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Highlights

- A new colorimetric aptasensor for the detection of CEA was developed.
- Aptamer-based colorimetric method with nanoparticle was used for the detection of CEA
- The colorimetric aptsensor was potential in other detecton of protein or nucleotides.

Simplified aptamer-based colorimetric method using unmodified gold nanoparticles for the detection of Carcinoma Embryonic Antigen

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Abstract

Here, we described a simple and sensitive colorimetric method for the detection of Carcinoma Embryonic Antigen (CEA), which was based on the phenomenon of salt-induced gold nanoparticles (AuNPs) aggregation and the conformation change of CEA's single-stranded DNA aptamer modified with sulfhydryl group. Because of the reaction between the thiol group and AuNPs, the aptamer could bind to the surface of AuNPs strongly. AuNPs modified with aptamer had a good stability in a high concentration of saline solution due to the repulsion of negative charge among the aptamer. In the presence of CEA, the aptamer trended to form a CEA-aptamer duplex instead of binding to the surface of AuNPs. As a result, the AuNPs changed the color from red to blue easily due to the salt-induced aggregation in the presence of CEA. Accordingly, the CEA was detected in this method. By adjusting the addition amount of the aptamer and NaCl, a sensitive linear range for CEA was found. Under the optimum conditions, the detection limit for CEA was 3 ng/mL. This rapid label-free

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assay using colorimetric method showed high sensitive for CEA detection, which made it a practical way in some cancer biomarker analysis.

Introduction

CEA is an intracellular adhesion glycoprotein known as the tumor-associated antigens[1]. As time goes by, nowadays CEA is usually used as tumor marker connected with much human cancer such as colorectal, gastric, pancreatic, and cervical[2-5]. According to the update research results, when the CEA level in the blood comes to 20 $ng \cdot mL^{-1}$, there is a big chance that the person suffers from a cancer[6]. Thus, a sensitive and precise method to detect the CEA is urgently needed.

To diagnose the disease as early as possible, many methods have been used for the determination of CEA including enzyme linked fluorescent assay[7], micropartical enzyme immunoassay[8], radioimmunoassays[9-10] and chemiluminescence assays[11]. In spite of high specificity and sensitivity, these methods are expensive, time consuming and laborious, which may be critical limitations in some environments. In order to improve biomedical applications, developing a simple, fast, and sensitive method to detect CEA is desirable. In particular, such a simple yes/no research answer would be practical for diagnosis of the cancer in developing area where healthcare facilities are relative inferior[12].

In this view, colorimetric detection is a very attractive method for its rapid visual assay on the spot without the need of any precise equipment[13]. Recently, the use of the gold nanoparticles (AuNPs) has opened a new window to provide a visual detection

of many diseases. AuNPs are spheres with a typical diameter range from 2 nm to 50 nm[14]. The surface plasmon absorption, which makes the AuNPs absorb and scatter more than those of conventional dyes, is responsible for their intense color change[15]. For the AuNPs, the color changes from red to steel-blue when the colloidal AuNPs is brought about by the transition from dispersed to aggregated states, with the corresponding shift of surface plasmon absorption, which can be observed by naked eyes[16-18]. These unique optical properties have allowed the application of AuNPs in simple and rapid colorimetric assays to offer more specificity and selectivity than the current methods. And their non-interference with other biomaterials makes them the most promising indicator for analytical and diagnostic studies.

Recently, aptamer has attracted strong interest because of its competitive advantages compared with other biological tools[19]. Aptamers are single-stranded oligonucleotides (DNA or RNA) that are selected by a process known as in vitro systematic evolution of ligands by exponential enrichment (SELEX)[20]. Moreover, they can bind to their targets with high affinity and specificity[21]. It has been proved that using either unlabeled or labeled AuNPs as colorimetric reporters, unfolded single strand deoxyribonucleic acid (ssDNA) can be well discriminated from the folded ssDNA or the double strand DNA (dsDNA)[22-23]. Hence, a variety of AuNPs-based colorimetic methods have been developed for the recognition and detection of target molecules including protein[24], drugs[25], small molecules[26], inorganic ions[27] and even cells. And compared with the aptamer-modified AuNPs-based methods that involve lengthy and complicated steps, such as modifying the aptamer onto the AuNPs and

separating the modified AuNPs from the unmodified AuNPs or excess aptamer, the unmodified AuNPs-based colorimetric assays that require no modification or separation steps is more attractive[28].

In the study, the method was based on the competing reaction of aptamer with the AuNPs and CEA. On the one hand, the aptamer with a thiol group at one end could bind to AuNPs, which increased the negative charge on the AuNPs colloid leading to a result that the repulsion among the particles was improved, preventing aggregation of AuNPs in a saline solution. On the other hand, the aptamer that was short single-stranded nucleic acid oligomers and could form a specific and firm three-dimensional structure to combine with CEA[29]. Therefore, when AuNPs were added to a solution containing CEA and aptamer, because of the combinative structure between CEA and aptamer, aptamer was not free to bind to AuNPs to stabilize the AuNPs. Under this condition, AuNPs cannot be stabilized at high concentration of NaCl and aggregates, accompanied with the color change from red to blue. While in the absence of CEA, aptamer was free to bind to AuNPs to stabilize the AuNPs, thus preventing their aggregation after adding NaCl, so the color remained red. The obvious distinction between the absence and presence of CEA made it rapid and naked visible to detect CEA. Although ELISA based methods have recently become very popular, they require a stable source of antibodies Moreover, preparation of antibody is laborious, expensive, and time-consuming. Compare with the ELISA, our method is one of the few that protein can be detected by the simple “mix and detect” fashion, which greatly decreases the operating difficulty. It is also the first time to

adopt a AuNPs-based colorimetric method to detect CEA, which may be popular in the real application because of its simple, fast, and economical properties.

Moreover, this assay may have application in diagnostic assay for other proteins or macromolecules by changing the aptamer, which provides another method in this field.

Materials and methods

2.1 Reagents and apparatus

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate, CEA and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (USA). All other reagents were of analytical grade. Oligonucleotides was synthesized and purified by Sangon (Shanghai, China). The base sequences of the oligonucleotides used in this work is listed as follow:

aptamer : 3'-SH-ATACCAGCTTATTCAATT-5'[30]

The solution containing CEA and aptamer was kept at 37□ in the Biochemical Incubator PSH500A (Yinhe, Chongqing), then stirred on the Magnetic Stirring Apparatus (Sile, Shanghai). Absorption spectra were recorded on a UV-3300 spectrophotometer (Japan) at a room temperature.

2.2 Preparation of gold nanoparticles

AuNPs were prepared according to the method reported previously[31-32]. Briefly, 100 mL aqueous solution of hydrogen tetrachloroaurate trihydrate (1 mM) was heated

to boiling while being string in a roung-bottom flask. Then 10 mL of trisodium citrate (38.8 mM) was added into the solution rapidly and the solution was boiled for another 15 min with color of the solution changing from dark blue to wine red. After that, heating the solution was stopped but was stirred until it cooled down to room temperature. Then the prepared colloid AuNPs were stored in brown glass bottles at 4℃. The final AuNPs had an average diameter of approximately 13 nm which was characterized by Transmission Electron Microscope (TEM) as shown in Fig. 2A.

2.3 The preparation of aptamer solution

To prepare the aptamer solution, the aptamer was dissolved in 0.1 M phosphate-buffered saline (PBS, PH=7.4, hybridization buffer) to 10 μM solution. Then 30 μL aptamer (10 μM) solutions and 15 μL TCEP (10 μM) were added into the samples containing different concentration that ranges from 0 to 120 ng CEA. Herein, TCEP is a reducing agent, intended to disrupt any disulfide bonds (-S-S-) and ensure that the free –SH groups are ready to react with the gold surface. To have an adequate combination of CEA and aptamer, after stirring the mixed solution until blended well, put them in the Biochemical Incubator PSH500A at 37 □ for 1 h.

2.4 AuNPs-based colorimetirc method

The mixed solution above was added into 500 μL AuNPs solution separately and stirred in an appropriate speed for 1 h. Different amount of aptamer bound to AuNPs to stabilize the AuNPs colloid. Followed by the addition of NaCl into these solutions,

these solutions presented color gradient from red to blue. 100 μL of solution was placed into a quartz cuvette. Then 200 μL of water was added before UV-vis measurement.

Result and discussion

3.1 Overall strategy

In our system, the AuNPs in solution were stabilized by adsorbed negative ions (citrate). Their repulsion prevented the strong van der Waals attraction between AuNPs from causing them to aggregate. However, high concentrations of salt will screen the charge on the surface of AuNPs, resulting the aggregation of AuNPs. It is reported that the aptamer with a thiol group at one end could bind to AuNPs[33], which increased the negative charge on the AuNPs colloid leading to a result that the repulsion among the particles was improved. The electrostatic repulsion prevented the strong van der Waals attraction and enhanced the stability of AuNPs against salt-induced aggregation, and thus the solution remained red under high-salt conditions. However, as depicted in Fig. 3. Upon the addition of CEA, the conformation change of CEA's aptamer changed from random coil structure to a specific and firm three-dimensional structure to combine with CEA. The three-dimensional structure prevented the exposure of the aptamer binding to AuNPs, and thus lost the ability of protecting the AuNPs under high-salt conditions. As a result, AuNPs aggregated under high-salt conditions and the color of AuNPs solution changed from red to blue. The overall strategy of this new colorimetric

method is presented in Fig. 3. The first step involves the aptamer modified with the thiol group at one end and CEA. The aptamer formed a three-dimensional structure to combine with CEA. When mixed with AuNPs, aptamer was not free to be bound to the unmodified AuNPs. While in the absence of CEA, aptamer was free to be bound to the surface of AuNPs as a result of the strong reaction of the thiol group and AuNPs. Importantly, the negative charges of aptamer on the surface of AuNPs made it difficult for AuNPs to change color due to salt-induced nanoparticle aggregation. In contrast, AuNPs was easily observed for color change caused by aggregation upon the addition of NaCl in the absence of the thiolated aptamer[20]. What's more, the more aptamer grated on AuNPs surface, the barrier which prevented each particle from aggregation was bigger. Thus, there was an appropriate addition of aptamer to make the distinction between the absence of CEA and the presence of CEA the most obvious. And the quantity of NaCl added into solution was also another important factor that influences the result. By controlling the quantity of aptamer and NaCl, the absorbance of the solution at 520 nm and 650 nm is in direct proportion to the quantity of CEA.

3.2 the result of aptamer-modified AuNPs on detection of CEA

In order to confirm the assumption that the thiolated aptamer bound to AuNPs was the only reason for prevention of salt-induced aggregation, the difference of the AuNPs with the addition of aptamer in saline solution was observed. The AuNPs without the aptamer modified on the surface had an instantaneous color change from red to blue after the addition of NaCl while the AuNPs solution containing the aptamer still remained red at identical conditions (Fig. 1 and Fig. 2). Absorption

spectroscopic analysis showed a more obvious distinction in the presence of aptamer, which confirmed the well dispersed state of AuNPs in this solution. To the contrary, a broad absorption spectrum bond appeared, suggesting that the AuNPs were easily aggregated in a saline solution. These different results lead to a fact that the aptamer binding to AuNPs enhanced the repulsion among AuNPs.

To test the CEA detection strategy, the aptamer was firstly added into the sample containing CEA. Then the solution was treated with AuNPs, followed by the addition of NaCl to observe the color change of AuNPs solution. The aptamer could form a three-dimensional structure[29] to combine with CEA, which was stronger than the Au-S bond. Thus the aptamer tended to combine with CEA in the presence of CEA, leading to a result that the AuNPs did not modified with the aptamer and easily aggregated in a saline solution. In contrast, the aptamer was free to bind to AuNPs in the absence of CEA. The abundant negative charges of the aptamer enhanced the repulsion among AuNPs, consequently leading to prevention of the salt-induced aggregation. As a result, the color of the AuNPs in the absence of CEA remained red after the addition of NaCl, while the the color of AuNPs changed in the presence of CEA (Fig. 1 and Fig. 2). Therefore, a method based on the color change visible to the naked eye is used to detect CEA.

3.3 The effect of concentration of aptamer and NaCl

The amount of NaCl added to the AuNPs solution was the factor to influence the aggregation of AuNPs. The color change of the solution was analyzed by measuring

absorption spectrum at 520 nm and 650 nm. An appropriate NaCl concentration was beneficial to improve sensibility to detect CEA (Fig. 4A). After the addition of 30 μL 10 μM aptamer, the data shows that 50 μL 0.5 M NaCl was the best choice to make the difference obvious between the presence and absence of CEA.

Also, the concentration of aptamer was an important factor to the sensibility. A high concentration of aptamer presented little difference in the presence of CEA, which was not sensitive to detect CEA. To the contrary, a low concentration of aptamer was also not sensitive to detect CEA, because the AuNPs in the condition was easily aggregated with CEA or without CEA. The effect of the different concentration of aptamer on AuNPs aggregation in a 40 $ng \cdot mL^{-1}$ CEA concentration was evaluated (Fig. 4B). 5 μL, 10 μL, 20 μL, 30 μL and 40 μL 10 μM aptamer were added into solutions containing 40 $ng \cdot mL^{-1}$ CEA accompanied with 15 μL 10 μM TCEP separately. After 1 h for reaction, these solutions were added to colloidal AuNPs solution separately and stirred for 1 h, followed by the addition of NaCl to induce aggregation of AuNPs. It was observed that the increasing in the concentration of aptamer led to an inducement aggregation, showing a good stability in a high NaCl concentration. So a proper concentration of aptamer in this colorimetric method was needed and we chose a 30 μL 10 uM aptamer as the final condition.

3.4 Direct detection of CEA

To evaluate the sensitivity of CEA dependent colorimetric sensor and dynamic working range of the proposed system, the absorbency at 520 nm and 650 nm

wavelength was monitored based on the above experiments. Firstly, the CEA samples ranging from 10 ng·mL^{-1} to 120 ng·mL^{-1} were mixed with 30 μL 10 μM aptamer and 15 μL 10 μM TCEP. After 1 h to react, these solution were added to 500 mL AuNPs and stirred for 1 h. Followed by the addition of 50 μL 0.5 M NaCl to promote aggregation of AuNPs, these solutions were analysed under the spectrophotometer. As shown in Fig. 4C, the absorbency at 520 nm and 650 nm wavelength was decreased with the increasing concentration of CEA. Linear relationship was found between the absorbency A_{650}/A_{520} and the concentration of CEA from 0 to 120 ng·mL^{-1} with correlation coefficient of 0.998 (Fig. 4C). and a detection limit of 3 ng·mL^{-1} was obtained using the method of 3σ. Thus a new colorimetric method of CEA was established. Here, it should be noted that the method is just potential applications and is still needed to be perfected to finish clinical diagnosis.

3.5 Selection of the assay

In order to study the selectivity of the optical sensor, several commonly existing proteins were tested using the sensing system in the identical condition, including myohemoglobin (MYO), mucoprotein (MUC) and bovine serum albumin (BSA). The amount of interfering proteins added to the sensing system was 200 ng. The sensing system treated with different proteins showed slight response from the absorption at 520 nm and 650 nm wavelength and visual observation (Fig. 4D). Meanwhile, though the amount of CEA was lower than other proteins, the distinct color change in the presence of CEA could be easily observed by naked eye. This colorimetric sensing

system showed a good selection, which was suitable for convenient differentiation between CEA and other materials.

3.6 Real sample analysis

To verify the practical application of the method, we tested spiked serum samples with different CEA concentration. The real samples had been diluted 50 times before each experiment in order to diminish false false positive contributions from the matrix. As shown in Table1, the recoveries of standard addition were between 94.4%, and 105.6%, which was a satisfactory result. These results reveal the potential application of this method for CEA detection in serum.

Table1

Analytical results for CEA in the serum sample

Serum sample[a]	Added(ng/ml)	Found(ng/ml)[b]	Recoveries(%)
1	8	7.6	95
2	16	16.9	105.6
3	24	23.3	97.1
4	32	30.2	94.4

a The serum sample was diluted 50 times.

b The average of three detections with this method.

Conclusions

In summary, this method was based on the competing reaction of aptamer with CEA and AuNPs. Through using the electrostatic repulsion caused by a large number of negative charges of aptamer which bound to the surface of AuNPs, the AuNPs were prevented from aggregating in a saline solution. Thus, the AuNPs solution still remained red in the absence of CEA, while the AuNPs solution changed from red to

blue in the presence of CEA due to the combination of CEA and aptamer. Thus, a new colorimetric method for the detection of CEA was developed. This is a quick and simple assay that does not require special instrumentation, making it a practical application in some cancer biomarker analysis. Moreover, the assay region, linear range and detection limit could be rational tuned by varying the amount of aptanmer and NaCl added to the sensing system. The tunable detection range and color change of the method were convenient and helpful in different detecting needs. Meanwhile, this method is potential to be extended in other detection of proteins or nucleotides by changing the aptamer.

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Figure Captions:

Fig. 1. Influence of aptamer-modified on the AuNPs. (1) AuNPS. (2) AuNPs in the 0.05 M NaCl solution. (3) Aptamer-modified AuNPs in the 0.05 M NaCl solution. (4) AuNPs mixed with the tube containing aptamer and CEA in the 0.05 M NaCl solution.

Fig. 2. (A) TEM images of AuNPs . (B) TEM images of AuNPs in the 0.05 M NaCl solution. (C) TEM images of Aptamer-modified AuNPs in the 0.05 M NaCl solution. (D) TEM images of AuNPs mixed with the tube containing aptamer and CEA in the 0.05 M NaCl solution.

Fig. 3. Schematic illustration of the colorimetric detection of CEA utilizing CEA's aptamer and unmodified AuNPs.

Fig. 4. (A) Influence of NaCl on the aggregation of AuNPs. The line a was for the solutions that did not contain CEA. The line b was for the solution containing CEA. (B) Influence of aptamer on the aggregation of AuNPs. The right columnar represented the solutions that did not contain CEA. The left columnar represented the solution containing CEA. 1 to 5 were added 5 μL, 10 μL, 20 μL, 30 μL, 40 μL 10 μM aptamer separately. (C) UV-vis absorption spectra of AuNPs sensing system treated with different amount of CEA (D) Absorbance ratio (A_{650}/A_{520}) of AuNPs sensing system treated with 30 μL 10 μM aptamer and 200 ng/mL interfering proteins.

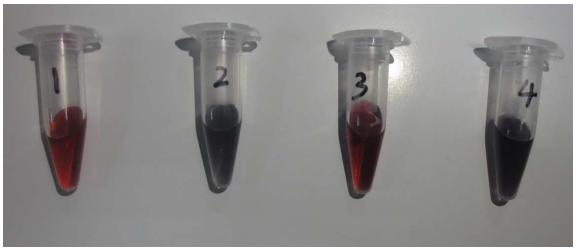
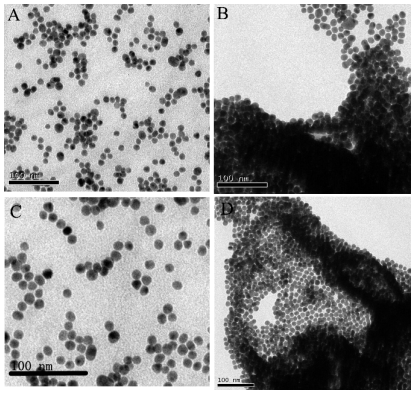


Fig. 1



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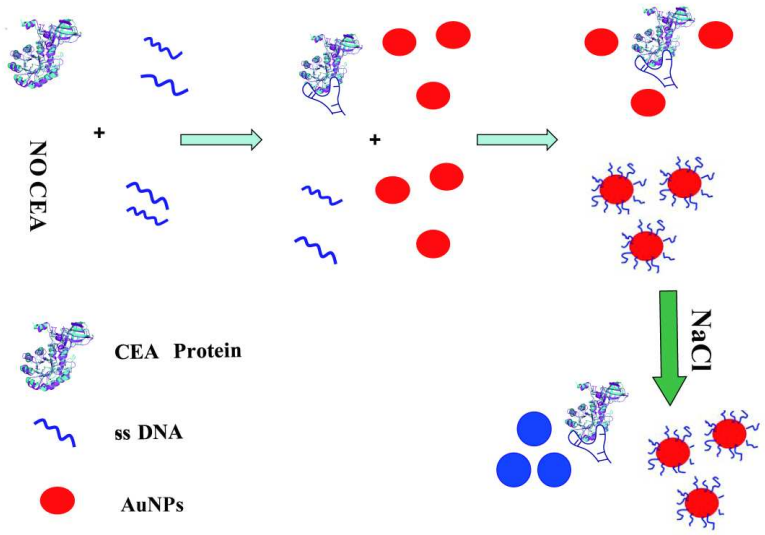

Fig. 3

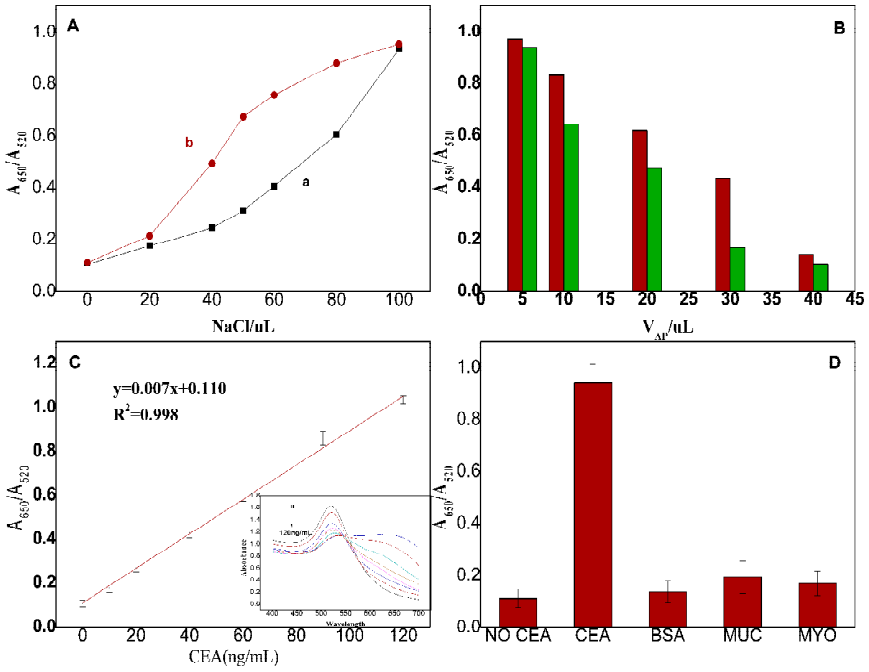

Fig. 4