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Cascade Signal Amplification Strategy for Subattomolar Protein Detection by Rolling Circle Amplification and Quantum Dots Tagging

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A cascade signal amplification strategy was proposed for detection of protein target at ultralow concentration by combining the rolling circle amplification (RCA) technique with oligonucleotide functionalized quantum dots (QDs), multiplex binding of the biotin−strepavidin system, and anodic stripping voltammetric detection. The RCA product containing tandem-repeat sequences could serve as excellent template for periodic assembly of QDs, which presented per protein recognition event to numerous quantum dot tags for electrochemical readout. Both the RCA and the multiplex binding system showed remarkable amplification efficiency, very little nonspecific adsorption, and low background signal. Using human vascular endothelial growth factor as a model protein, the designed strategy could quantitatively detect protein down to 16 molecules in a 100 μL sample with a linear calibration range from 1 aM to 1 pM and was amenable to quantification of protein target in complex biological matrixes. The proposed cascade signal amplification strategy would become a powerful tool for proteomics research and clinical diagnostics.

The ultrasensitive techniques for protein detection play essential roles in the elucidation of life processes and molecular mechanisms of many diseases, because a few molecules of protein are sufficient to affect the biological functions of cells and trigger pathophysiological processes. However, it seems impossible to use conventional methods to detect these important protein biomarkers due to their low abundance in body fluids or tissues.[1] Thus, it is very urgent to develop ultrasensitive methods for detection of low abundant protein.

Recently, DNA amplification techniques based on polymerase chain reaction (PCR) have been explored as important strategies for sensitive protein assays.[2] Different assay formats such as liposome-PCR,[3] phage display mediated immuno-PCR,[4] and aptamer-based affinity PCR[5] have been developed to increase the abundance of the detection probes and significantly lower the limit of detection for protein analysis. However, they require thermal cycling and strict laboratory conditions to avoid contamination or false results. Due to the unique optical, electronic, and mechanic properties of nanomaterials such as gold nanoparticles, carbon nanotubes, nanowires, magnetic nanobeads, and quantum dots (QDs), a series of nanoparticle probes have been developed to enhance recognition events of targets and significantly lower the detection limit for protein analysis.[6−10] These nanomaterials were used as carriers by biofunctionalization with antibodies or aptamers for recognization of targets or/and immobilizing enzymes or nucleic acids for signal amplification. Most of them are at the proof-of-concept stage and require additional tests of the matrix effect. Furthermore, the variability of the probes often affects the reproducibility and quantification, especially for the real samples.[2]

Aiming at the detection need of low abundant proteins and the problems existing in the amplification techniques by PCR and nanoparticle probes, this work integrated an advanced amplification technique, rolling circle amplification (RCA), with quantum dot tags as well as two general signal amplification methods, the multiplex binding of the biotin−strepavidin system and anodic stripping voltammetric detection (ASV), to design a cascade signal amplification strategy for ultrasensitive detection of proteins. RCA is an isothermal DNA amplification procedure[11] and can generate a linear concatenated DNA molecule containing up to 1000 complementary copies of the circular DNA in 1 h.[12] Compared with PCR, this technique possesses many advantages such as the

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isothermal amplification procedure and the linear kinetic model.[13] Therefore, it has gained considerable attention as a novel tool to amplify the recognition events for ultrasensitive detections of DNA,[14] RNA,[15] ATP,[16] and proteins.[1,17−19] Here, the integration of a series of amplification techniques further extended the application of RCA, particularly, in convenient detection of extremely low abundant proteins. It could present a protein recognition event to a large number of quantum dot tags, which then released numerous detectable reporters for electrochemical readout.

Human vascular endothelial growth factor (VEGF) is an important angiogenic factor that plays a crucial role in tumor growth and metastasis, which is overexpressed in a vast majority of human tumors such as lung, thyroid, breast, gastrointestinal tract, kidney, bladder, and ovary.[20−22] The importance of VEGF in tumor genesis and progression makes it an attractive biomarker for tumor diagnostics and significant target for the development of anticancer therapies.[23,24] However, VEGF is present in biological samples at very low concentration.[23,25] Therefore, this work used VEGF as a model protein to verify the practicability of the proposed strategy. This cascade signal amplification led to an extremely low limit of detection for VEGF down to a subattomolar level with a wide dynamic range of 6 orders of magnitude. It was, thus, a very powerful tool for quantitation of extremely low abundant proteins and elucidation of molecular mechanisms of some diseases.

EXPERIMENTAL SECTION

Materials and Reagents. The enzyme-immunoassay kit for VEGF was purchased from Boster Biological Technology Co., Ltd. (China). It contained a 96-well microplate coated with antihuman VEGF monoclonal antibody, a vial of human VEGF reference standard solution, and a vial of biotin labeled antihuman VEGF polyclonal antibody. The oligonucleotides with the following sequences[1] were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China): biotinylated primer, 5′-biotin-AAA AAA AAA AAA AAA CAC AGC TGA GGA TAG GAC AT-3′; circular template, 5′-p-CTC AGC TGT GTA ACA ACA TGA AGA TTG TAG GTC AGA ACT CAC CTG TTA GAA ACT GTG AAG ATC GCT TAT TAT GTC CTA TC-3′; thiolated DNA probe, 5′-thiol-$(CH_2)_6$-TCA GAA CTC ACC TGT TAG-3′.

Phi29 DNA polymerase, T4 DNA ligase, and dNTP were obtained from Fermentas (Lithuania). Streptavidin was obtained from Promega (USA). Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$) was purchased from Alfa Aesar China Ltd. Mercaptopropionic acid (MPA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), salmon sperm DNA, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA). Cell lysis buffer was obtained from Beyotime Institute Biotechnoliogy (China). All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water ($\geq$18 MΩ, Milli-Q, Millipore).

Apparatus. The UV−vis absorption and photoluminescence spectra were recorded with an UV-3600 UV−vis−near infrared (NIR) spectrophotometer (Shimadzu, Japan) and a F900 fluorescence spectrometer (Edinburgh Instruments Ltd., UK), respectively. The transmission electron microscopic (TEM) image was observed under a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). The fluorescence image was taken by a TE2000-U inverted fluorescence microscope (Nikon, Japan). All electrochemical measurements were performed on a CHI 660 electrochemical analyzer (Co. CHI, TX) with a conventional three-electrode system composed of platinum wire as auxiliary, saturated calomel electrode as reference, and a mercury film modified glassy carbon electrode as working electrode.

Preparation of CdTe QD−DNA Probe. The synthesis of water-soluble MPA-modified CdTe QDs referred to the method reported previously.[26] The obtained QD solution was subjected to ultrafiltration using Vivaspin concentrator (Sartorius, 10 000 MW) at 15 000*g* for 10 min to remove excessive MPA. The upper phase was washed twice with water and diluted to a certain concentration with water. The resulting CdTe QDs solution could be stable for 3 months, and its concentration and the size of QDs were detected with the UV−vis absorption.[27] MPA-modified CdTe QDs (0.12 nmol) were mixed with 0.6 nmol of thiolated DNA probe in 500 μL of 50 mM, pH 7.4 Tris−HCl buffer and left overnight under shaking and free of light. The resulting mixture was ultrafiltrated using Vivaspin concentrator (10 000 MW) at 10 000*g* for 10 min at 4 °C to remove the nonconjugated DNA. The obtained conjugates were washed thrice with 50 mM Tris−HCl buffer by ultrafiltration to obtain CdTe QD−DNA probe (~300 nM), which was kept at 4 °C prior to use.

Circularization of DNA Template. Ten nanomoles of circular template oligonucleotide and 10 nanomoles of biotinylated primer oligonucleotide were mixed in 100 μL of ligation buffer (50 mM, pH 7.5 Tris−HCl buffer, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM ATP) and incubated at 37 °C for 30 min. Then, 0.2 units of T4 DNA ligase was added and incubated at 22 °C for 1 h. After ligation, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. The resulting mixture could be used directly or stored at −20 °C.

Sandwich Immunoreaction in Microplate Wells. VEGF analyte (100 μL) at variable concentrations were added in microplate wells coated with antihuman VEGF monoclonal antibody and incubated for 1.5 h at 37 °C, respectively. After the reaction solutions were removed, 100 μL of biotin labeled anti-

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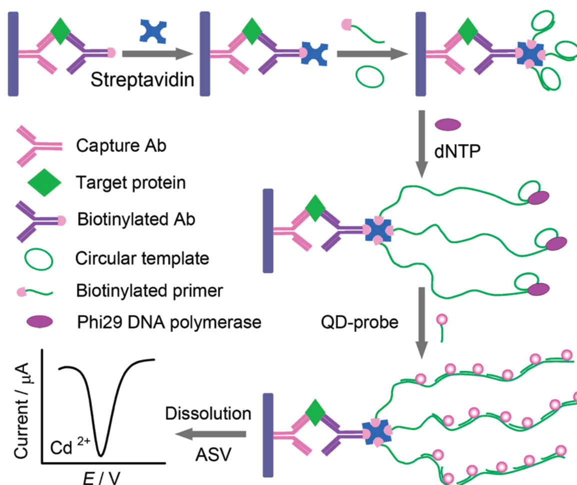
VEGF polyclonal antibody was added in each well and incubated at 37 °C for 1 h. The resulting microplate wells were washed thrice with 200 μL of washing buffer (50 mM, pH 7.5 Tris−HCl buffer and 0.05% Tween 20) and used for the following operation.

Rolling Circle Amplification and QD Tagging. After the sandwich immunoreaction, the microplate wells were incubated with 100 μL of 1 nM streptavidin solution containing 0.2% BSA and 100 μg/mL salmon sperm DNA per well at 37 °C for 30 min. After washing with 200 μL of washing buffer, 100 μL of circularization mixture containing 1 nM circular template DNA and 1 nM biotinylated primer DNA was added to each well and incubated at 37 °C for 30 min. After the microplate wells were washed thrice with 200 μL of washing buffer, RCA reaction was initiated by addition of 0.2 units of phi29 DNA polymerase in 100 μL of reaction buffer (50 mM, pH 7.5 Tris−HCl buffer, 10 mM magnesium acetate, 33 mM potassium acetate, 1 mM dithiothreitol, 10 mM dNTP, and 0.1% Tween 20) and continued for 1 h at 37 °C. After the microplate wells were carefully washed thrice with 200 μL of washing buffer, 100 μL of 20 nM QD−DNA probe was added to each well and hybridized at 37 °C for 30 min. After the microplates were carefully washed thrice with 200 μL of washing buffer to remove the nontagged QD−DNA probe, 100 μL of 0.1 M HNO_3 was added to each well and incubated for 1 h under sonication to dissolve the tagged QDs. The resulting Cd^{2+} solutions were used for electrochemical detection.

Electrochemical Detection of Cadmic Cation Released from Tagged QDs. The obtained solutions of Cd^{2+} were mixed with 900 μL of 0.2 M, pH 4.6 HAc−NaAc buffer to perform ASV analysis with a mercury film modified glassy carbon electrode.[28] The working electrode was prepared by 4 cycles of alternate deposition at −1.0 V for 40 s and scan from −0.9 to −0.3 at 0.1 V/s in 0.2 M, pH 4.6 HAc−NaAc buffer containing 40 μg/mL Hg^{2+} under N_2 atmosphere. The ASV detection was carried out by electrodepositing cadmium at −1.1 V for 10 min and stripping from −0.9 to −0.3 V under N_2 atmosphere using a square wave with 25 mV amplitude, 4 mV potential step, and 25 Hz frequency.

Detection of VEGF in Complex Matrixes. T47D human mammary adenocarcinoma cells were cultured in a flask in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS, Sigma), penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. After culture for 12 h, the cell culture fluid was collected and centrifuged at 1000 rpm for 10 min to remove cell debris. The obtained supernatant and fresh culture fluid were 1000 time diluted to perform the detection of VEGF with the designed method. The VEGF in the supernatant was also quantified with a conventional ELISA method according to the manufacturer's instruction. To detect the VEGF in a cell lysate, the monolayer of T47D cells in a flask was rinsed three times with PBS, the cells were brought into 5 mL of ice-cold PBS containing 0.5 mM EDTA with a cell scraper, the suspended cells were then collected and centrifuged at 1500 rpm for 10 min at 4 °C, and the sediment containing approximately 10^5 cells was finally resuspended in 1 mL of ice-cold lysis buffer. After incubation on ice for 30 min, the cell lysate was obtained by centrifuging at 12 000 rpm for 10 min at 4 °C to collect the supernatant, which was used as a cell lysate for VEGF detection.

Scheme 1. Schematic Representation of the Cascade Signal Amplification Strategy for Protein Detection



QD−Streptavidin Method. MPA-modified CdTe QDs (0.2 nmol) were mixed with 0.05 mg of EDC and 0.1 nmol of streptavidin in 500 μL of 50 mM, pH 7.4 Tris−HCl buffer. After incubation for 3 h at 25 °C under shaking and free of light, the resulting mixture was ultrafiltrated using Vivaspin concentrator (50 000 MW) at 3000*g* for 12 min at 4 °C to remove the nonconjugated QDs and byproduct. The obtained QD−streptavidin conjugates were washed with 50 mM, pH 7.4 Tris−HCl buffer three times by ultrafiltration. The product (~200 nM) was kept at 4 °C.

As control, after the sandwich immunological reaction, the obtained microplate wells were incubated with 100 μL of 20 nM QD−streptavidin conjugates at 37 °C for 30 min. After the microplate wells were carefully washed thrice with 200 μL of washing buffer to remove the nontagged QD−streptavidin, 100 μL of 0.1 M HNO_3 was added to each well and incubated for 1 h under sonication to dissolve the tagged QDs. The resulting solutions containing Cd^{2+} were then used for electrochemical detection.

RESULTS AND DISCUSSION

Design of Strategy. The VEGF, a target protein, was first captured on an anti-VEGF monoclonal antibody-coated microplate well to form a sandwich immunocomplex with biotinylated anti-VEGF polyclonal antibody. The streptavidin was then bound to the biotinylated immunocomplex to act as a bridge for multiplex anchoring of biotinylated single-strand oligonucleotide. As seen in Scheme 1, one streptavidin-bound immunocomplex could anchor three biotinylated oligonucleotide strands to act as RCA primers for binding of the circular template. In the presence of nucleotides and phi29 DNA polymerase, the RCA was initiated to produce micrometer-long single-strand DNA, which contained hundreds of tandem-repeat sequences for linear periodic assembly of a large number of CdTe QD−DNA probes for enhancement of recognition event. The cadmic component of the resulting QD-tagged rolling circle amplified DNA (QD−RCA) product was finally dissolved and quantified by anodic stripping voltammetry. The nanoparticle-based amplification resulted from the large

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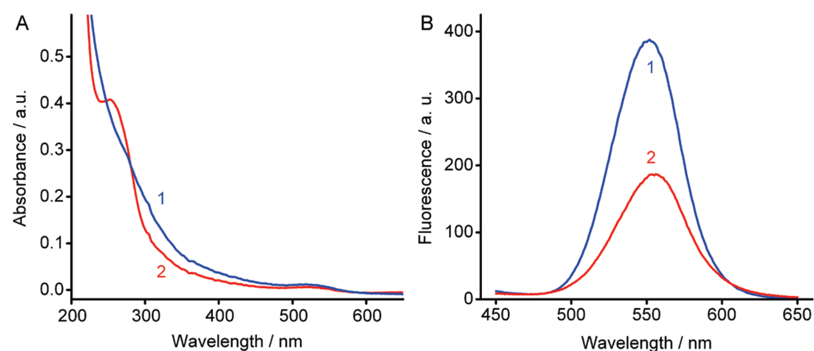

Figure 1. (A) Absorption and (B) emission spectra of water-soluble MPA-capped CdTe QDs (1) and QDs functionalized with thiolated oligonucleotide probe (2).

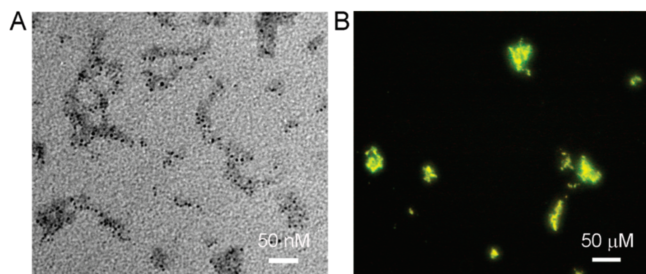

Figure 2. (A) TEM and (B) fluorescence microscopic images of QD-tagged RCA product.

number of detection reporters in a QD nanoparticle, and the anodic concentration of the cadmic component further enhanced the sensitivity of the proposed protocol for monitoring the recognition events of low abundant protein.

Characterization of QD−DNA Probe. From the UV−vis absorption spectrum of the resulting MPA-capped CdTe QDs solution, the size of QDs was detected to be 2.8 nm.[27] For tagging QDs to the tandem-repeat sequences, the CdTe QDs were first biofunctionalized with thiolated oligonucleotide probe, which possessed a complementary sequence with part of the tandem-repeat sequence. The obtained QD−DNA probe could be confirmed by absorption and emission spectra (Figure 1). The appearance of a new absorption peak at 260 nM indicated the successful biofunctionalization of QDs with the oligonucleotide probe. The decrease in fluorescence intensity after the biofunctionalization step indicated the decrease in the quantum yield, which was attributed to additional surface defects upon the association of the oligonucleotides.[29] The average surface coverage of the oligonucleotide probes was estimated to be ~3 per nanoparticle, measured by determining the amount of nontagged oligonucleotide probe spectroscopically.

QD-Tagged RCA Product. The tagging of QDs on RCA product could be confirmed by TEM and fluorescence microscopy. The TEM image showed that the diameter of CdTe QDs was approximately 3 nm (Figure 2A), which was slightly greater than the size of unfunctionalized QDs and consistent with the result obtained from the UV−vis absorption. Furthermore, the uniformly conjugated CdTe nanoparticle smaller than the length of 18 bp oligonucleotide probe (about 6 nm) made it be easily tagged on the linearly concatenated RCA product. It could be seen from Figure 2A that a large number of QDs regularly aligned on the stretched RCA products could reach micrometer in length. The result was comparable with the previous report of Au nanoparticles assembly on a rolling circle amplified DNA strand.[30] Thus, the RCA procedure produced the tandem-repeat oligonucleotide sequences complementary with the QD−DNA probe, leading to the tagging of QDs on RCA product.

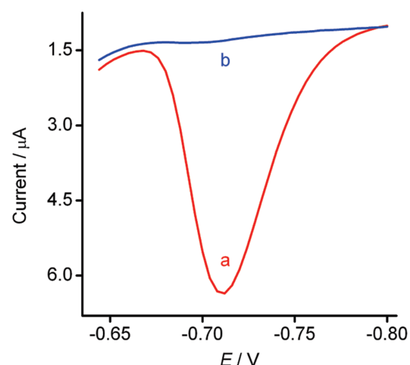

Figure 3. Anodic stripping voltammograms of cadmic cation responding to 1 fM of VEGF with (a) and without (b) RCA.

In aqueous solution, the QD-tagged RCA products spontaneously collapsed into a random coil of DNA and showed micrometer-sized fluorescent objects in the fluorescence microscope (Figure 2B). Their fluorescence intensity was about 40 times stronger than the single QD−DNA probe in surrounding solution. This result further proved the predominant amplification capability of RCA and significant labeling efficiency of QD−DNA probe. Thus, the RCA product containing tandem-repeat sequences could serve as excellent template for periodic assembly of QDs and substantial signal amplification.

ASV Detection of Cadmic Cation Reporter. After the CdTe QDs were dissolved with HNO_3 from the QD-tagged RCA products, the dissolved cadmic cation reporter could be conveniently detected by ASV. A distinct stripping voltammetric peak of cadmic cation reporter responding to 1 fM of VEGF target could be observed at −0.71 V in 0.2 M, pH 4.6 HAc−NaAc buffer after rolling circle amplification (Figure 3a). This peak also demonstrated the QD-tagged RCA product could be easily conjugated with sandwich immunocomplex by the biotin−streptavidin system.

The stripping voltammogram without RCA as control showed a very low electrochemical response of the cadmic cation reporter (Figure 3b). This weak response indicated very little nonspecific adsorption of QD−DNA probe on the wall of microplate well and

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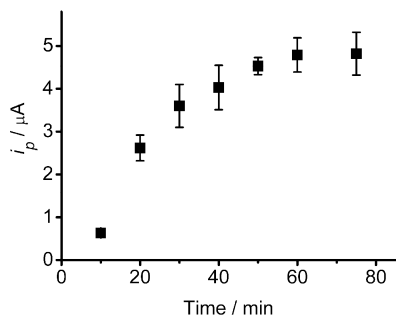

Figure 4. Influence of RCA reaction time on the anodic stripping voltammetric signal responding to 1 fM of VEGF.

biotin−streptavidin conjugated immunocomplex, which was attributed to the blocking of BSA and salmon sperm DNA in the streptavidin incubation step. The blocking efficiency could be further verified by comparing the responses with and without blocking. After blocking with 0.2% BSA and 100 μg/mL salmon sperm DNA, the background response decreased from 2.01 to 0.89 μA, while the peak current responding to 10 aM VEGF target decreased slightly from 2.45 to 2.25 μA, which was significantly higher than the background response. The elimination of the nonspecific adsorption was directly beneficial to improving the sensitivity of the designed method.

Time-Dependent Signal Amplification of RCA. A long RCA reaction time is expected to generate more complementary copies of the circular template for yielding enhanced signal amplification. The effect of RCA reaction time on the electrochemical signal was shown in Figure 4. At the initial 10 min, the electrochemical response was very weak, indicating a relatively slow rate of RCA reaction. The peak current increased rapidly with the increasing RCA reaction time after 10 min and trended to a constant value at 60 min, indicating the saturation of RCA product due to the exhaustion of RCA substrates or inactivation of the phi29 DNA polymerase. Thus, 60 min was selected as the optimum time for the RCA reaction.

Signal Amplification Performance of QD−RCA Strategy. To further test the signal amplification of the QD−RCA strategy, a QD-based signal amplification method was designed as a control using QD-labeled streptavidin as signal amplification device to conjugate with the sandwich immunocomplex. The QD−streptavidin method showed a limit of detection of about 10 fM for VEGF (Figure 5), which was comparable with the previous method using QDs as electrochemical label.[31] The responses of the QD−RCA method for VEGF at the same concentrations were significantly higher than those of the QD−streptavidin method. More interestingly, these methods showed similar responses to the VEGF-free sample, indicating that the RCA process did not visibly increase the background response. The responses could be attributed to the nonspecific adsorption of biotin labeled antihuman VEGF polyclonal antibody.

After subtracting the response to blank solution, the peak currents obtained with the QD−RCA method for 100 and 10 fM of VEGF were about 3 and 12 times those with the QD−streptavidin method, respectively. Moreover, the signal for 1 fM of VEGF obtained with the QD−RCA method was even higher than that for 100 fM VEGF obtained with the QD−streptavidin method,

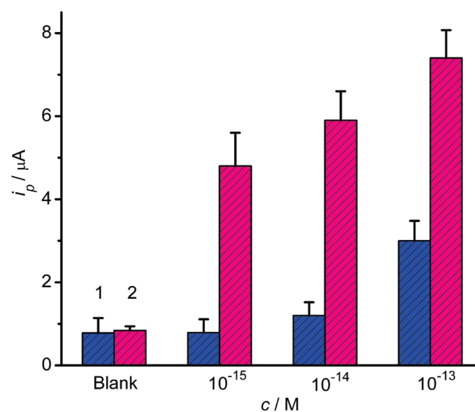

Figure 5. Anodic stripping voltammetric signals using QD-labeled streptavidin (1) and QD-tagged RCA product (2) as signal amplification devices for detection of VEGF.

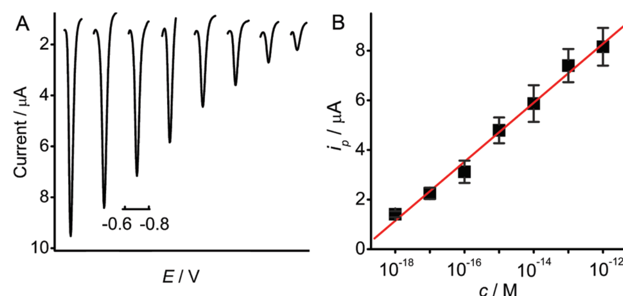

Figure 6. (A) Anodic stripping voltammograms of cadmic cation responding to 10^{-12}, 10^{-13}, 10^{-14}, 10^{-15}, 10^{-16}, 10^{-17}, 10^{-18}, and 0 M VEGF (left to right). (B) The quantitative dynamic range of the designed method.

showing remarkable amplification performance. A more large-scale QD-tagged RCA product could be generated at lower analyte concentration due to the excess substrates and decreasing steric inhibition. Thus, the amplification efficiency increased with the decreasing protein concentration which greatly improved the sensitivity of the designed strategy for detection of low abundant protein.

Analytical Performance of QD−RCA Strategy. In view of the outstanding ability for signal amplification, the dynamic range of the designed method for detection of VEGF analyte was examined. The stripping peak current of cadmic cation was proportional to the logarithm value of VEGF concentration over a 6-decade range from 1 aM to 1 pM with a linear correlation coefficient of 0.996 (Figure 6). The broad dynamic range was attributed to the linear kinetic model of RCA. The detection limit was 0.27 aM in a 3 σ rule, which was lower than those of 3 aM for biobarcode assay coupling with PCR,[6] 7 aM for gold-nanocatalyst sensitized electrochemical assay,[8] 0.08 fg mL^{-1} (2.3 aM) for DNA-encapsulating liposome-based RCA immunoassay,[18] and about 10^3-fold lower than those for nanomaterial-based assays.[31,32] In other words, the QD−RCA method was able to detect about 16 molecules of VEGF in a 100 μL sample. The ultrahigh sensitivity was attributed to the cascade signal amplification, very little nonspecific adsorption, and low background signal.

To estimate the reproducibility of the QD−RCA strategy, the intra-assay imprecision of five different microplate wells at one

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assay and interassay imprecision at five different assays for detection of 100 aM VEGF were examined. The intra-assay peak currents differed by 7.0%, and the coefficient of variation of interassay peak current was 14.3%, indicating that the QD−RCA strategy was robust and could be used for protein detection with acceptable reproducibility.

The utility of the QD−RCA approach for analytical purpose was further demonstrated with a complex biological matrix. The VEGF concentration in a diluted sample of cell culture supernatant was detected to be 2.70 pM, which was in good agreement with 3.18 pM obtained with a conventional ELISA method. While the VEGF in the fresh culture fluid was not detectable as the culture analyzed with both methods. The proposed approach was also used to detect VEGF with a wide range of concentrations in cell lysates, which were prepared by spiking 5, 50, and 500 fM of purified VEGF in initial cell lysate, respectively. The peak current increased propotionally with the increasing concentration of added VEGF, which was coincident with the calibration curve obtained with standard VEGF solutions. According to the calibration curve, the VEGF content in the initial cell lysate was obtained to be 1.6 fM, and the recovery of VEGF spiked in cell lysate was from 93.5% to 112.4%. Thus, the designed method allowed precise quantification of protein in complex biological matrixes without the matrix effect, which often exists in many other signal amplification methods.

CONCLUSIONS

A versatile method is developed with a breakthrough for detection of subattomolar protein (down to 16 protein molecules) by combining the rolling circle amplification technique with the biofunctionalization of quantum dots, the multiplex binding of the biotin−strepavidin system, and anodic stripping voltammetric detection for cascade signal amplification. The QD-tagged rolling circle amplified DNA as signal amplification device offers an enormous ratio of metal component to target molecule and dramatically increases the sensitivity. The method does not require complicated conjugation chemistry and thermal cycling and shows a broad dynamic range and acceptable reproducibility. Using more pairs of RCA substrates and different QDs, the method has the potential for multiplex detection. By integrating immunoreaction, molecular biotechnology, nanobiotechnology, and electrochemical detection, this primary research opens new horizons for integrating different disciplines and will promote the basic and clinical research. The proposed QD−RCA strategy would become a powerful tool for proteomics research and clinical diagnostics.

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