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Gold nanoparticle-based exonuclease III signal amplification for highly sensitive colorimetric detection of folate receptor†

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By combining terminal protection of small molecule (folate)-capped DNA probes, exonuclease III signal amplification and gold nanoparticles, we developed a simple and label-free colorimetric assay for highly sensitive detection of folate receptor (FR). A detection limit of 50 fM FR was obtained using UV-vis spectrometry and 10 pM FR could be visualized by the naked eye.

Proteins are important molecules that play key roles in regulating a wide range of physiological functions. Abnormal expressions of proteins have profound implications in many diseases[1] such as cancer,[2] acquired immunodeficiency syndrome[3] and Alzheimer's disease.[4] Hence, the development of highly sensitive assays for accurate quantification of extremely low levels of protein biomarkers is of significant importance for early diagnostics and therapy of diseases. Until now, many conventional approaches, including enzyme-linked immunosorbent assay and western blotting, have been routinely utilized in protein analysis.[5] Although these conventional strategies provide accurate detection of proteins, they are, however, accompanied by many disadvantages, such as enzyme labelling, time-consuming assay procedures, and the need for sophisticated equipment. Aptamers, which are DNA or RNA oligonucleotides isolated from random sequence libraries by '*in vitro* selection' or SELEX through an iterative process of adsorption, recovery, and amplification,[6] have been recognized as promising alternatives to antibodies in protein recognition and detection. Nevertheless, the small library of well-established aptamers restricts their extensive applications in protein assays. Therefore, it is highly desirable to develop simple, sensitive, and selective amplification strategies for their applications in protein analysis and disease diagnostics.

Small molecule–protein interactions[7] have attracted extensive attention in molecular diagnostics and therapeutics due to their importance in chemical genetics, molecular diagnostics, and drug development. Inspired by this concept, the utilization of small molecule-capped DNA terminally tethered to proteins is a novel approach to overcome some of the above mentioned shortcomings in protein assays.[8] For example, Wu's group developed a method based on terminal protection of small molecule-linked DNA for sensitive detection of protein.[8b] On the other hand, various target recycling methods used for the detection of DNA[9] have inspired and promoted the development of terminal protection-based protein assays. For example, Tan and co-workers developed an enzyme-free terminal protection protein method using a DNAzyme as a signal amplifier.[8a] Based on small molecule-terminated DNA, Li's group constructed a nicking endonuclease-assisted amplification system for the detection of protein biomarkers.[8e] However, the DNA labelling process involved in these systems restricts their widespread applications. Exonuclease III (Exo III) which does not require any specific enzymatic recognition sequence and catalyses the stepwise removal of mononucleotides of DNA duplexes was advocated for amplified detection of target DNA.[10] Herein, leveraging on the merits offered by Exo III signal amplification and the terminal small molecule-capped DNA, we developed a gold nanoparticle (AuNP)-based simple, highly sensitive and selective colorimetric method for the detection of protein taking folate receptor (FR) as a model protein.

FR is a highly selective biomarker that is overexpressed by many primary and metastatic types of cancer.[11] Therefore, quantitative analysis of FR is especially important in early cancer diagnosis. The working principle of our detection strategy is schematically represented in Scheme 1. Folate-capped single-stranded DNA (DNA-1) is hydrolysed into mononucleotides by Exo I from its 3′-end. After the introduction of FR, the protein can specifically bind to the folate capped at the 3′-end of DNA with strong affinity. The protein tethered at the 3′-end in a 1 : 1 ratio can protect DNA-1 from the hydrolysis by Exo I because of significant steric hindrance. Therefore, an equal

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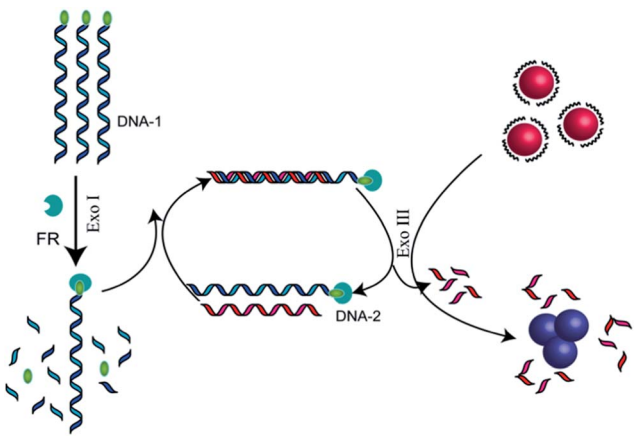
amount of DNA-1 could enter the next amplification process, hybridising with its complementary DNA strand (DNA-2) and acting as a trigger strand to induce the digestion of DNA-2. Exo III can digest double-stranded DNA with blunt or recessed end from the 3′-terminus, while it is inactive to single-stranded DNA or 3′-protruding termini of double-stranded DNA. Therefore, to avoid the digestion of DNA-1 by Exo III, an overhang at the 3′-terminal of single-stranded DNA-1 was designed. After the addition of Exo III, the duplex DNA is degraded from the 3′-hydroxyl terminal of DNA-2, releasing DNA-1. The released DNA-1 then hybridises with another DNA-2 to initiate a new cycle. In our system, single-stranded DNA-2 acts as a stabilizer of AuNPs. With the digestion of DNA-2, the stability of the AuNPs decreases, eventually leading to the aggregation of the AuNPs and resulting in colour change in a high ionic strength medium. Therefore, the concentration of FR could be determined by the colour change of the AuNPs.

The as-prepared AuNPs are negatively charged and wine red in colour due to their specific surface plasmon resonance absorption. Addition of an appropriate amount of salt could screen the electrostatic repulsion between the negatively charged AuNPs and result in their aggregation. However, with the protection of DNA-2, the salt-induced aggregation is efficiently suppressed and the solution retains its original colour. To achieve the best performance, the salt concentration was first optimized (Fig. S1†). It was found that the stability of AuNPs/DNA-2 is strongly dependent on the salt concentration. It was also found that 200 mM NaCl is optimal for the development of highly sensitive FR assay. We then investigated the effect of the DNA-2 concentration on the stability of the AuNPs. It was observed that the protective effect of DNA-2 is monotonically decreased with the decrease in DNA-2 concentration and a rapid wine red-to-pale purple colour change was observed at low concentrations of DNA-2. This phenomenon was further confirmed by UV-vis spectrometry (Fig. S2†). This DNA-2 concentration dependent AuNP aggregation prominently facilitates the implementation of the FR detection.



Scheme 1 Schematic illustration of highly sensitive colorimetric detection of folate receptor by using terminal protected DNA and Exo III cyclic amplification.

To verify the feasibility of this strategy, the trigger DNA-1 induced Exo III cyclic amplification was performed (Fig. S3†). The colour change of the AuNPs/DNA-2 solution was not obvious only in the presence of 1.0 nM trigger DNA-1. In contrast, the colour changed rapidly from wine red to pale purple at the same trigger DNA-1 concentration when Exo III was introduced into the solution. Previous studies have demonstrated that DNA duplexes have little stability effect toward AuNPs in the presence of a high salt concentration.[12] When the concentration of trigger DNA-1 is lower, less DNA-2 is consumed to form DNA duplex, thus showing little effect on the stability of the AuNPs. While in the presence of Exo III, trigger DNA-1 binds to DNA-2 and consequently leads to the digestion of DNA-2, subsequently releasing the trigger DNA-1 strand to bind to another DNA-2 strand to initiate the next round of cleavage. This cyclic reaction repeats over and over again until all DNA-2 strands are consumed, resulting in significant signal amplification and eventually the AuNP aggregation. Next we tested the concentration dependence of the AuNP aggregation on DNA-1. As shown in Fig. 1, with the increasing concentration of DNA-1, the absorbance at 650 nm increased consequently whereas the absorbance at 520 nm decreased accompanying a gradual red-shift. Accordingly, the absorbance ratio at 650 and 520 nm could be employed for the quantification of target DNA. It was found that there is a logarithmic dependence of the A_{650}/A_{520} ratio on the trigger DNA-1 concentration in the range from 0.1 to 10 pM. Meanwhile, the colour of the AuNP solution gradually changed from wine red to pale purple with the increasing amount of trigger DNA-1 and the colour of the solution was easily visualized at 5 pM.

To evaluate the sensitivity of the proposed FR assay, sample solutions with different concentrations of FR were incubated with folate-capped DNA-1 and Exo I before the Exo III cyclic amplification followed by the addition of the AuNPs and salt. As

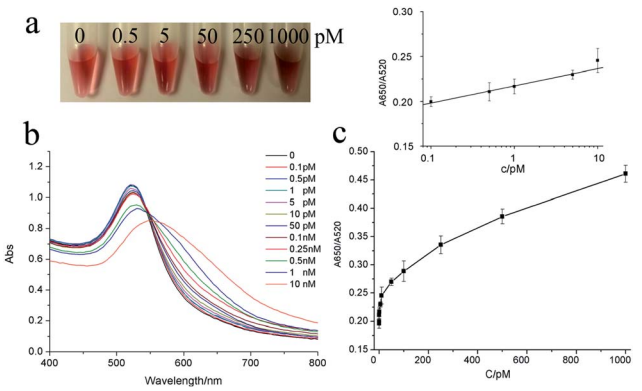


Fig. 1 (a) Photograph showing colorimetric response of the detection system in the presence of various concentrations of trigger DNA-1; (b) absorption spectra of different concentrations of trigger DNA-1 (from 0–10 nM) induced signal amplification; (c) plot of DNA concentration *vs.* absorbance (A_{650}/A_{520}) for trigger DNA-1 assay. Inset: the calibration curve for DNA-1 concentrations from 0.1 pM to 10 pM. (The concentrations of DNA-2, Exo III and AuNPs are 0.1 μM, 50 U mL^{-1} and 4 nM, respectively.)

the concentration of FR increased, more DNA-1 strands were protected from the Exo I digestion and entered the cyclic amplification process, leaving less DNA-2 to stabilize the AuNPs. This was accompanied by a decreased absorption at 520 nm and an increased absorption at 650 nm in the UV-vis spectrum (Fig. 2a). The ratio of A_{650}/A_{520} (Fig. 2b and c) increased with the concentration of FR and showed a semi-logarithmic dependence on the FR concentration in the range from 0.1 to 10 pM. Under optimized conditions of 2 h Exo III digestion at 37 °C in pH 7.4 10 mM phosphate buffer containing 1.0 mM $MgCl_2$, a detection limit of 50 fM, estimated according to the 3σ criterion, was obtained in UV-vis spectrometry and 10 pM for direct visualization by the naked eye was achieved. Such sensitivity is comparable to or even better than most terminal-protected protein assays.

The FR-induced aggregation of the AuNPs was further confirmed by transmission electron microscopy (TEM). As shown in Fig. 3a, the AuNPs were nearly monodispersed in the absence of FR after and in the presence of a high salt concentration of 200 mM, a direct consequence of the protective effect of the high concentration of DNA-2. While in the presence of FR, the AuNPs aggregated after the addition of salt (Fig. 3b). The TEM results were consistent with the red-shift of the UV-vis absorption band as well as the colour change of the AuNP solution in the absence and the presence of FR, further confirming that the designed colorimetric detection system is indeed appropriate for highly sensitive detection of FR.

To validate the specificity of the detection system, three other proteins, namely, BSA, IgG and avidin, were tested. As shown in Fig. 4, 1.0 nM FR led to an obvious absorption change and purple-coloured solution, while the presence of other proteins at 10 nM exhibited no observable change of the UV-vis spectra as well as the colour of the AuNP solution, suggesting that there is no effect on the stability of the AuNPs/DNA-2 system. It is well known that the FR-folate interaction is specific. In this case, FR produced a protein protected 3′-terminus, resulting in the protection of trigger DNA-A from enzyme digestion, thus leading to the digestion of DNA-2 in the Exo III cyclic amplification and finally bringing obvious changes in the UV-vis absorption spectrum. Therefore, the proposed colorimetric assay presents remarkably high selectivity. The excellent selectivity of the assay presents itself as a promising technique for protein biomarker detection.

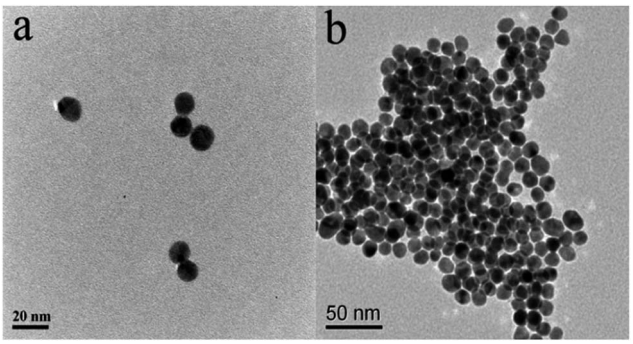


Fig. 3 TEM images of the colorimetric system (a) in the absence and (b) in the presence of 1.0 nM FR.

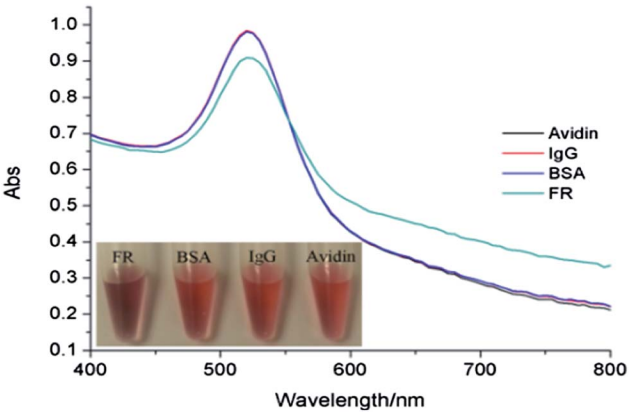


Fig. 4 Absorption spectra of the AuNP solutions in the presence of 10 nM avidin, 10 nM IgG, 10 nM BSA and 1.0 nM FR, respectively.

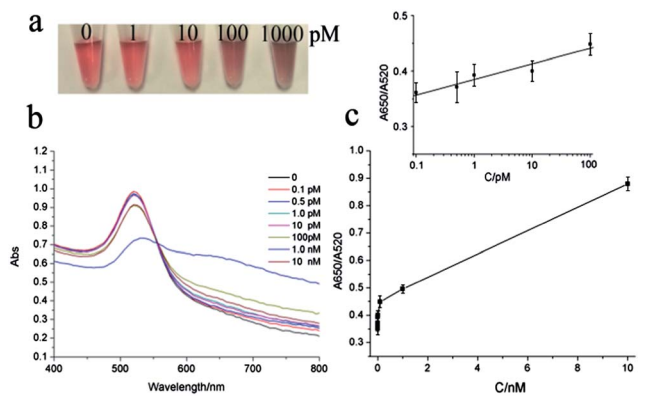


Fig. 2 (a) Photograph showing the colorimetric response in the presence of various concentrations of FR; (b) absorption spectra obtained for FR at different concentrations (from 0.0 to 10 nM); (c) plot of protein concentration *vs.* absorbance ratio (A_{650}/A_{520}). Inset: the calibration curve for FR concentrations from 0.1 pM to 10 pM. (The concentrations of DNA-2, Exo I, Exo III and AuNPs were 0.1 μM, 50 U mL^{-1}, 50 U mL^{-1} and 4.0 nM, respectively.)

In summary, we have developed a simple and high sensitive colorimetric assay for the detection of FR. The assay combines the terminal small molecule-protection of DNA with enzyme-assisted DNA recycling signal amplification to achieve ultrasensitive detection of FR with high selectivity. A determination limit of 50 fM FR was obtained by UV-vis spectrometry and 10 pM FR was able to be visualized by the naked eye. We expect that this strategy may offer new opportunities for developing a wide variety of low cost, simple, and sensitive assays for proteins which are highly relevant to clinical diagnosis.

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