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Horseradish peroxidase and aptamer dual-functionalized nanoprobe for the amplification detection of alpha-methylacyl-CoA racemase

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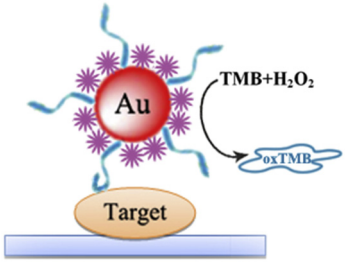
HIGHLIGHTS

- A simple, high sensitive and effective assay was developed based on HRP and aptamer dual-functionalized nanoprobe.
- The detection limit is about 4.6 pg mL^{-1}, which is three orders of magnitude lower than that of fluorescence aptasensor.
- This assay shows high selectivity toward AMACR against other non-specific proteins.
- The assay avoids the conjugation between recognition element and the catalytic enzyme, making it more simple and effective.

GRAPHICAL ABSTRACT



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ABSTRACT

Alpha-methylacyl-CoA racemase (AMACR) is over-expressed in many cancer types and can serve as a novel diagnostic biomarker. Development of convenient and sensitive detection methods of AMACR is of particular importance for cancer diagnosis. Aptamers are a type of recognition elements, which possess many advantages over antibody, making them suitable for applications in biosensing and biotechnology. In this work, we use the efficient surface modification of gold nanoparticles (AuNPs) to prepare the horseradish peroxidase (HRP) and aptamer dual-functionalized nanoprobe. The immobilization of HRP and thiol-terminated aptamer on the surface of AuNPs can be achieved through electrostatic interaction and the formation of Au–S bond, respectively. This nanoprobe, which is used as discriminating and catalytic probe, can be combined with enzyme immunoassay method to increase the detection sensitivity of AMACR. The detection limit can reach as low as 4.6 pg mL^{-1} due to the dual signal amplification from enzymatic cycling and the high loading of enzymes on AuNPs. This sensitivity is about three orders of magnitude higher than that of AMACR aptamer based fluorescence method, which is also comparable to or one order of magnitude higher than that of ELISA. Furthermore, this method is more simple and effective, which not only avoids the conjugation between recognition element and the catalytic enzyme,

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but also achieves greater signal amplification. This assay could be used as a sensitive and selective platform for the detection of target protein.

1. Introduction

AMACR is a mitochondrial and peroxisomal enzyme that plays an important role in beta-oxidation of branched-chained fatty acids through the inter conversion between the (2R)- and (2S)- methyl branched-chain fatty acyl-CoAs [1–3]. It is reported that AMACR is abundantly expressed in prostate cancer, the concentration of which is even increased in premalignant lesions [4–6]. Some other studies have reported that the overexpression of AMACR can also occurs in hepatocellular carcinoma [7–9], breast cancer [10], renal cancer [11] and other cancers [12]. Since it has been proven that AMACR can serve as a novel diagnostic biomarker of cancer, the sensitive and selective determination of AMACR is of particular importance in cancer diagnosis.

The most commonly used methods for protein detection is based on the specific interaction between antibody and antigen [13,14], such as ELISA (enzyme-linked immunosorbent assay). However, antibodies are usually costly and may easily degrade or inactivate under unfavorable environmental conditions. Aptamers are another type of recognizing molecules to the protein, which are isolated from random-sequence DNA or RNA libraries by an in vitro selection process termed SELEX (systematic evolution of ligands by exponential enrichment) [15,16]. Compared to antibodies, aptamers possess a number of advantages, such as simple synthesis, good stability, flexible design and wide applicability, making them suitable for applications in biosensing and biotechnology [17–20]. For example, Chen and co-workers have selected DNA aptamers targeting AMACR by single-bead SELEX, which can be further applied to the fluorescent detection of AMACR [6]. In recent years, many highly sensitive aptamer-based assays have been proposed for the detection of protein via different enzymes based signal amplification [21–24]. Although exhibiting high sensitivity, these assays require complicated experimental design or operation. Furthermore, they are often based on aptamer conformational changes, which may easily lead to false-positive results.

With the rapid development of nanotechnology, nanomaterials have received more and more attention over the past decade [25–27]. AuNPs are one of the most typical nanomaterials, which have advantages such as easy and rapid synthesis, efficient surface modification and excellent biocompatibility [28–30]. The properties such as surface chemistry, optical and electrochemical properties have made AuNPs to be widely applied in biomedicine and biosensing. Despite numerous advances have been made in AuNPs based assays, a number of constraints still exist in these methods, such as limited sensitivity and selectivity. To overcome these drawbacks, the incorporation of enzymes into the detection systems has been widely studied [31,32].

It is worth mentioning that the special surface chemistry of AuNPs makes them easily functionalized with other biomolecules containing special functional groups such as thiol and amine [32–34]. For example, AuNPs allow special immobilization protocols via Au–S binding for thiolated aptamers, proteins and low molecular mercaptans. Meanwhile, proteins can be absorbed on the surface of AuNPs and retains their biochemical activity. Thus, AuNPs can be also used to load high amounts of enzymes for biosensing [35–37]. Among these enzymes, horseradish peroxidase (HRP) has been commercially used in various assays, which is even the cheapest one. In this paper, we use the efficient surface modification of AuNPs to prepare the HRP and aptamer dual-functionalized nanoprobe, which can be combined with enzyme immunoassay method to develop a new assay for the amplification detection of AMACR.

2. Materials and methods

2.1. Reagents and apparatus

HRP, 3, 3′, 5, 5′-tetramethylbenzidine (TMB), Chloroauric acid ($HAuCl_4$), bovine serum albumin (BSA), Hemoglobin (Hb), Human serum albumin (HSA) and Tween-20 were purchased from Sigma–Aldrich Chemical Co. (USA). 96 microplates were purchased from Costar Co. (USA). AMACR was purchased from Origene Technologies Inc. (USA). Glypican-3 (GPC3) was purchased from Sino biological Inc. (China). Phosphate buffer solution (6.7 mM PBS, pH 7.4) using for protein dilution and coating was supplied by Hyclone Co. (USA). All other reagents were of analytical grade and obtained from Sinopharm Chemical Reagent co., Ltd. (China). Ultrapure water obtained from a Millipore water purification system (Milli-Q) was used throughout all the experiments. The thiol-terminated aptamer designed in this study was synthesized by Shanghai Sangon Biotechnology Co. (China), which was purified by high-performance liquid chromatography (HPLC). The sequence of thiol-terminated aptamer is as follows: 5′-CCC TAC GGC GCT AAC CCA TGC TAC GAA TTC GTT GTT AAA CAA TAG GCC ACC GTG CTA CAA-A_{18}-SH-3′. It should be noted that the underlined letters of this sequence are the aptamer sequence of AMACR. The extra bases added to the 5′- end of this sequence are designed to reduce the impact of steric hindrance of aptamer recognition.

The absorbance signals of the system were all measured by microplate reader (Spectra Max M5, Molecular Devices). The morphology of the AuNPs was observed by using a JEM-2010 electron microscope (JEOL, Japan). The particle size was determined by dynamic light scattering (DLS) using the Zetasizer Nano ZS (Malvern Instruments, UK).

2.2. Preparation of AuNPs and dual-functionalized AuNPs

Firstly, AuNPs with approximately 13 nm in diameter were prepared using methods as previously described. In brief, 50 mL of 1 mM $HAuCl_4$ aqueous solution were heated to boiling and vigorously stirred. Then, 5 mL of 38.8 mM trisodium citrate were added quickly into this solution, resulting in the color change from pale yellow to deep red. The solution was kept boiling and stirring for another 20 min, and then allowed to slowly cool down to room temperature. The morphology and size of AuNPs was verified by TEM and DLS. The concentration of AuNPs was estimated to be 13.8 nM by UV–Vis spectroscopy, which was calculated based on an extinction coefficient of 2.7×10^8 M^{-1} cm^{-1} at 520 nm for 13 nm AuNPs.

Aptamer and HRP dual-functionalized AuNPs were prepared as follows. Firstly, the pH value of AuNPs solution was adjusted to 9.0 by adding Na_2CO_3. Then, 20 μL of 1 mg mL^{-1} HRP were added to 1.0 mL pH-adjusted AuNPs solution. After reaction for 1 h at room temperature, this mixture was concentrated 5-fold and then