ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2015, **51**, 3387

Received 28th December 2014,
Accepted 13th January 2015

DOI: 10.1039/c4cc10363g

www.rsc.org/chemcomm

An electrochemical peptide cleavage-based biosensor for prostate specific antigen detection *via* host–guest interaction between ferrocene and β-cyclodextrin†

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Here we have developed a "signal-on" peptide cleavage-based assay to directly transduce the peptide cleavage events into electrochemical signals *via* the host–guest interaction between ferrocene (Fc) and β-cyclodextrin (β-CD) for prostate specific antigen (PSA) detection.

Prostate cancer is a major cause of death in the male population aged over 50 years.[1] Precise and early diagnosis of tumor markers offers the best hope for the treatment of the deadly malignancy.[2] Prostate specific antigen (PSA), a glycoprotein in human serum, has been proved to be the most reliable and specific biomarker for screening prostate cancer.[3] Up to now, the most commonly used detection method for PSA has been based on immunoassays.[4] Although immunoassays exhibit promising sensitivity and specificity, they mostly involved a monoclonal or a polyclonal antibody of PSA tagged with enzymes, fluorophores or nanomaterials.[5] These approaches with complicated preparation and detection processes are often time consuming, inconvenient, expensive and labor-intensive.[6] Furthermore, the antibodies with the inherent drawbacks such as easily denaturing with temperature changes, hard to prepare and difficulty in exposing active sites for antigen binding restrict their application to some extent.[7]

To overcome the disadvantages of traditional antibodies, a short peptide has recently emerged as a more compelling choice than an antibody for protein assays.[8] Peptide showed many advantages over antibodies, such as facile production through chemical synthesis, resistant to harsh environments, small size and cost-effective.[9] Since a specific peptide with the sequence HSSKLQ has been identified as a substrate for measuring PSA enzymatic activity with a high degree of specificity and good stability,[10] several peptide-based methods have been developed for the detection of PSA, such as fluorescence,[11] electrochemiluminescence,[12] and electrochemical assays.[13] Electrochemical methods have been of great interest for applications in peptide-based detection assays owing to their distinct advantages such as speed, sensitivity, and convenience. For instance, Revzin[14] and Kelley[15] groups have constructed some electrochemical sensors in which the peptides are labeled at one end with a redox reporter, most frequently ferrocene or methylene blue (MB), for detecting various biological proteins. These strategies allow the monitoring of the target in a simple and effective fashion. However, the electrochemical measurement monotonously relied on target induced cleavage of the redox tag labeling peptide on an electrode, and such signal-off assays may have an intrinsic limitation on sensitivity and easily get false results as the true ones. Thus, it is necessary to develop a more sensitive and reliable "signal-on" approach for the determination of PSA.

Currently employed signal readout techniques for "signal-on" electrochemical approaches based on peptide-cleavage are rarely developed.[16] It has been a bottleneck for peptide-cleavage assays to transduce the protein amount into an increasing response. To solve this problem, we turned our eyes to the supramolecular host–guest recognition technology. Because the recognition motifs are specific and bioorthogonal, the host–guest interaction has been extensively applied in bioanalysis,[17] and host–guest interaction between β-CD and Fc is the most frequently used one.[18] Since Harada and Takahashi reported the host–guest interaction between β-CD and Fc,[19] Fc and its derivatives have been concentrated on developing inclusion complexes with β-CD, which could enhance their solubility, electro-stability and bioavailability.[20] On the basis of the construction of a simple and feasible electrochemical technique, we used the β-CD to capture the redox tag Fc, which was related to the concentration of the target protein, to construct a "signal-on" electrochemical approach *via* the host–guest interaction.

In this work, we directly converted the peptide cleavage events into electrochemical signals *via* the host–guest interaction between

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† Electronic supplementary information (ESI) available: Experimental section; characterization of the Fc-peptide modified Fe_3O_4@Au biocomplex; and the electroactive surface area of the CNT-PAMAM modified electrode. See DOI: 10.1039/c4cc10363g

Fc and β-CD. The Fc labeling peptide was immobilized onto Fe_3O_4@Au magnetic beads first. In the presence of the target, PSA selectively cut the probing peptide to release much Fc containing peptide fragments, which were further separated from the magnetic beads with a magnet. Through the formation of a host–guest complex with β-CD, the released Fc molecules can be concentrated on the electrode surface to give a detectable electrochemical signal. Moreover, by adopting MWCNT-polyamidoamine dendrimers (CNT-PAMAM) nanohybrids as sensor platforms can not only enhance the immobilization of β-CD for capturing the released Fc, but also facilitate electron transfer from Fc to the electrode surface. Therefore, the proposed biosensor has exhibited excellent performance for the electrochemical detection of PSA with high sensitivity and specificity. The schematic diagram of a stepwise assembled procedure of the sensor and the electrochemical detection principle are shown in Scheme 1 (see ESI† for more experimental details).

A typical electrochemical assay for PSA detection was performed as follows: first, 20 μL of prepared Fe_3O_4@Au nanocomposites was transferred into a 1.5 mL eppendorf tube and mixed with 20 μL of Fc-peptide conjugate for 16 h to obtain the Fe_3O_4@Au–Fc-peptide biocomplex. After magnetic separation to remove the excess reagents, the Fe_3O_4@Au–Fc-peptide biocomplex was incubated with 20 μL of PBS solution containing different concentrations of PSA at room temperature for 50 min. Subsequently, the mixture solution was separated from the biocomplex in a magnetic field, and then dropped onto the prepared GCE/CNT-PAMAM/β-CD functionalized electrode for 1 h. After rinsing with ultrapure water, the electrode was used for the electrochemical measurements.

The CV measurement has been employed to characterize the assembly steps of the sensing interface. As can be seen in Fig. 1A, a well-defined redox peak of $[Fe(CN)_6]^{3-/4-}$ was observed at the bare electrode (curve a). When the electrode was modified with CNT-PAMAM nanohybrids, the peak current was markedly increased, indicating that the CNT-PAMAM nanohybrids could greatly promote the electron transfer. After the CNT-PAMAM nanohybrids were activated by the non-conductive glutaraldehyde (GA) (c), the peak current clearly decreased, implying that the CNT-PAMAM was activated for the covalent coupling of amine terminated β-CD. Subsequently, with the introduction of β-CD, a further decrease of peak current (curve d) was observed, indicating that β-CD was assembled successfully. The differences of the CVs in different modified stages clearly reflected the changes in each stage of the electrode surface.

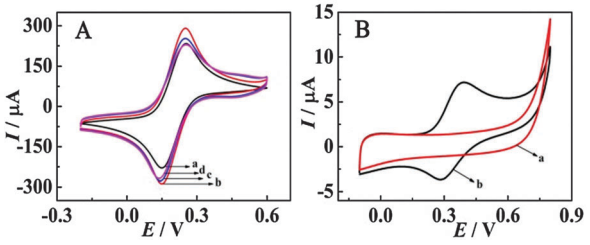
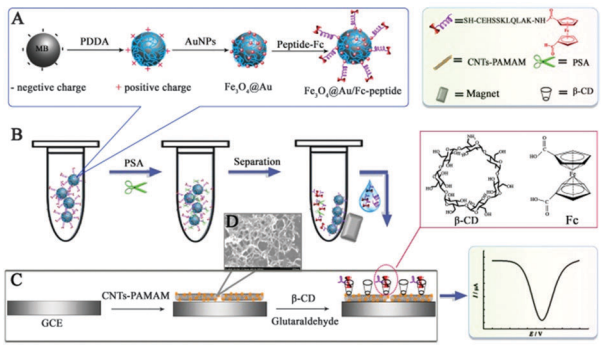


Fig. 1 The electrochemical characterization of the stepwise modified electrodes: CVs of (a) bare GCE; (b) GCE/CNT-PAMAM; (c) GCE/CNT-PAMAM/GA; (d) GCE/CNT-PAMAM/GA/β-CD in 2 mL 5 mM $Fe(CN)_6^{3-/4-}$ solution, scan rate: 100 mV s^{-1} (A); (a) CVs of GCE/CNT-PAMAM/GA/β-CD, (b) response for analyzing 1 ng mL^{-1} PSA in 2 mL PBS (pH 7.4) (B).

Curve a in Fig. 1B shows the CV current of the modified GCE/CNT-PAMAM/GA/β-CD electrode in PBS at 100 mV s^{-1}. After the prepared electrode was incubated with the mixture solution of Fc containing peptide fragments, a stable and obvious peak current (curve b) of Fc was obtained, indicating that Fc molecules were successfully captured onto the electrode surface. This result has confirmed the formation of the Fc–β-CD complex and the supramolecular recognition capability of the β-CD immobilized on the electrode surface to Fc.

To evaluate the sensitivity and the potential quantitative application of the proposal platform, we performed the designed strategy with different concentrations of PSA and the electrochemical responses were recorded by DPV. From Fig. 2A we could see that the reduction peak current increased monotonically with the increasing concentration of PSA, implying that Fc-labeled peptides were digested by PSA and the electroactive molecules (Fc), which were related to the concentration of the target protein, were captured on the electrode by β-CD successfully. The standard calibration curve for PSA detection is shown in Fig. 2B. The dose response curve showed a strong linear dependence of current on the logarithm (lg) of PSA concentration in the range from 0.001 to 30 ng mL^{-1} with a detection limit of 0.78 pg mL^{-1}. The regression equation was $I = -0.94431 \lg c - 8.0889$ with a correlation coefficient of



Scheme 1 Schematic illustration of the electrochemical peptide cleavage-based method for PSA detection using the as-prepared β-CD functionalized electrode: the preparation of the Fe_3O_4@Au–Fc-peptide biocomplex (A); the magnetic separation process (B); the preparation of the functionalized electrode and electrochemical detection of PSA (C); SEM image of CNT-PAMAM nanohybrids (D).

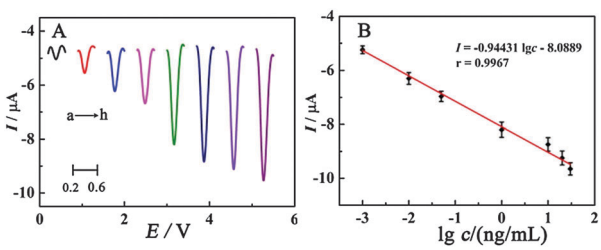


Fig. 2 The DPV of PSA detection at different concentrations (from a to h: 0 ng mL^{-1}, 0.001 ng mL^{-1}, 0.01 ng mL^{-1}, 0.05 ng mL^{-1}, 1 ng mL^{-1}, 10 ng mL^{-1}, 20 ng mL^{-1}, 30 ng mL^{-1}), detection buffer: PBS (pH 7.4) (A); the calibration plot of current intensity *vs.* lg c_{PSA} (B).

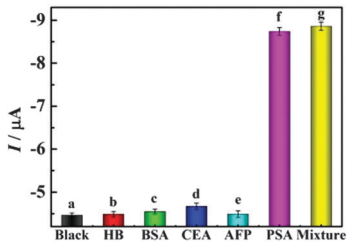


Fig. 3 Specificity of the biosensor toward (a) zero analyte, (b) 10 ng mL^{-1} HB, (c) 10 ng mL^{-1} BSA, (d) 10 ng mL^{-1} CEA, (e) 10 ng mL^{-1} AFP, (f) 1 ng mL^{-1} PSA, (g) 10 ng mL^{-1} BSA + 10 ng mL^{-1} HB + 10 ng mL^{-1} CEA + 10 ng mL^{-1} AFP + 1 ng mL^{-1} PSA.

0.9967. We have also compared the detection limits of the present work with those of some other reported methods. As is shown in Table S1 (see ESI†), our method has a lower detection limit in comparison to the reported methods, which has reconfirmed its high sensitivity. Furthermore, the simple fabrication process is another advantage compared to different PSA immunosensors using special reagents and multistep amplification methods.

To test the specificity of the proposed method, several 10 ng mL^{-1} non-target proteins including BSA, hemoglobin (HB), CEA and AFP were assayed to investigate the specificity of the method. Due to PSA selective cleavage of the probing peptide, the electrochemical signal for the sensing system presented high selectivity as shown in Fig. 3. Only the target protein PSA (1 ng mL^{-1}) could induce a remarkable signal, whereas other potential interferences, which even 10-fold higher concentration than that of PSA, were induced negligible responses. Moreover, the cross-sensitivity of the biosensor in a mixture of the four different substances containing PSA was also examined. The obtained response from the mixture was almost the same as the response obtained from PSA only. The result indicated that the developed method possessed high specificity for PSA detection.

The stability of the proposed assay was evaluated by successive cyclic scans. After the continuous CV measurement of 50 cycles, the response current decreased only about 3.1% of its initial response (Fig. S6, see ESI†). The results suggested that the prepared GCE/CNT-PAMAM/GA/β-CD electrode could maintain favorable electroactivity of Fc and the developed assay used for PSA analysis has satisfying stability. To evaluate the reproducibility of the sensor, each concentration has been measured at least three times. The relative standard deviations could all be within 5%, implying the quite good reproducibility of our method.

The applicability of the proposed method in real samples has also been investigated. The assay of the target PSA in a series of real samples was investigated by detecting PSA in human serum. The standard addition method was employed to evaluate the applicability of the proposed method. Herein, a series of samples were prepared by adding PSA of different concentrations to 10-fold-diluted normal human serum (obtained from Ninth People's Hospital of Chong qing, China). The recoveries were calculated by the found amount/added amount ratio, and the data are given in Table S2. From Table S2 (see ESI†), we could see that the recovery (between 101.2% and 106.3%) and relative standard deviation values (between 4.32% and 6.57%) were acceptable. Notably, the signals measured in serum are somewhat higher than those measured in buffer, which may reveal the presence of PAS in normal human serum (normal value: less than 4 ng mL^{-1} in serum). The measured data indicated that the method may be competent for PSA assay clinical applications.

In conclusion, in our method, the direct transduction of peptide cleavage events into electrochemical signals *via* the host–guest interaction of Fc with β-CD provided a simple, sensitive method for detecting PSA. In addition, the adoption of the β-CD/CNT-PAMAM modified electrode can greatly facilitate the signal acquisition for electrochemical detection, which not only takes advantage of good conductivity but also profits from the strong and selective supramolecular recognition ability of β-CD to Fc. Moreover, this "signal-on" approach could minimize false positive results which were caused by electrode stripping or fouling. Therefore, our sensor has displayed excellent performance in the electrochemical detection of PSA in both reaction buffer and complex serum samples. And thus the proposed method can be expected to hold great potential in clinical prognosis and monitoring.

This work was financially supported by the NNSF of China (21275119, 51473136), the Ministry of Education of China (Project 708073), the Natural Science Foundation of Chongqing City (CSTC-2011BA7003), the China Postdoctoral Science Foundation (2014M550454), the Chongqing Postdoctoral Science Foundation (Xm2014022) and the Fundamental Research Funds for the Central Universities (XDJK2013A27, XDJK2014A012, XDJK2014C138), and China Postdoctoral Science Foundation (2014M550454), China.

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