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Nanoparticle-catalyzed reductive bleaching for fabricating turn-off and enzyme-free amplified colorimetric bioassays

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ABSTRACT

Nanoparticle-catalyzed reductive bleaching reactions of colored substrates are emerging as a class of novel indicator reactions for fabricating enzyme-free amplified colorimetric biosensing (turn-off mode), which are exactly opposite to the commonly used oxidative coloring processes of colorless substrates in traditional enzyme-catalyzed amplified colorimetric bioassays (turn-on mode). In this work, a simple theoretical analysis shows that the sensitivity of this colorimetric bioassay can be improved by increasing the amplification factor ($k_{cat}\Delta t$), or enhancing the binding affinity between analyte and receptor (K_d), or selecting the colored substrates with high extinction coefficients (ε). Based on this novel strategy, we have developed a turn-off and cost-effective amplified colorimetric thrombin aptasensor. This aptasensor made full use of sandwich binding of two affinity aptamers for increased specificity, magnetic particles for easy separation and enrichment, and gold nanoparticle (AuNP)-catalyzed reductive bleaching reaction to generate the amplified colorimetric signal. With 4-nitrophenol (4-NP) as the non-dye colored substrate, colorimetric bioassay of thrombin was achieved by the endpoint method with a detection limit of 91 pM. In particular, when using methylene blue (MB) as the substrate, for the first time, a more convenient and efficient kinetic-based colorimetric thrombin bioassay was achieved without the steps of acidification termination and magnetic removal of particles, with a low detection limit of 10 pM, which was superior to the majority of the existing colorimetric thrombin aptasensors. The proposed colorimetric protocol is expected to hold great promise in field analysis and point-of-care applications.

1. Introduction

The development of highly sensitive and selective methods to detect disease-related protein biomarkers at low concentrations is crucial for clinical diagnoses and treatments (Gubala et al., 2012). Among a wide variety of analytical methods or techniques for protein detection (Jans and Huo, 2012; Saha et al., 2012; Wang et al., 2010; Zhang et al., 2007; Zhang and Zhou, 2012), colorimetric protein sensing has attracted much attention due to its simplicity, easy naked-eye observation, and low cost without any advanced or complicated instrumentation (Song et al., 2011; Zhao et al., 2008). The key challenge for colorimetric biosensing is transforming the target binding events into color changes. In this aspect, natural enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase (ALP) have been widely used in the colorimetric bioassays since they can catalyze specific chromogenic substrates to produce a detectable color change (Ambrosi et al., 2010; Li et al., 2011; Porstmann and Kiessig, 1992). However, the inherent instability and variability of protein enzymes and their complicated preparation and purification procedures greatly hamper their applications (Selvaraju et al., 2008; Wei and Wang, 2008).

To address this issue, many efforts have been made to develop enzyme-free colorimetric bioassays by employing various nanomaterials (Song et al., 2010; Wang, 2005). For example, the color change upon aggregation of gold nanoparticles (AuNPs) has been increasingly utilized for colorimetric sensing of various analytes (Zhao et al., 2008). However, this principle often suffers from relatively poor sensitivity because of the inherent lack of signal amplification (Nam et al., 2007). Obviously, it is highly desirable to develop nanomaterial-based amplified colorimetric approaches. To this end, two main amplification strategies are usually used for colorimetric biosensing. One is the well-known DNA-based amplification strategy, where nanomaterials have very large surface areas and hence serve as carriers for accommodating numerous DNA reporter molecules. For example, in bio-barcode assays (BCA) (Nam et al., 2007), the detection of target analyte is converted into the detection of the DNA barcode probes, which are initially immobilized onto and then released from the nanoparticles, and finally detected in solution or on a surface by a color change (Brakmann, 2004). However, practical applications of this strategy

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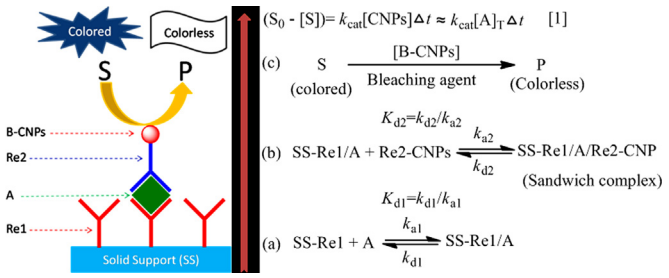
are restricted due to the complex conjugation chemistries and detection procedures (Das et al., 2006). In contrast, the other more straightforward strategy is nanomaterial-catalyzed signal amplification. For instance, many nanomaterial-based artificial enzymes with intrinsic peroxidase-like activity have been extensively applied to achieve the catalytically amplified colorimetric bioassays (Gao et al., 2007; Song et al., 2011). Similarly, artificial peroxidase-like DNAzymes with hemin as a cofactor have been employed as well (Kosman and Juskowiak, 2011; Shlyahovsky et al., 2007). However, the use of these enzyme mimics relies heavily on H_2O_2-mediated catalytic oxidation of the conventional peroxidase substrates, especially 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 3,3′,5,5′-tetramethylbenzidine (TMB). Additionally, nanoparticle-induced metal (Au, Ag) deposition processes through catalysis are also frequently used for amplified colorimetric bioassays (Song et al., 2010). But this colorimetric technique is commonly applied onto solid substrates and requires rigorous control of the deposition time (Selvaraju et al., 2008).

Theoretically, regardless of formation (turn-on mode) or destruction (turn-off mode) of colored molecules, any catalytic indicator reaction that changes color in response to a biorecognition event can be applied to fabricate an amplified colorimetric bioassay. Inspired by this thought, recently it has been demonstrated that $NaBH_4$-mediated decolorization reaction of methyl orange (MO) could be exploited as an indicator reaction for highly sensitive colorimetric bioassays (Li et al., 2013). In that study, AuNPs decolorized numerous colored MO dye molecules to colorless molecules through catalysis in the presence of $NaBH_4$, exactly opposite to the formation of a large number of colored molecules in traditional enzyme-catalyzed colorimetric bioassays. Importantly, this novel colorimetric strategy eliminated the employment of various enzymes and metal deposition processes, and thus minimized the existing constraints of limited enzyme–substrate systems to a certain extent. However, there are numerous unexplored possibilities to expand its applicability. Therefore, herein our study mainly focused on (1) obtaining an in-depth insight into its fundamental principle and amplification factor theoretically; (2) seeking applicable non-dye colored substrates and the corresponding termination approaches for enabling endpoint-based colorimetric detection; (3) improving the detection efficiency by applying a distinct colored substrate for kinetic-based colorimetric detection, which can eliminate the acidification termination and particle removal steps required for the endpoint detection method. For the proof-of-concept demonstration, we chose thrombin as the target protein. It can catalyze many coagulation-related reactions responsible for blood clotting and involves two well-studied DNA aptamers (apt15 and apt29) for enabling a sandwich-type bioassay (Bock et al., 1992; Tasset et al., 1997). Combining the effective separation and enrichment properties of apt15-conjugated paramagnetic particles (apt15-PMPs) with good catalytic activities of apt29-functionalized AuNPs (apt29-AuNPs) toward reductive bleaching of 4-nitrophenol 4-(NP) and methylene blue (MB), we have developed a turn-off and enzyme-free amplified colorimetric aptasensor that permitted thrombin detection by the endpoint or the kinetic method (Scheme 1S).

2. Experimental

2.1. Materials and chemicals

All the oligonucleotides used in this work were synthesized and purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences of two thrombin-binding aptamers are as follows: 5′-biotin-A_{15}-GGT TGG TGT GGT TGG-3′ (apt15) and 5′-HS-A_{15}-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′ (apt29). Human



Scheme 1. Fundamental principle of catalytic nanoparticle (CNP)-enabled amplified colorimetric bioassay using a reductive bleaching process.

serum albumin (HSA), immunoglobulin A (IgA), immunoglobulin G (IgG) and lysozyme (Lys) were purchased from Biosharp, Japan. Human α-thrombin was ordered from Sigma-Aldrich, Co. LLC., USA. Streptavidin-coated paramagnetic particles (PMPs, ca., 1.0 μm) were purchased from Promega Corporation (Madison WI, USA). Details of buffer solutions were found in Appendix A. Ultrapure water was used throughout the experiments. All other reagents were of analytical reagent grade.

2.2. Preparation of apt29-AuNPs and apt15-PMPs

AuNPs were synthesized by the classical citrate reduction method and observed with a JEM-200CX transmission electron microscope (TEM) (Fig. S1). Apt29-AuNPs and apt15-PMPs were prepared according to the published methods (Li et al., 2013).

2.3. Investigation of catalytic activities of apt29-AuNPs toward reductive bleaching of 4-NP, MB and HCF (III) by $NaBH_4$

For AuNP-catalyzed reductive bleaching of 4-NP, a mixture containing 4-NP (800 μL, 0.35 mM) and $NaBH_4$ (2200 μL, 58 mM) was first prepared in a quartz cuvette. Then 200 μL of apt29-AuNP solution was rapidly added to the above solution. Similarly, for the reduction of MB, 50 μL of apt29-AuNP solution was rapidly added to the aqueous mixture of MB (685 μL, 0.25 mM) and $NaBH_4$ (2465 μL, 16.6 mM). For the reduction of potassium hexacyanoferrate (III) (HCF(III)), 180 μL of apt29-AuNP solution was added to the aqueous mixture of HCF(III) (1600 μL, 2.81 mM) and $NaBH_4$ (1420 μL, 160 mM). For the uncatalyzed reaction without AuNP catalysts, apt29-AuNP solution was replaced by an equal volume of $NaBH_4$ solution. A Thermo Scientific Nanodrop 2000c spectrophotometer (USA) was used to monitor their reaction kinetics by measuring their time-dependent absorption spectra.

2.4. Endpoint-based colorimetric detection of thrombin using AuNP-catalyzed reductive bleaching of 4-NP

For endpoint-based colorimetric thrombin measurement, the formed sandwich complex (also called AuNP-coated PMPs, Scheme 1S) was first dispersed in 40 μL of washing buffer, and then a reaction mixture containing 4-NP (50 μL, 0.35 mM) and $NaBH_4$ (100 μL, 80 mM) was added. After reaction for 8 min, HCl (20 μL, 1.6 M) and NaOH (20 μL, 4 M) solutions were added in proper sequence to stop the catalytic reaction. Afterwards, the mixture was magnetically separated and the supernatant (200 μL) was collected for absorbance measurement at 400 nm using a Synergy NEO HTS microplate reader (BioTek, USA).

2.5. Kinetic-based colorimetric detection of thrombin using AuNP-catalyzed reductive bleaching of MB

For kinetic-based colorimetric thrombin measurement, a reaction mixture containing MB (50 μL, 0.25 mM) and $NaBH_4$ (50 μL, 40 mM) was first added to 100 μL of the AuNP-coated PMP solution. Then,