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Colorimetric assay for protein detection based on "nano-pumpkin" induced aggregation of peptide-decorated gold nanoparticles

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ABSTRACT

Small peptide can be used as an effective biological recognition element and provide an alternative tool for protein detection. However, the development of peptide-based detecting strategy still remains elusive due to the difficulty of signal transduction. Herein, we report a peptide-based colorimetric strategy for the detection of disease biomarker by using vascular endothelial growth factor receptor 1 (Flt-1) as an example. In this strategy, N-terminal aromatic residue-containing peptide modified gold nanoparticles (GNPs) can form bulky aggregate by the introduction of cucurbit[8]uril (CB[8]) that can selectively accommodate two N-terminal aromatic residue of peptides simultaneously regardless of their sequences. However, in the presence of Flt-1, the peptide can specifically bind to the protein molecule and the N-terminal aromatic residue will be occupied, resulting in little aggregation of GNPs. By taking advantage of the highly affinitive peptide and efficiency cross-linking effect of CB[8] to GNPs, colorimetric assay for protein detection can be achieved with a detection limit of 0.2 nM, which is comparable with traditional methods. The feasibility of our method has also been demonstrated in spiked serum sample, indicating potential application in the future.

1. Introduction

As is well known, the practical approaches for protein detection are still mainly dominated by enzyme-linked immunosorbent assay (ELISA) (Brennan et al., 2010; Hu et al., 2012; De la Rica and Stevens, 2013). However, this technique is largely limited by the selection and production of high-quality antibodies, which are expensive, low yield and have easy denaturation. Recently, *in vitro* screen technique has been used to obtain amounts of small peptides that show high specificity and affinity toward a wide spectrum of proteins. Compared with antibodies, these small peptides have their unique advantages as recognition elements (Li et al., 2013a, 2013b, 2013c, 2014b). For example, small peptide could be easily, economically, and with high-throughput obtained by a solid phase peptide synthesis (SPPS) technique, while the acquisition and purity of antibody is rather cumbersome and time-consuming. Besides, Small peptide, with simple and defined chemical structure, is easy to modify according to given circumstances, while antibody, which is a biological macromolecule usually cannot be arbitrarily modified without impairing its affinity (Akter et al., 2012). In addition, small peptide is highly stable and easily keeps its activity in many harsh conditions (high temperature, unfavorable pH, etc.). On the other hand, although aptamers may be a strong and versatile alternative to antibodies, they have been selected in limited quantities (Song et al., 2012), which have greatly restricted their practical application. By contrast, a great many peptides have been available which can bind to certain proteins with extremely high specificity and affinity (Rice et al., 2006).

Gold nanoparticle (GNP), a nanomaterial with excellent biocompatibility and extremely high extinction coefficient, has been demonstrated to be a highly competitive biosensing platform due to its interesting optical properties. In this laboratory, a collection of work based on GNPs for the detection of DNA, proteins, and small molecules has been reported (Li et al., 2014a; Liu et al., 2010; Gao et al., 2013). Despite the impressive superiority of the GNP-based strategies, unfortunately, the development of the colorimetric assays using peptides as recognition elements still remains challenging (Pavan and Berti, 2012), owing to the two facts: (1) it is still hard to select multiple peptides that specifically bind to one certain protein target, thus hardly employing the traditional "sandwich" structure for peptide-based colorimetric strategy; (2) it is still hard to precisely assemble peptides-based nanostructure, so many ripe DNA-based strategies are not suitable for

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peptide-based colorimetric assays. Therefore, there is still a bottleneck for peptide elements being integrated into simple color-based methods, which largely restrict their further application in biological detection.

Herein, we propose a novel peptide-based colorimetric approach for protein detection, which may provide a solution to the above mentioned problems. Cucurbit[n]uril (CB[*n*]s), a pumpkin-shaped macrocyclic molecule, has attracted considerable attention on account of their unique chemical structure and exceptional molecular recognition properties (Zhang et al., 2012; Wittenberg et al., 2013). As one member of the CB[*n*]s family, CB[8] can selectively accommodate two N-terminal aromatic residues of peptides regardless of the sequence (Heitmann et al., 2006). Based on this characteristic, it is reasonable to consider that CB[8] can induce aggregation of N-terminal aromatic residue-containing peptide modified GNPs and then a colorimetric assay may be established. Specifically, in the presence of CB[8], peptides with N-terminal aromatic residue will be dimerized and then lead to the aggregation of peptide-coated GNPs. However, when the peptides bind to corresponding protein targets, the aromatic residue sites will be occupied, leading to the failure of CB[8] interaction and the corresponding aggregation of GNPs. In this work, we have also employed a well-recognized tumor marker, vascular endothelial growth factor receptor 1 (Flt-1) (An et al., 2004), to demonstrate the detection capability of our proposed method. Flt-1 is not only a well predictor for treatment response of bevacizumab, which is the first validated angiogenesis inhibitor and has proven to effectively suppress metastatic disease progression (Lambrechts et al., 2013), but also a well-recognized protein marker which is overexpressed in Myelodysplastic syndromes (MDS) (Hu et al., 2004; Kristýna et al., 2012; Schuch et al., 2002). Current approaches for the detection of Flt-1 are based on immunological methods, thus these methods use antibodies to capture the protein and fluorescent labels or enzymatic amplification to visualize the antibody–protein binding. The high-quality antibodies are expensive, low yield, have easy denaturation and the detection of Flt-1 *via* fluorescent labels or enzymatic amplification needs expensive reagents and measurement instruments (Kristýna et al., 2012). Our peptide-based colorimetric approach presents a powerful optical biosensor technology which avoids those problems. In addition, our method may also have universal applicability, since the basic requirement of our method is the formation of supramolecular complex conducted by CB[8], which can be achieved just by employing peptides with aromatic residue at its amino end.

2. Experimental section

2.1. Materials

Peptide [H_2N-WHSDMEWWYLLGGGGGC-COOH, Lyophilized powder, purity $>95\%$] was synthesized by China Peptides Co., Ltd. Recombinant human Flt-1 was purchased from Sino Biological Inc. Cucurbit[8]urils was obtained from Sigma-Aldrich Chemical Co. $HAuCl_4 \cdot 3H_2O$ was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Other chemicals used in this work were of analytical grade and used as received. The stock solution of both peptide and Flt-1 was prepared by dissolving the powder with 0.1 M phosphate buffer solution (PBS, pH 6.2). All aqueous solutions were made using ultrapure water, which was prepared with a Milli-Q purification system to a specific resistance of $>18\ M\Omega$ cm.

For the detection of Flt-1 in complex biological sample, venous blood samples from healthy volunteers were collected. This investigation was approved by the local ethics committee. Blood samples were separated by centrifugation and the serum was collected and diluted. The final serum sample (1% in 0.1 M PBS) was spiked with different concentrations of Flt-1 (1 nM, 10 nM and 100 nM respectively).

2.2. Preparation of GNPs and peptide-modified GNPs

Gold nanoparticles with an average diameter of 13 ± 2 nm were prepared by citrate reduction of $HAuCl_4$. Trisodium citrate (5 mL, 38.8 mM) was first added rapidly to boiling solution of $HAuCl_4$ (50 mL, 1 mM) with fierce stirring. The change in the color of the solution can be observed from pale yellow to deep red within 1 min. Then the solution was boiled while stirring for another 30 min to ensure that the reaction is complete. Finally, the solution was allowed slowly to cool to room temperature and was ready for use.

The prepared gold nanoparticles were incubated with peptide stock solution (the final concentration is 0.25 mM) containing 0.1% w/v BSA at 4 °C overnight. BSA was used to stabilize GNPs and minimize any non-specific adsorption. To remove any unbound peptides, the solution was centrifuged three times at 13,500*g* for 30 min. The red oily precipitate was then dispersed in 1 mL solution (0.1 M PBS at pH 6.2) and stored at 4 °C.

2.3. Colorimetric analysis

Peptide-modified GNPs were incubated with target protein Flt-1 with different concentrations in 0.1 M PBS (pH 6.2) for 0.5 h. Afterward, CB[8] solution was added to the mixture (the final concentration is 0.32 mM) for 1.5 h. Then UV–vis absorbance measurements can be performed. All experimental procedures were carried out at room temperature.

2.4. Instruments

All absorbance spectra were performed by UV–vis spectroscopy (UV 2450, Shimadzu, Japan). The TEM measurements were performed using a JEM-200CX transmission electron microscope operating at 200 kV (JEOL, Japan).

3. Results and discussion

3.1. Sensor design

The peptide (H_2N-WHSDMEWWYLLG-GGGGC-COOH) employed in this work contains three functional regions: 1) Flt-1 binding motif (WHSDMEWWYLLG) at the N-terminus; 2) hydrophobic spacing region (GGGG) in the middle, reducing the steric hindrance between protein molecule and gold surface; and 3) a cysteine residue (C) bearing one thiol group at the C-terminus, facilitating the modification onto GNPs. The basic principle of our method is shown in Scheme 1. In the presence of CB[8], two aromatic residues from the peptide can be selectively accommodated in the CB[8] cavity, resulting in the aggregation of the peptide-coated GNPs. However, when the target protein is pre-incubated with the GNPs and is then exposed to the CB[8], no aggregation can be formed and the colloid still remains red. This is because aromatic residue sites are occupied due to the specific binding between Flt-1 and peptide, thus no free aromatic residues can induce the aggregation events. Therefore, a novel method for protein assay based on peptide and GNPs can be developed.

UV–vis absorption spectra are firstly used to verify the feasibility of the method, and the color changes in different conditions are shown in Fig. 1. Compared with the typical characteristic absorption peak of bare GNPs (Fig. 1A, curve a), an 8 nm shift (from 520 nm to 528 nm) can be observed after introducing peptide to