-NaAc buffer (pH 5.2) and electrochemically measured using anodic stripping voltammetry (ASV) on a CHI660D workstation (CH Instruments). For the measurements, a mercury film–modified glassy carbon electrode was used as the working electrode, while a saturated calomel electrode (SCE) and a platinum electrode were used as the reference and counter electrode, respectively. The procedure for ASV measurements involved a 6-min electrodeposition of Cd^{2+} at − 1.2 V, followed by square wave voltammetric (SWV) scanning conducted from − 1.0 to − 0.5 V with a 15-Hz frequency and 50-mV amplitude. Before measurements, all test solutions were bubbled with high-purity nitrogen for 15 min to yield an oxygen-free environment.

Results and discussion

### Principle of the detection method

Figure 1 may illustrate the principle of the electrochemical method for IL-8 detection. As depicted, anti-IL-8@MBs are first prepared via the condensation reaction of the carboxyl group and amino group, while samples with or without target IL-8 are treated with TCEP to yield active thiols (SH). After specific immune-recognition and magnetic separation, TCEP-treated IL-8 are enriched onto MBs, which are easily coupled with maleimide-modified DNA-QDs (Mal-DNA-QDs) through the Michael addition reaction between the active thiol and maleimide group. In this state, DNA-QDs are linked to the surface of MBs and serve as electrochemical reporters. Because the incorporation of DNA-QDs is only triggered by the presence of TCEP-treated IL-8, detection of IL-8 can then be achieved by tracing electrochemical responses of DNA-QDs.

Validation of feasibility of the detection method

Deemed to be the critical step of the detection method, TCEP treatment–induced formation of active thiols has first been confirmed. As well known, Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid, DTNB) is the favorite reagent for measurement of active thiols within proteins because it can react with them to produce highly yellow-colored 5-thiol-2-nitrobenzoic acid that has a typical absorption peak at 412 nm [26]. Figure S1 (see ESM) displays Ellman's assay results for IL-8 without and with TCEP treatment. It can be seen that the absorption peak at 412 nm is negative for natural IL-8 but significantly obvious for TCEP-treated IL-8, clearly demonstrating the new formation of active thiols after TCEP treatment.

After that, fluorescence microscopy has been employed to investigate the occurrence of immune-recognition and Michael addition on the surface of anti-IL-8@MBs. For this purpose, Cy5-labeled DNA probes (Cy5-labeled Mal-Template and Cy5-labeled Template) were designed to act as signaling probes. As shown in Fig. 2 A and B, no fluorescence is obtained when IL-8 is not pretreated with TCEP while bright red fluorescence can be observed upon the presence of TCEP-treated IL-8. These results are reasonable and consistent with Fig. S1 (see ESM), because natural IL-8 has no inherent active thiols but TCEP treatment can transform the disulfide bonds within natural IL-8 into active thiols, which can then be conjugated to Cy5-labeled Mal-Primer. Further control experiments have been conducted in the absence of target IL-8 or maleimide group. As expected, no obvious fluorescence is obtained in both two cases (Fig. 2C, D), eliminating the possibility of non-specific adsorption of Cy5-labeled DNA probes. All these results not only demonstrate that Mal-Template can only be linked to MBs surface by the way of Michael addition, but also prove that TCEP treatment has no

Fig. 1 Schematic representation of electrochemical detection of IL-8 based on facile incorporation of DNA-QDs
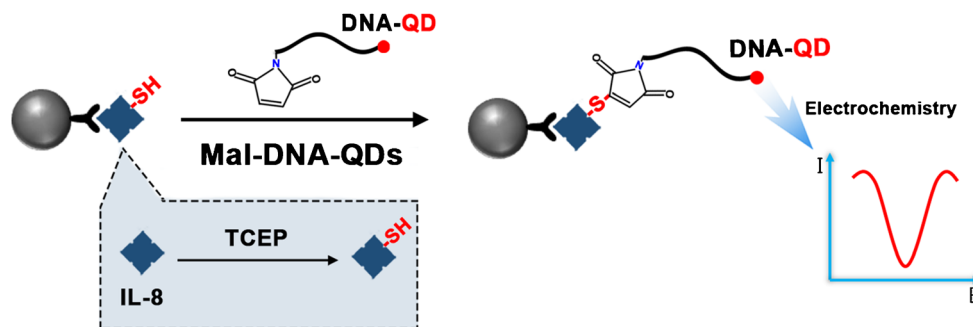
effect on the immune-recognition between IL-8 and its antibody, which are in agreement with our design.

DNA-QDs are designed to act as signal reporters in the detection method, so their preparation and usability have also been examined. Figure 3A shows a typical transmission electron microscopy (TEM) image of DNA-QDs, from which we can see that the DNA-QDs are uniform and well-dispersed. Dynamic light scattering (DLS) result confirms that the DNA-QDs have a good size distribution with an average hydrodynamic diameter of about 5 nm (Fig. 3B). Fluorescence spectrum displays that the DNA-QDs feature a characteristic fluorescence emission peak at 645 nm (Fig. 3C). These results are coherent with literatures and validate the successful preparation of DNA-QDs [25]. Furthermore, electrochemical readout of DNA-QDs has been performed using ASV. To this end, DNA-QDs were first treated with HNO_3 to release Cd^{2+}. The obtained Cd^{2+} was then concentrated onto a mercury film–modified glassy carbon electrode by a 6-min electrodeposition at -1.2 V, which could eliminate the interference of other molecules such as DNA. Afterward, SWV measurement

was conducted to quantify the deposited cadmium. As shown in Fig. 3D, a typical peak of Cd/Cd^{2+} at around -0.77 V [27] is obtained for DNA-QDs, proving the usability of DNA-QDs as signal reporters.

Having confirmed the TCEP treatment, immune-recognition, Michael addition, and preparation of DNA-QDs, we have then applied them to detect IL-8. Figure 4 represents the electrochemical responses obtained under different conditions. As shown in Fig. 4A curve a, an apparent peak is observed in the presence of 10,000 fg/mL IL-8, while only a small one is observed in the absence of IL-8 (Fig. 4A curve b). The clear comparison proves the feasibility of the method for IL-8 detection. Control experiment has also been conducted in the absence of the maleimide group, and the result verified that DNA-QDs were indeed conjugated to TCEP-treated IL-8 via the Michael addition (Fig. 4A, curve c). However, it should be noted that the ratio of electrochemical responses for 10,000 and 0 fg/mL IL-8 is only 5.14, which would make the detection sensitivity unsatisfactory. To solve this problem, RCA is employed in the detection concept by replacing Mal-

Fig. 2 Fluorescence microscopy images of anti-IL-8@MBs after incubation with **A** IL-8 and Cy5-labeled Mal-Template, **B** TCEP-treated IL-8 and Cy5-labeled Mal-Template, **C** TCEP and Cy5-labeled Mal-Template, and **D** TCEP-treated IL-8 and Cy5-labeled Template
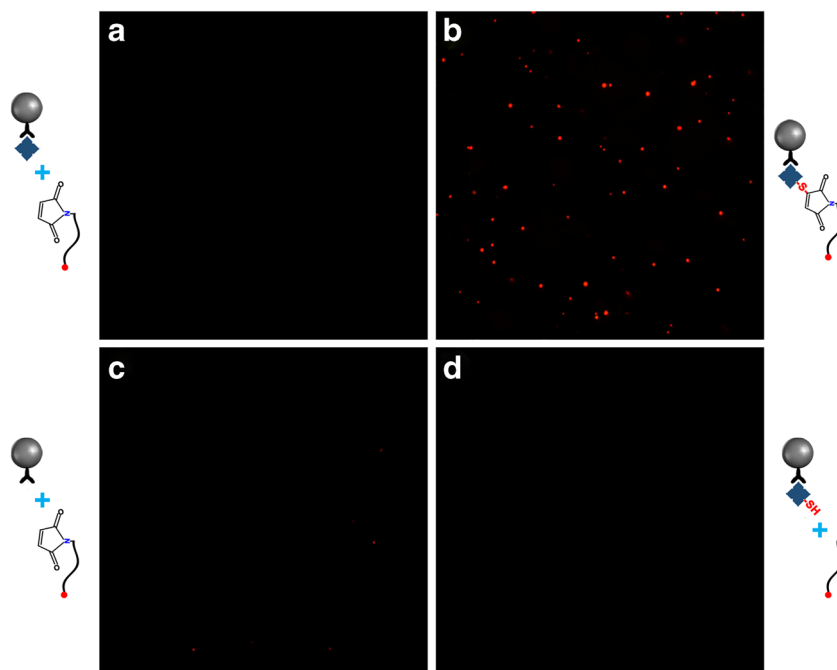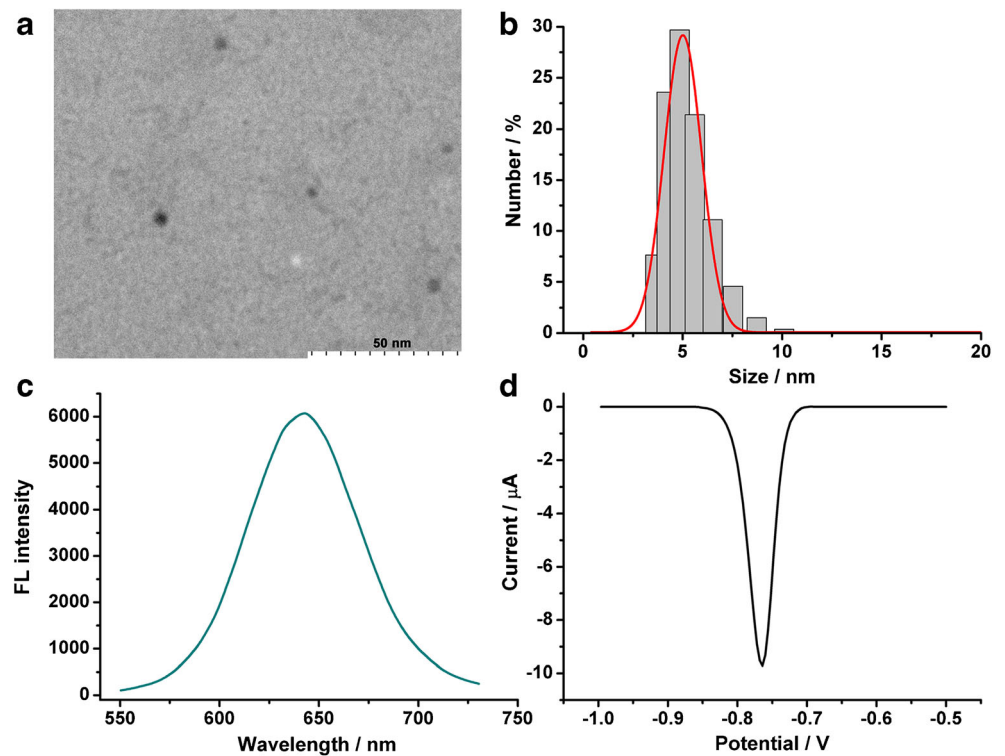
Fig. 3 Characterization of DNA-QDs: **A** TEM image, **B** DLS result, **C** fluorescence spectrum, and **D** electrochemical response

DNA-QDs with Mal-Primer (Fig. 4B, part a). It can be seen from Fig. 4B (part b) that a much higher response is obtained with RCA for detecting 10,000 fg/mL IL-8. This is reasonable because the process of RCA can generate long-range DNA strands on the surface of MBs, subsequently recruiting large numbers of DNA-QDs through base pairing.

Analytical performance of the detection method

Encouraged by the feasibility of the detection method, we have proceeded to perform quantitative detection of IL-8. To realize a desired performance, several important factors involved in the detection procedure, such as the durations of
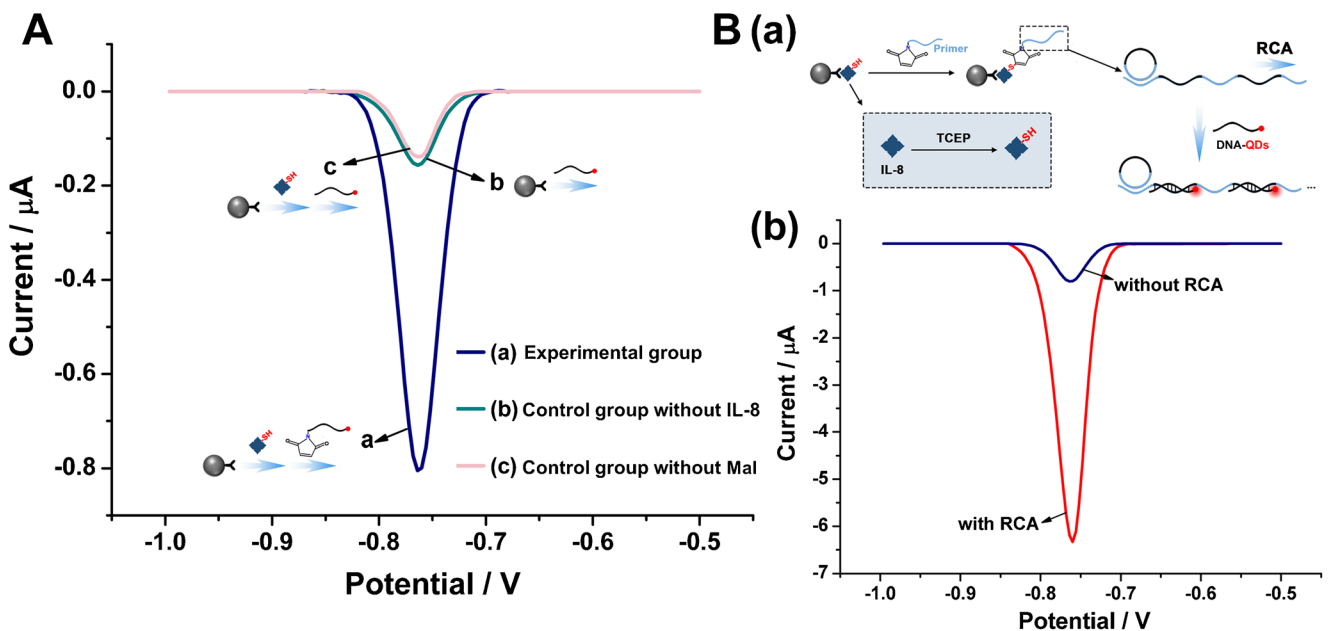

Fig. 4 A Electrochemical responses for the detection of (a) 10,000 and (b) 0 fg/mL IL-8. Curve c corresponds to the control group, in which 10,000 fg/mL IL-8 are detected in the absence of the maleimide group. **B** Part a is the schematic representation of electrochemical detection of IL-8 with the RCA process; part b is the electrochemical responses for the detection of 10,000 fg/mL IL-8 with (a) or without (b) RCA process

TCEP treatment, Michael addition, and RCA process, have been optimized beforehand (ESM Figs. S2–S4). Under the optimized conditions, a series of different concentrations of IL-8 have then been detected using our method. Figure 5A displays the obtained electrochemical responses, which show a gradual increase with the addition of IL-8. This is reasonable, because more IL-8 would produce more active thiols after TCEP treatment, causing the linkage of increased amounts of Mal-Primer onto MB surface. As a result, more long-range DNA strands are produced, which recruit more DNA-QDs to release more Cd^{2+} after acid treatment, giving a higher response. It is noted that the response emerges at 5 fg/mL IL-8 with a current of 0.76 μA, which is near the peak current of electrochemical response obtained upon detecting 10,000 fg/mL IL-8 without RCA (~ 0.8 μA; Fig. 4A). That is in this case, an amplification factor achieved by using RCA can thereby be calculated to be about 2000 (10,000/5). Figure 5B shows the relationship between the change in the peak current of electrochemical response (I-I_0, where I and I_0 are the peak currents of electrochemical responses obtained in the presence and absence of IL-8) and IL-8 concentration. Obviously, the I-I_0 increases linearly with the logarithm of IL-8 concentration over the range from 5 to 5000 fg/mL. The regression equation is $y = 1.784 \lg x - 0.85$ ($R^2 = 0.999$), where y is the I-I_0 (μA) and x is the logarithm of IL-8 concentration (fg/mL). The limit of detection (LOD), defined as a signal-to-noise ratio of 3, is estimated to be 3.36 fg/mL, which displays a favorable and improved sensitivity compared with previous reports (ESM Table S2). Although the LOD is a bit higher than that of an electrochemical immunosensor based on the conductive carbon black and star PGMA polymer composite material (3.36 fg/mL vs 3.3 fg/mL) [8], our method avoids the complicated processes of nanomaterial synthesis and electrode modification and thus might have a better application prospect. Moreover, considering that the cutoff value of

serum IL-8 in oral cancer patients is 22.5 pg/mL [28], the LOD of this method can be sufficient for clinical applications. In addition, detection of IL-8 with each concentration has been repeated for at least three times and the relative standard deviations (RSD) are all within 4.1%, implying a satisfactory reproducibility of the detection method.

Selectivity of the method has then been evaluated. For this purpose, several non-specific proteins, including oral cancer biomarkers (CA-125, IL-6, and CYFRA-21), saliva protein (LPO), and blood protein (BSA), have been detected using the method. The peak currents of obtained electrochemical responses are compared with those of target IL-8 and blank control and are shown in Fig. 6. It can be found that a high peak current is obtained in the presence of 5000 fg/mL IL-8, while the peak currents for non-specific proteins are only be similar as that of blank control. These data demonstrates a desirable selectivity of the detection method.

Finally, the potential applicability of the method in the non-invasive diagnosis of oral cancer has been investigated. To this end, three different amounts of IL-8 (with final concentrations of 5 fg/mL, 20 fg/mL, and 100 fg/mL) were spiked into 100 μL of 10-times diluted normal human serum ordered from AmyJet Scientific Inc. (Wuhan, China) and detected as outlined above. These experiments were approved by the Scientific Ethical Committee of Shanghai University and performed in accordance with the ethical standards. Electrochemical responses of these serum samples were used to calculate the values of detected concentrations by interpolation in the calibration for standards shown in Fig. 5B. Table S3 (see ESM) compares the added concentration of IL-8 and the detected concentration in these serum samples. Delightedly, the recoveries are between 97.20 and 104.4% and RSDs are all within 5%. So, the method can be used to detect IL-8 in serum samples with high accuracy, indicating a great potential for non-invasive diagnosis.
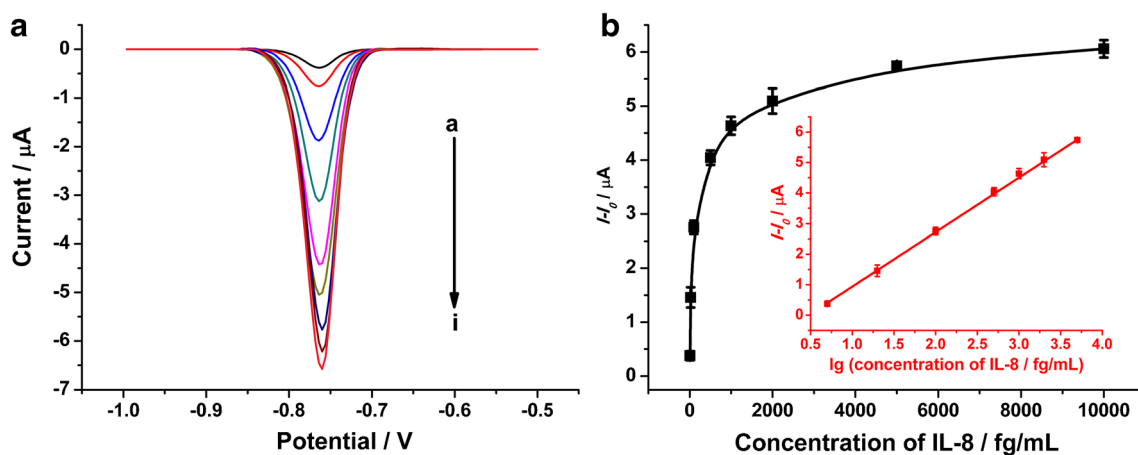

Fig. 5 A Electrochemical responses for the detection of different concentrations of IL-8: (a) 0 fg/mL, (b) 5 fg/mL, (c) 20 fg/mL, (d) 100 fg/mL, (e) 500 fg/mL, (f) 1000 fg/mL, (g) 2000 fg/mL, (h) 5000 fg/mL, and (i) 10,000 fg/mL. **B** The resulting calibration curve for the electrochemical detection of IL-8. Inset shows the linear relationship between the I-I_0 and the logarithm of IL-8 concentration
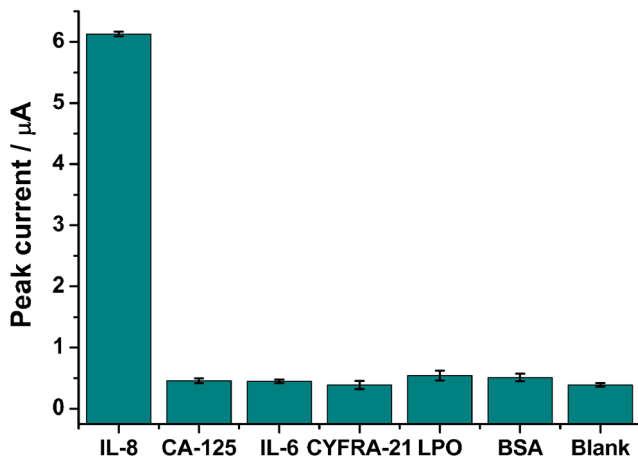
Fig. 6 Peak currents of electrochemical responses obtained upon detecting different proteins. From left to right 5000 fg/mL IL-8, 5 ng/mL CA-125, 5 ng/mL IL-6, 5 ng/mL CYFRA-21, 5 ng/mL LPO, 5 ng/mL BSA, and the blank control that contains no IL-8 or non-specific proteins

Conclusions

In conclusion, a new electrochemical method was developed to detect IL-8. The use of TCEP treatment provided a simple and effective way to incorporate DNA-QDs and RCA process into the detection procedure, thereby achieving a satisfactory detection limit of 3.36 fg/mL. Experiments also demonstrated that the method displayed desirable selectivity, reproducibility, and applicability in complex serum samples. Moreover, the method may have inherent capability of being extended to detect other disulfide-contained biomarkers by simply changing the used antibody. In view of these features, we expect that the method provides an invaluable tool for IL-8 detection and have a great potential in biomedical applications in the future.

Compliance with ethical standards

These experiments were approved by the Scientific Ethical Committee of Shanghai University and performed in accordance with the ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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