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Highly sensitive protein detection based on a novel probe with catalytic activity combined with a signal amplification strategy: assay of MDM2 for cancer staging†

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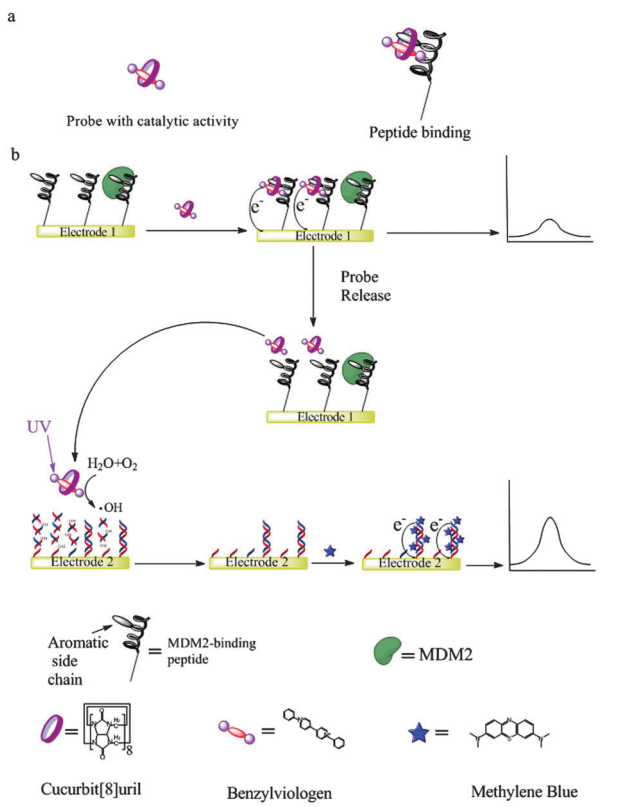
A novel probe with catalytic activity is used for highly sensitive MDM2 detection.

The importance of protein detection in clinical practice and biomedical research has promoted efforts to develop highly sensitive methods for protein assays.[1] Traditionally, antibodies are used as protein-binding elements in such assays. More recently, chemically less complex agents have been proposed as targeting ligands with improved properties.[2] Ligands such as peptides, oligonucleotides and small molecule drugs can be functionally equivalent to antibodies, but the less complex structure of these ligands facilitates chemical synthesis and site-specific modification that are desirable for developing novel sensing methods. Moreover, new detection methods based on these molecules will enable more cost-efficient and sensitive protein assays.

In this work, peptides, a recently emerged type of targeting ligand,[3] are equipped with a novel probe with catalytic activity to achieve high sensitivity of protein detection. In contrast to biologically produced antibodies, peptides are obtained *via* artificial processes such as combinatorial screening and rational design based on molecular structure of proteins. Peptides can selectively recognize and exhibit high affinity to a wide range of targets, including proteins and low molecular weight species. So on the one hand, peptides can serve as protein-binding elements.[3] On the other hand, peptides can also generate signal readout *via* binding with low molecular weight probes.[4] In this study, the probe is designed as an initiator of a signal amplification process, such as a catalytic reaction, so that many strategies can be used to amplify signal readout without interference in the original protein–peptide binding affinities. Here we report the use of a host–guest complex as the probe with catalytic activity (Scheme 1). First, the complex probe can bind strongly and selectively with a peptide *via* host–guest interaction with the aromatic side chain of the peptide.[5] Second, the complex probe is a photocatalyst that can efficiently catalyze the cleavage of dsDNAs under UV excitation.[6] So binding of the target protein can be catalytically transduced into amplified signal readout by



Scheme 1 (a) The probe with catalytic activity used in this work and its binding with the peptide. (b) The principle of MDM2 detection. Not drawn to scale.

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quantifying the remainder of dsDNAs immobilized on an electrode surface after catalytic photo-cleavage. Compared with *G*-quadruplex-based artificial peroxidase and horseradish peroxidase (HRP), the cucurbituril inclusion complex packs both the functions of peptide binding and signal amplification into a much smaller molecular framework.

As a proof-of-concept, Murine Double Minute 2 (MDM2) has been selected as the model target. It is known that deregulation of MDM2 in tumors of variant origins may promote their malignant transformation by overriding normal cell cycle signaling,[7] and the oncogenic function of MDM2 is realized by binding with p53. Studies on the MDM2–p53 interaction have yielded an MDM2-binding peptide particularly suitable for our proposal.[8] As shown in Scheme 1b, the MDM2-binding peptide contains both the binding site of MDM2 and the binding site of the probe. But the latter site is within the former, so the MDM2-binding peptide cannot simultaneously bind with MDM2 and the probe. In the detection procedure, the peptides are first immobilized on an electrode surface following our previously established method.[9] After incubation with the test sample containing MDM2, a fraction of the peptides become MDM2-bound. Then, the MDM2-free peptides bind with the probes *via* host–guest interaction, and the electrochemical response of the probes can be directly used to detect MDM2, the signal readout is inversely proportional to the amount of MDM2.

To obtain signals positively correlated with MDM2 concentration and to improve the sensitivity of MDM2 detection, the photocatalytic properties of the probe are then exploited in this study. Firstly, the probes bound with the MDM2-free peptides are electrochemically detached and collected in a solution. Under UV excitation, the probes with catalytic activity collected in the solution can generate hydroxyl free radicals, which can efficiently cleave the dsDNAs immobilized on another electrode surface. Consequently, by using methylene blue as the electroactive species, the target protein can be quantified based on the detection of the remaining dsDNAs. Because the amount of the immobilized dsDNAs is fixed, and the amount of probes bound with the MDM2-free peptides is inversely proportional to the amount of MDM2, the amount of remaining dsDNAs after cleavage is in turn proportional to the amount of MDM2. Moreover, since a little variance in the MDM2 amount can be catalytically amplified into a major difference in the readout signal of methylene blue, a highly sensitive method for the detection of the target protein is possible.

To realize the above depicted assay, binding of the probes with the peptides is first verified (Fig. S1a, ESI†). The concentration of the probe and the incubation time of the peptide modified electrode with the probe have been optimized using square wave voltammetry (Fig. S2 and S3, ESI†). So, 16 h incubation time with 750 μM probe is selected for the subsequent experiments. Incubation time for MDM2 binding has been optimized as well (Fig. S4, ESI†), and 2 h incubation is sufficient for MDM2–peptide binding. Under the optimized conditions, the readout signal of the probes bound with the MDM2-free peptides is inversely proportional to MDM2 concentration (Fig. S1b, ESI†), in accord with the above-described principle of detection. And Fig. S1b (ESI†) also shows that the detection without amplification is in the ng ml^{-1} range.

The catalytic properties of the probe are then employed to enable more sensitive detection of the target protein. To efficiently catalyze DNA photo-cleavage, the probes are electrochemically detached from the peptide-modified electrode surface and collected in a solution. For the accuracy of the assay, it is crucial that the probes collected in solution can still replicate the inverse proportion between MDM2 concentration and the amount of probes bound with the MDM2-free peptides. So, it has been first of all confirmed by the obtained SWVs of the probes in solution, after thorough electrochemical detachment (Fig. S1c, ESI†).

To make a good use of the probe, the catalytic photo-cleavage of DNA by the probe is thus investigated in more detail. Extensive catalytic photo-cleavage can greatly reduce the background response; however, since UV excitation can spontaneously generate low levels of hydroxyl radicals, extensive catalytic photo-cleavage is also accompanied by greater false negative (Fig. S5, ESI†). To this end, the cleavage time is adjusted to provide the lowest possible background with no apparent false negative (Fig. S6a and b, ESI†). As shown in Fig. S6c (ESI†), after 40 min, catalytic photo-cleavage is accomplished, while the false negative is insignificant, so 40 min cleavage time is selected for the subsequent experiments.

By using the catalytic photo-cleavage activity of the probe to amplify the readout signal, the peak currents of the obtained SWV responses show a gradual increase along with a very small amount of MDM2, and a linear calibration range can be established from 10 pg ml^{-1} to 60 pg ml^{-1} (Fig. 1). So, the high sensitivity of our method can be of great help to its application by reducing the amount of test sample required. The standard deviations of repetitions are all below 3%, therefore the repeatability of our method is satisfactory. Besides, our method also has good selectivity, since all control species result in response nearly the same as the background response (Fig. 2). Specifically, interference of a biological matrix is investigated. Since our assay is for MDM2 quantification in

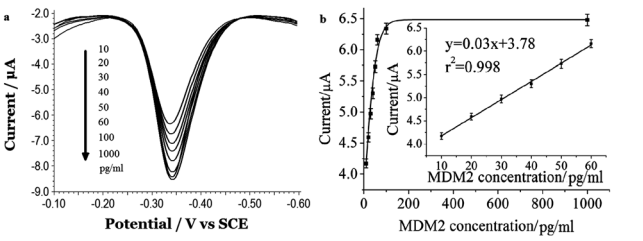

Fig. 1 (a) Square wave voltammograms (SWVs) obtained at the dsDNA-modified electrode for the measurement of MDM2 concentration. The dsDNA-modified electrode first underwent catalytic photo-cleavage and was then treated with 0.1 mM methylene blue. The photo-cleavage is catalyzed by the probes electrochemically detached from a peptide-modified electrode that has been previously treated in sequence with different concentrations of MDM2 and 750 μM probe. Panel (b) shows the peak currents of SWVs in (a) as a function of MDM2 concentration. The inset shows the linear range and the corresponding regression equation. Error bars indicate standard deviation from average (n = 3). (It is worth noting that although the response of 10 pg ml^{-1} is evidently much higher than the background shown in Fig. 2, lower concentration has a deviation >3%, due to dilution error inevitable at such low concentrations, so these data are unreliable and discarded.)

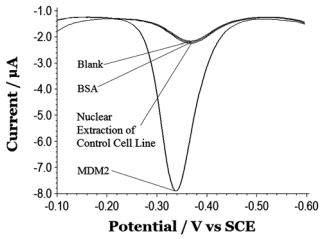

Fig. 2 SWVs obtained at the dsDNA modified electrode to show the interference of control species. All conditions are the same as in Fig. 1a, except that a standard MDM2 sample and several control samples are incubated with the peptide-modified electrode. BSA and MDM2 are 1000 pg ml^{-1}. 10 mM PBS (pH 7.4) is used as the blank control.

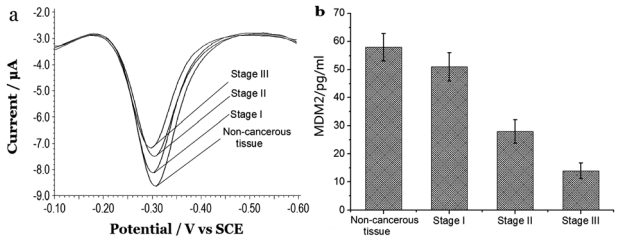

Fig. 3 (a) SWVs obtained at the dsDNA modified electrode for the measurement of MDM2 in real biological samples. All conditions are the same as in Fig. 1a, except that real biological samples are incubated with the peptide-modified electrode. The biological sample is fractioned from tissues of NSCLC victims at different stages ($n \geq 3$). (b) Expression of MDM2 in different stages of NSCLC, non-cancerous tissue is also sampled as reference. The amount of MDM2 is calculated from the peak current in panel (a) *via* the calibration curve in Fig. 2(b).

non-small cell lung cancer (NSCLC), the nuclear fraction of MDM2-silenced NSCLC cells has been used as a control target. Our result shows that the blank nuclear extraction has no significant interference in our method.

The efficiency of our method in cancer staging has also been evaluated by quantifying MDM2 in cancerous biopsy samples from NSCLC victims. As shown in Fig. 3, advancement of NSCLC inversely correlates with MDM2 expression, consistent with previous reports using antibody-based methods.[7] It is worth noting that in the 100× diluted tissue samples used in our assay, the amount of MDM2 is very low. Especially in the samples from advanced stage NSCLC, MDM2 quantity is significantly lower than the limit of detection of the commercially available ELISA kit (129 pg ml^{-1}).[10] But our method can still properly determine it by virtue of the probe and the amplification strategy. Therefore, compared with the conventional methods, our method requires much less sample, which can greatly alleviate the pain of the cancer victim in biopsy.

In this work, a novel probe with catalytic activity is proposed for protein assay. The probe is combined with a signal amplification strategy to achieve highly sensitive detection of the target protein. The probe can bind strongly and specifically with the protein-binding peptide and amplify the signal readout by catalyzing a photo-cleavage reaction. The catalytic signal amplification can provide a pg ml^{-1} detection range which is unattainable using commercially available ELISA assays for MDM2 assay. Additionally, since the only requirement for the binding of the probe with the protein-binding peptide is an aromatic amino acid, which exists in almost all protein-binding peptides, the proposed method possesses the potential as a universally applicable strategy for sensitive detection of many kinds of proteins.

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