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Ultrasensitive immunoassay based on dual signal amplification of the electrically heated carbon electrode and quantum dots functionalized labels for the detection of matrix metalloproteinase-9†

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A dual signal amplification strategy was designed for electrochemical detection of matrix metalloproteinase-9 with the integration of electrically heated carbon electrode technique and quantum dots labels.

Matrix metalloproteinases (MMPs) belong to a multigene family of zinc-dependent endopeptidases that collectively degrade all components of the extracellular matrix. They play well-established roles in normal physiological and pathological processes.[1,2] The overexpression of MMPs commonly associates with a variety of cell malignancy, including tumors of dermal, intestinal, pulmonary, ovarian, gastric, pancreatic, and mammary origin. Particularly, MMP-9 has been previously described as one of the most important enzymes related to the invasiveness and metastatic potency of human malignant tumors. Therefore, the accurate and sensitive detection of MMP-9 has become an intriguing subject in the study of disease mechanism, pathogenesis and treatment. Up to now, the expression of MMP-9 in diseased tissues is usually detected by ELISA, Western Blot analysis and zymography.[3] Although promising, most of them, unfortunately, may be time-consuming, labor-intensive, require high technical expertise and sophisticated instrumentation. Meanwhile, the poor detection limit (ng mL^{-1}) of these methods restricts further application for some special diseases, especially during the early stage of diseases. Hence, it is beneficial to develop a highly sensitive and convenient detection approach for MMP-9.

Recently, electrochemical technique has been applied to monitor MMP-9 activity.[4–6] For example, Andrew *et al.*[4] developed an electrochemical impedance spectroscopy method where the detection limit of MMP-9 was 1.1 nM; Kizek *et al.*[5] created a chronopotentiometric stripping analysis with a 100 pg mL^{-1} detection limit.[5] Although all of these work were remarkable, the sensitivity and selectivity still need improving. Therefore, a dual signal amplification strategy has been designed for electrochemical detection of MMP-9, which integrates electrically heated carbon electrode (HCPE) technique with quantum dots (QDs) labels. The HCPE technique is a fantastic way to accelerate reaction kinetics and improve the mass transport by changing the temperature of electrode, thus leading to an enhanced electrochemical signal together with a higher signal-to-background ratio.[7–9] Furthermore, the heated-electrode technique only heats the electrode but leaves the bulk solution temperature unchanged, which is suitable for clinical disease diagnostics. On the other hand, QDs are well recognized as electroactive labels for signal amplification in immunoassays with the great performance of exhibiting sharp and well-resolved stripping voltammetric signals.[10,11] Particularly, when QDs are assembled on the surface of various nanocarriers, the signal amplification feature is dramatically enhanced.[12–14] Consequently, the approach shows high sensitivity due to taking advantage of the specific technique of HCPE and QDs.

In addition, the fabrication of immunosensor needs the immobilization of a "receptor site", which selectively recognizes the analyte.[15] In this work, carbon nanotube-doped polypyrrole (MWNTs-PPy) synthesized by electrodeposition was chosen as an immobilization scaffold of proteins, which possesses properties of the individual components with synergistic effects.[16] PPy has perfect conductive properties, thermal stability and biocompatibility in combination with biomaterials.[17,18] PPy is, moreover, easily modified by proteins,[15] and molecular imprints of high[19–21] and low[22] molecular weight. These properties make the PPy extremely useful for the design of biosensors. The prepared carbon nanotube-doped polypyrrole not only maintains all excellent properties of PPy, but also keeps excellent electrical and mechanical properties of carbon nanotubes which evaluates the surface-active groups-to-volume ratio and superb thermal stability of MWNTs-PPy.[23–28] Thereby, MWNTs-PPy is considered as the perfect material for immunosensors.

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Herein, we developed a novel dual amplification strategy for sandwich immunosensor through combining the heated-electrode technique with QDs for the detection of MMP-9. A superbiocompatible and thermal steady material, carbon nanotube-doped polypyrrole (MWNTs-PPy) nanocomposite, was chosen to immobilize proteins. The proposed biosensor exhibited attractive advantages such as ease of performance, high sensitivity and specificity in the detection of MMP-9, revealing great potential towards early evaluation of cancer therapeutic effects.

The preparation procedure for CdTe QDs functionalized labels (anti-MMP-9/PS@PDA/CdTe-QDs) is shown in Fig. 1A. Firstly, polydopamine (PDA) was spontaneously deposited on the polyethylene sphere (PS) surface through self-oxidative polymerization of dopamine as reported previously.[29] Then the achieved polydopamine coated polyethylene sphere (PS@PDA) nanoparticles were further incorporated with poly(diallyldimethylammonium chloride) (PDDA) *via* stirring for 20 min. Residual PDDA was removed by high-speed centrifugation and washed by ultrapure water three times. Subsequently, CdTe QDs solution was added into a dispersion of PS@PDA/PDDA, and the mixture was sonicated for 20 min. Excess particles were removed by subsequent centrifugation and then redispersion in water. This process resulted in the formation of homogeneous PS@PDA/PDDA/CdTe-QDs nanoparticles. Then, EDC and NHS were used as coupling agents to modify the resulting mixture with signal antibody by the formation of an amide link between the amino of anti-MMP-9 and the carboxylic of QDs. Finally, this anti-MMP-9/PS@PDA/PDDA/CdTe-QDs bioconjugates were stored in PBS (pH 7.4) with 3% BSA at 4 °C before use. Fig. 2D is the HRTEM image of PS@PDA/PDDA/CdTe showing the uniformity of the coating and the presence of the CdTe nanocrystals. Additionally, layer-by-layer (LBL) assembly process for Ab_2/PS@PDA/PDDA/CdTe bioconjugates was further verified by microelectrophoresis measurements (expressed as zeta-potential) in Fig. S4.†

Fig. 1B depicts the stepwise procedure of the immunosensor fabrication. In the fabrication, the CNT nanotube-doped PPy was first synthesized through electropolymerization for the immobilization of proteins, which showed good biocompatibility (Fig. S2†) and remarkably conductivity (Fig. S3†). Then, the

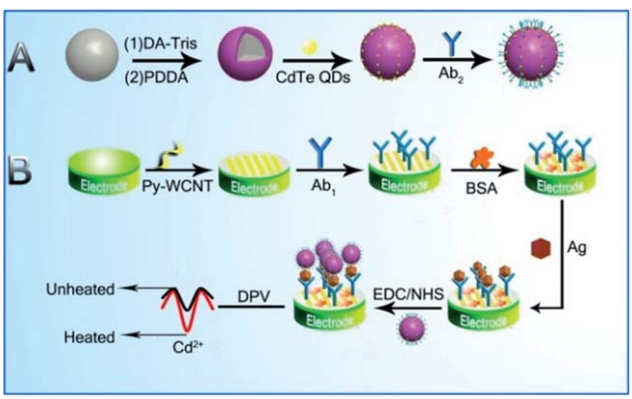

Fig. 1 (A) Assembly procedure of anti-MMP-9/PS@PDA/CdTe-QDs bionanolabel. (B) Schematic representation of the fabrication and measurement process of the sandwich-type immunosensor.

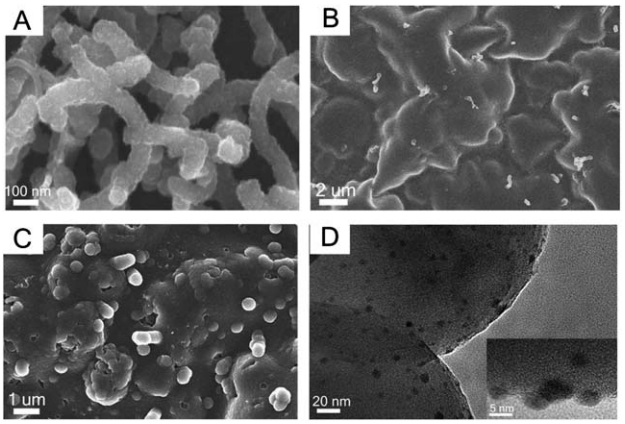

Fig. 2 SEM images of MWCNTs-PPy (A), Ab_1/MWCNTs-PPy (B), Ab_2/Ag/Ab_1/MWCNTs-PPy (C), without ITO chips and TEM image of the prepared PS@PDA/PDDA/CdTe-QDs (D), bottom inset: enlarged HRTEM image of PS@PDA/PDDA/CdTe-QDs. Scale bar: 5 nm.

MWNTs-PPy modified GCE was put into 100 ng mL^{-1} Ab_1 for 12 h. After washing with PBS buffer, the resulting immunosensor was incubated with blocking solution for 1 h to eliminate nonspecific binding and block excess active groups. The fabricated immunosensor was stored at 4 °C when not in use.

The detection was based on the typical procedure for sandwich-type immunoreactions. First, the immunosensor was incubated with a 10 μL drop of Ag standard solution or serum samples with different concentrations for 40 min. After the binding reaction between Ab_1 and Ag was carried out, the immunosensor was incubated with Ab_2/PS@PDA/PDDA/CdTe bioconjugates for 40 min, and washed thoroughly with PBS to remove nonspecifically binding secondary antibodies. Following, the captured labels were dissolved in HNO_3, and the cadmic component acting as the detector target was quantified by differential pulse voltammetry (DPV). Finally, the heated-electrode technique was introduced in the stripping analysis for signal amplification.

The fabrication process was first characterized by cyclic voltammetry (CV) as shown in Fig. 3C. Compared with bare glass carbon electrode (GCE) as shown in Fig. 3C, curve a, the peak current of MWCNTs-PPy/GCE was obviously increased, indicating the MWCNTs-PPy film facilitated the diffusion of the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe towards the electrode surface. When Ab_1 (c), Ag (d) and Ab_2 conjunction (e) were absorbed on the MWNTs-PPy surface, the peak currents were decreased consecutively, which could be ascribed to the successive modifications of insulating proteins against the electron transfer on the electrode. SEM images can give further information on the morphology changes in the modification process (Fig. 2A–C). The MWNTs-PPy nanocomposites were mostly in the form of small bundles or single tubes on GCE (Fig. 2A). After being immersed into Ab_1 for 12 h, the surface became conglobation due to the protein aggregation. Furthermore, when Ab_2 conjugation attached to Ab_1/MWCNTs-PPy, the surface turned to be much rougher and appeared to have some decoration of the anti-MMP-9/PS@PDA/CdTe-QDs bionanolabel.

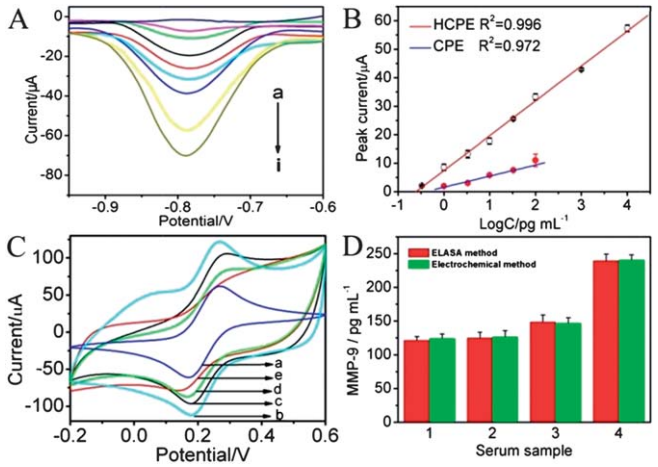

Fig. 3 (A) Typical DPV responses of the immunosensor recorded on HCPE at temperature of 49 °C with MMP-9 concentration ((a)–(i): 0, 0.33, 1, 3.3, 10, 33, 100, 1000, 10 000 pg mL^{-1}). (B) The resulting calibration curves of protein plotted on a semi-log scale (the red line is heated at 49 °C and the blue line is at room temperature about 25 °C). (C) Cyclic voltammograms of bare GCE (a), MWCNTs-PPy/GCE (b), MWCNTs-PPy/GCE/Ab_1 (c), MWCNTs-PPy/GCE/Ab1/BSA/Ag (d), and MWCNTsPPy/GCE/Ab_1/BSA/Ag/Ab_2 (e), in 2 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. (D) Comparison of serum MMP-9 levels determined using electrochemical immunoassay and ELISA method. Property of MWNTs-PPy film.

To assess the sensitivity and quantitative range of the proposed immunoassay, we measured routine samples of different MMP-9 concentrations using the developed sandwich-type format based on HCPE at a temperature of 49 °C. The peak current of the immunosensor on HCPE increased with the increasing MMP-9 concentration in the incubation solution (Fig. 3A). The calibration plots showed a good linear relationship in the range from 0.3 to 10 000 pg mL^{-1} with a correlation coefficient of 0.996. The detection limit (LOD) for MMP-9 was 0.033 pg mL^{-1} (0.36 fM) at a signal-to-noise of 3 (Fig. 3B, the red line). As a comparison, the amperometric response of the developed immunosensor was recorded at room temperature, about 25 °C, by carbon paste electrode (CPE). However, the linear range was only 1.0–100 pg mL^{-1} with LOD of 0.159 pg mL^{-1} (1.73 fM). Particularly, its correlation coefficient reduced to 0.972. Additionally, the magnification range of HPCE was from 2.8 to 5.7 times, compared with CPE in the linear range. The high sensitivity and wide linear range of the proposed immunosensor resulted from the following: (1) the dual signal amplification of QDs nanoprobe and temperature-based signal enhancement using heated-electrode technique, and (2) the high protein binding capability and electrical property of MWNTs-PPy film. Thus, this novel ultra-sensitive immunosensor was enough for practical applications.

The analytical reliability and application potential of the designed immunoassay was investigated by analyzing real samples, in comparison with the ELISA method. The results are listed in Fig. 3D, which shows acceptable results with the RSD of less than 2.1%, indicating feasibility of the proposed method for serum sample. The selectivity, reproducibility and stability of the immunosensor were also evaluated as shown in Fig. S6 (ESI†), indicating good analytical performance.

Conclusions

In summary, a novel ultrasensitive electrochemical immunoassay based on heated electrode technique was successfully developed. Combination of MWNTs-PPy nanocomposites and QDs nanoprobe, as well as the dual amplification technique of HCPE, and the proposed electrochemical immunoassay, exhibits acceptable stability, reproducibility, and accuracy, and excellent performance for the detection of MMP-9. Moreover, the smart method shows attractive performance for accurate clinical disease diagnostics, suggesting potential applications towards the early evaluation of tumor diseases.

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