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Convenient Colorimetric Detection of Thrombin via Aptamer-Mediated Inhibition and Restoration of the Oxidase Activity of Nanoceria

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Cerium oxide nanoparticles, also called nanoceria, have recently gained much attention as oxidase-mimicking nanozymes that catalyze the oxidation of chromogenic substrates for color generation without the addition of H_2O_2. Herein, we have developed a unique colorimetric biosensor for thrombin in human blood plasma, which relies on thrombin-binding aptamer (TBA)-mediated inhibition of the oxidase activity of nanoceria and its restoration by very selective interactions of TBA with target thrombin. In this system, nanoceria were first incubated with TBA, resulting in quick reduction of the oxidase activity of nanoceria via the adsorption of single-stranded (ss)DNA-type TBA on nanoceria. By the addition of sample solutions containing target thrombin, TBA bound on the nanoceria would strongly interact with free thrombin and be detached from the nanoceria, thereby increasing the available surface area of the nanoceria and consequently enhancing the oxidase activity. Using this strategy, target thrombin was successfully detected at concentrations as low as 100 pM over a wide linear range from 0.1 to 10 nM. The diagnostic capability of this method has been demonstrated by detecting thrombin in human blood plasma, showing its great potential in the practical applications.

Keywords: Nanoceria, Thrombin, Thrombin-Binding Aptamer, Oxidase Mimetics.

1. INTRODUCTION

Thrombin is an important enzyme that plays significant roles in the process of blood coagulation. When vascular injury occurs, thrombin is generated by the proteolytic cleavage of prothrombin and catalyzes the conversion of soluble fibrinogen into insoluble strands of fibrin to prevent bleeding and promote blood coagulation.[1,2] Furthermore, thrombin significantly contributes to inflammatory reactions, and its presence at an abnormal level is closely related to many types of diseases.[3] For these reasons, several diagnostic methods to determine thrombin levels have been developed and already commercialized, mostly based on enzyme-linked immunosorbent assay (ELISA) procedures to generate colorimetric, fluorogenic, or electrochemical products.[4] These assays offer high sensitivity and selectivity to determine thrombin levels; however, relatively complex experimental procedures for establishing antigen-antibody interactions critically limit their widespread applications. Thus, the development of simpler and more rapid protocols that are suitable for facility-limited or point-of-care testing environments remains a significant goal.

Recently, nanomaterials with enzyme-like characteristics (nanozymes) have been intensively studied for the development of novel biosensors enabling convenient and sensitive detection of target molecules.[5,6] They are generally stable and robust even in harsh environments and exhibit catalytic activities to produce relevant signals corresponding to the target molecules. Among them, cerium oxide nanoparticles, or nanoceria, have unique oxidase-like activity that no H_2O_2 is needed to oxidize colorimetric substrates such as 3,3′,5,5′-tetramethylbenzydine (TMB) or 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid).[7] Moreover, compared with other nanozymes that generally

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require several tens of minutes to clearly obtain a colorimetric response, color generation catalyzed by nanoceria generally takes place very rapidly within a few minutes.[7,8] Based on these catalytic advantages, nanoceria can be efficiently used in place of horseradish peroxidase in ELISA because they are not limited by instability.[9] We also reported an ultrafast colorimetric method for the detection of nucleic acids that is based on the inhibition of the oxidase activity of nanoceria by the quick adsorption of DNA molecules on the surface of nanoceria.[8] Although these examples demonstrate the potential of oxidase-mimicking nanoceria in various biosensing applications, there have been few studies on the practical utility of nanoceria for the detection of clinically important target molecules, such as thrombin, without any sophisticated experimental procedures.

Thus, in order to accelerate the use of nanoceria in practical detection in clinical fields, we have developed an efficient colorimetric strategy for thrombin assay based on thrombin-binding aptamer (TBA)-induced suppression of the oxidase activity of nanoceria and its restoration corresponding to target thrombin. Nanoceria have been previously employed for chemiluminescence-based detection of thrombin.[10] However, aptamer-mediated control of the catalytic activity of nanoceria has not yet been investigated for the development of convenient colorimetric assay procedures. The sensitivity of the constructed assay system was determined and its clinical utility in detecting various levels of thrombin in human blood samples was also assessed.

2. EXPERIMENTAL DETAILS

2.1. Materials

Thrombin, nanoceria, sodium acetate, trizma hydrochloride, sodium chloride, potassium chloride, magnesium chloride, dimethyl sulfoxide, ethanol, acetic acid, human serum, and TMB were purchased from Sigma-Aldrich. TBA (5′-GGT TGG TGT GGT TGG-3′) was synthesized from Integrated DNA Technologies. All chemicals used in this study were of analytical grade or higher.

2.2. Characterization of Nanoceria

Samples for transmission electron microscopy (TEM) analysis were prepared by drop-casting the particle suspensions on a carbon-coated copper TEM grid (Electron Microscopy Sciences, USA) followed by drying at room temperature. All samples were observed using field-emission TEM (Tecnai, FEI, Netherlands) with accelerating voltages up to 200 kV. Dynamic light scattering measurements were performed on a Malvern particle size analyzer (Malvern, UK). To find the diameter, 2 mL of nanoceria (0.1 mg/mL) solution was added in a polystyrene cuvette and the sample was scanned for 5 min (eight runs) to obtain one set of raw data. The average values for the diameters were determined with at least three repeated measurements per sample. Zeta potential measurements were performed on a Zetasizer Nano-ZS (Malvern Co., UK).

2.3. Aptamer-Mediated Colorimetric Assay for Thrombin Detection

In a typical assay, 40 μL of TBA solution was first added to 10 μL of nanoceria solution (10% (w/v) colloidal dispersion in 2.5% acetic acid). Subsequently, 150 μL of reaction buffer (20 mM Tris-HCl containing 140 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$, pH 7.5) was added and incubated for 10 min at room temperature using a rotator to induce TBA adsorption on nanoceria. After this, unreacted TBA was removed by centrifugation at 10,000× g for 1 min and TBA-nanoceria composite was obtained. Then, 200 μL of sample solution containing target thrombin (50 μL) dispersed in 150 μL of the reaction buffer was additionally applied to the separated TBA-nanoceria composite. After 10 min of incubation using a rotator at room temperature followed by separation of nanoceria by centrifugation at 10,000× g for 1 min, 200 μL of buffer solution containing TMB substrate (500 μM) in sodium acetate (200 mM, pH 4.0) was finally added and incubated for 1 min. After the colorimetric reaction, the solution was treated with centrifugation at 10,000× g for 1 min to separate the nanoceria from the solution. The supernatant was used to capture color images representing the progress of the reaction and also transferred to a transparent 96-well plate to measure the corresponding absorption intensity. Spectrophotometric measurements were conducted at a scanning mode or at 652 nm using a microplate reader (Synergy H1, BioTek, VT).

2.4. Thrombin Detection in Human Serum

To prepare different levels of thrombin in human serum, a predetermined amount of thrombin was added into human serum to make spiked samples.[11] The concentration of thrombin in each spiked sample (50 μL, 50-fold dilution) was determined by the same procedure as described above. The recovery rate [recovery (%) = measured value/actual value × 100] and the coefficient of variation [CV (%) = SD/average × 100] were determined to evaluate the precision and reproducibility of the assays.

3. RESULTS AND DISCUSSION

The overall strategy involved in designing the colorimetric detection of thrombin is illustrated in Figure 1. The assay procedures involve initial incubation of TBA with nanoceria, which might allow TBA to adsorb on the surface of the nanoceria and thereby reduce their oxidase activity. Then, sample solutions were added to the nanoceria with TBA adsorbed on their surface. In the presence of target thrombin in sample solutions, TBA would strongly

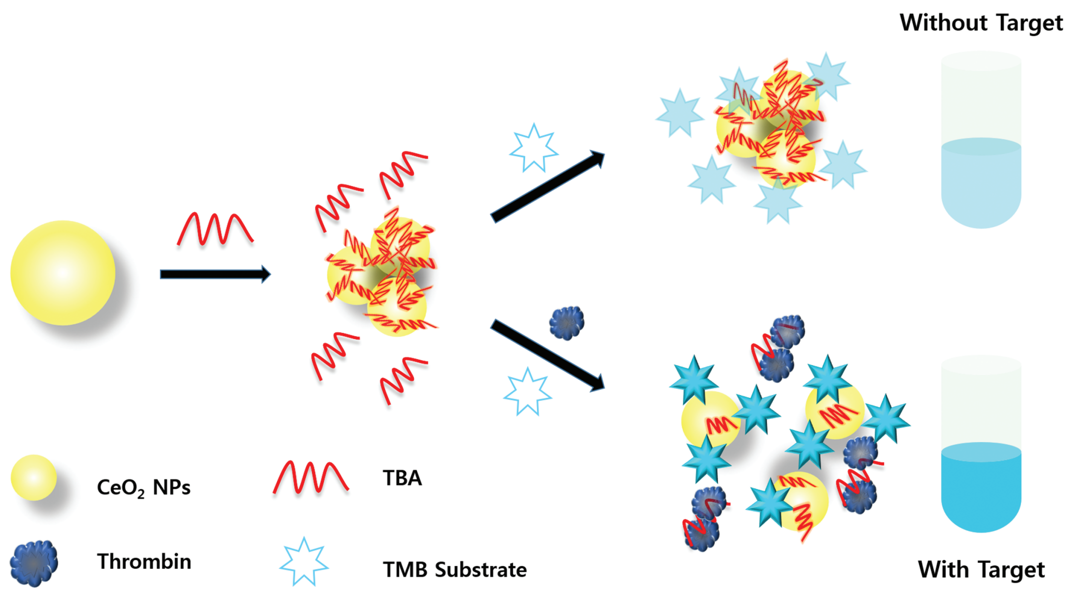

Figure 1. Schematic illustration of aptamer-mediated inhibition and thrombin-induced restoration of the oxidase activity of nanoceria for the determination of thrombin level.

interact with free thrombin and be detached from the surface of nanoceria, resulting in an increase in the available surface area for catalytic events and a concomitant increase in the oxidase activity of nanoceria. On the contrary, when thrombin is not present in sample solutions, TBA would not be detached and the oxidase activity of nanoceria is not enhanced because of shielding by the bound TBA on the surface of the nanoceria. Overall, assay samples that contain thrombin displayed an intense colorimetric response while those without thrombin exhibited a significantly lowered colorimetric response. We envisioned that the increase in the colorimetric signal in response to thrombin could be utilized to construct a convenient colorimetric assay system for thrombin detection.

The feasibility of reducing the oxidase activity of nanoceria via interactions with TBA was first evaluated. As shown in Figure 2, control samples without TBA yielded the highest colorimetric signal resulting from TMB oxidation by the nanoceria. On the other hand, a significant and gradually reduced colorimetric signal was generated from the samples that contained TBA. The extent of signal reduction with 100 nM TBA was determined to be *ca.*

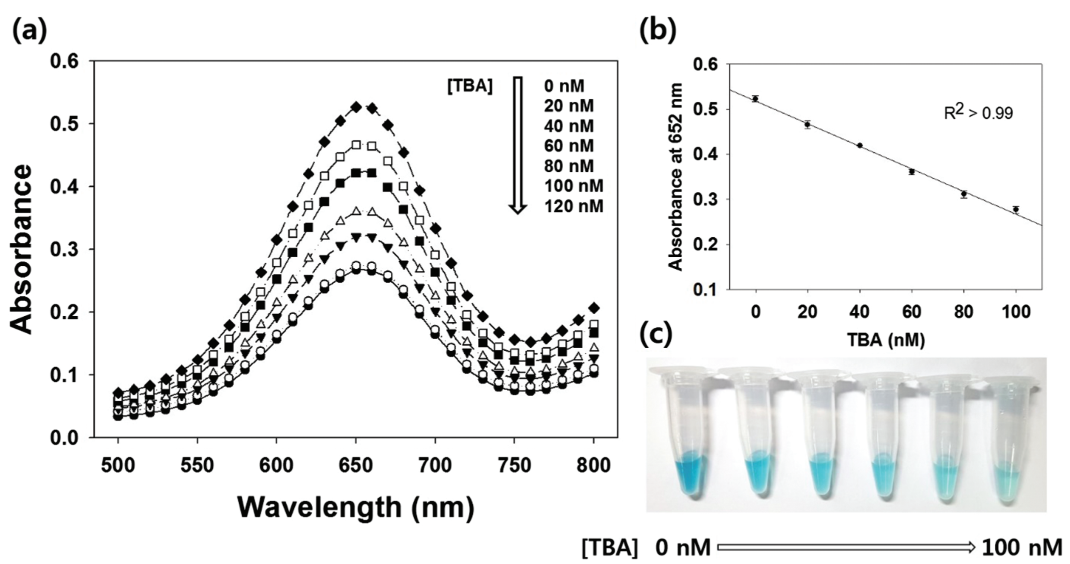

Figure 2. (a) UV-vis absorption spectra obtained from nanoceria-based colorimetric oxidation of TMB in the presence of TBA at various concentrations. (b) Plot of the absorbance at 652 nm versus the concentration of TBA. Error bars represent the standard deviation of three independent measurements. (c) Real images corresponding to (a).

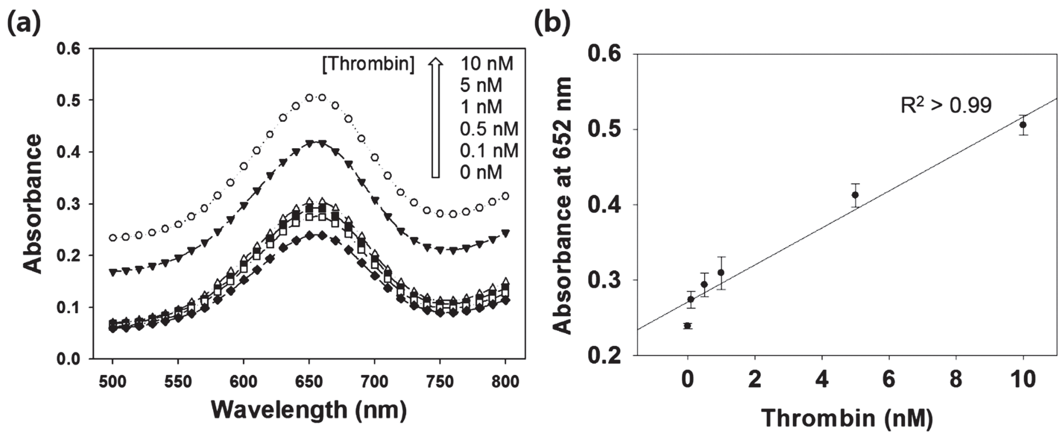

Figure 3. (a) UV-vis absorption spectra obtained from nanoceria-based assay system for thrombin at various concentrations. TBA at 100 nM was used in this experiment. (b) Plot of the absorbance at 652 nm versus the concentrations of thrombin. Error bars represent the standard deviation of three independent measurements.

50% based on a comparison of the absorption intensities at 652 nm, which corresponds to the maximum absorption of the oxidized TMB. Further addition of TBA (120 nM) did not result in a significant decrease in the absorption intensities at 652 nm. Moreover, plots of the intensities of colorimetric signals versus TBA concentration displayed excellent linearity from 0 to 100 nM of TBA. These observations revealed that TBA significantly reduced the catalytic activity of nanoceria in proportion to the amount of TBA, as expected.

We then demonstrated the restoration of the oxidase activity of nanoceria by the addition of target thrombin. The results show that the colorimetric response, which was significantly reduced by the addition of TBA, was gradually recovered as the concentrations of added thrombin increased up to 10 nM (Fig. 3(a)). An excellent linear relationship ($R^2 > 0.99$) existed in the calibration plot in the range of 0.1–10 nM and the limit of detection (LOD) was determined to be approximately 100 pM, which was calculated from the mean of blank data plus three times the standard deviation (Fig. 3(b)). This LOD and the linear range values are among the best results of those recently reported that describe colorimetric methods for thrombin detection.[12,13]

We speculated the reasons for the TBA-mediated inhibition and thrombin-induced restoration of the oxidase activity of nanoceria. It was reported that positively charged nanoceria form large aggregates with negatively charged DNA, which significantly contributes to the reduction in the catalytic activity of the nanoparticles.[8] We also assumed that the addition of target thrombin relieves the aggregation of nanoceria via strong interactions with TBA, thereby inducing the recovery of the initial colorimetric response without any TBA. TEM images of nanoceria in the absence and presence of TBA clearly confirmed that the addition of DNA induced the aggregation of nanoceria to yield lower catalytic activity (Fig. 4). Importantly, with the addition of target thrombin, the aggregation phenomenon was relieved and a relatively dispersed state of nanoceria, which truly contributes to the restoration of the initial colorimetric activity, was observed. TEM analyses were well coordinated with the particle size distributions

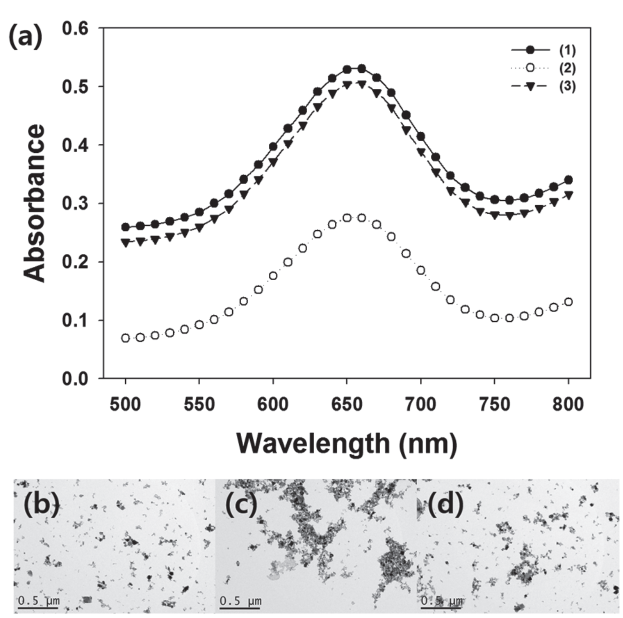

Figure 4. (a) UV-vis absorption spectra obtained from the TMB oxidation by nanoceria only (1, b), nanoceria interacting with TBA (2, c), and nanoceria in the presence of both TBA and thrombin (3, d). TBA at 100 nM and thrombin at 10 nM were used in this experiment.

Table I. Mean diameter (nm) and zeta potential (mV) measurements of nanoceria at pH 7.5 in the (a) absence and (b) presence of TBA by dynamic light scattering measurements.

	(a) Nanoceria in the absence of TBA	(b) Nanoceria in the presence of TBA
Mean diameter (nm)	205.04	2284.1
Zeta potential (mV)	27.75	−21.67

Table II. Detection precision of the current assay system for the determination of thrombin levels in spiked human serum samples. Actual value was determined by the added amount of thrombin in diluted human serum (50-fold). The recovery rate [recovery (%) = measured value/actual value × 100] and the coefficient of variation [CV (%) = SD/average × 100] were determined to evaluate the precision and reproducibility of the assays.

[Thrombin] spiked (nM)	Measured (nM)	SD	CV (%)	Recovery (%)
0.5	0.528	0.02	3.79	105.57
1	0.971	0.09	9.27	97.18
5	5.157	0.23	4.46	103.14

measured by dynamic light scattering (Table I). The combined results demonstrated that TBA was adsorbed onto surfaces and induced the aggregation of nanoceria, resulting in the inhibition of the oxidase activity of nanoceria. In addition, thrombin induced the re-dispersion of nanoceria, resulting in the restoration of the oxidase activity of nanoceria.

Finally, the proposed method was employed for the assay of thrombin in human plasma. In this experiment, human plasma was appropriately diluted with a buffer to fall within the linear range and the amount of spiked thrombin was quantitatively determined. As presented in Table II, this method yielded good recovery rates of 97.2–105.6% and CVs of 3.8–9.3%, confirming the excellent reliability and reproducibility of the present method for the determination of thrombin levels in clinical applications.

4. CONCLUSION

In summary, a novel strategy for colorimetric detection of thrombin was successfully developed. The assay relies on the inhibition of the oxidase activity of nanoceria, which is triggered by TBA, and subsequent restoration of the catalytic activity of nanoceria by the target thrombin. To the best of our knowledge, this is the first report to exploit the interesting tendency of thrombin and its aptamer to control the oxidase activity of nanoceria for thrombin detection. This assay can be easily conducted by monitoring the colorimetric transition of the samples with the naked eye or using a spectrophotometer. The clinical utility of this simple yet highly efficient colorimetric strategy was successfully verified by reliably quantifying thrombin content in human plasma. On the basis of its performance, this biosensor prepared using the catalytic activity of nanozymes and target-specific affinity of aptamers may provide a new avenue for the rapid, convenient, and cost-effective detection of clinically important biomolecules in practical applications.

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