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RAPID COMMUNICATION

Colorimetric detection of thrombin based on signal amplification by transcription-reverse transcription concerted reaction using non-crosslinking aggregation of gold nanoparticles

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Abstract
Gold nanoparticles (AuNPs) have been used as colorimetric biosensors by utilizing the difference in color between the dispersed (red) and aggregated (blue) states. We previously developed a biosensor that converts sandwich-type thrombin recognition to RNA amplification and color difference of AuNPs. But the sensitivity was insufficient because of the linear signal amplification mechanism. In this study, we designed an exponential signal amplification biosensor based on transcription-reverse transcription concerted (TRC) reaction.

Keywords Aptamer · Thrombin · Gold nanoparticle · Signal amplification · Transcription-reverse transcription concerted (TRC) reaction

Gold nanoparticles (AuNPs) have recently been applied to a variety of biosensors because of their unique electrochemical and optical properties [1–4]. AuNPs with a diameter of less than 100 nm dispersed in a solution show a brilliant red color, but when aggregated, they change to purple/blue because of a red shift in absorption wavelength [5]. Using this large difference in color caused by the dispersion and aggregation of AuNPs, sensors that can detect various molecules have been constructed [6–10].

Because the color change of AuNPs can be observed only when there is a significant difference in aggregation or dispersion [11], the measurement sensitivity has been improved by combining it with signal amplification reactions that mainly utilize the structural change of aptamers [12–16]. These aptamer conformational change-based systems are not applied to ligands such as antibodies and lectins, which are frequently used in the field of bioassays. Therefore, we have developed a colorimetric sensor based on a sandwich-type assay that does not depend on the conformational change of aptamers [17]. However, our sensor mechanism was a linear signal amplification system using cyclic reactions of an enzyme, and therefore, its sensitivity was lower than that of an exponential signal amplification system using Hyperbranched rolling circle amplification (HRCA) [12], or others. HRCA is one of the methods for exponential amplification of long DNA strands using cyclic single-stranded DNA as a template and strand displacement DNA polymerase.

In this study, we designed an exponential signal-amplified sandwich biosensor that does not require a conformational change in the ligand using the color differences of AuNP solutions. For exponential signal amplification, we used the transcription-reverse transcription concerted (TRC) reaction [18], which can achieve exponential RNA amplification by the concerted action of reverse transcriptase and RNA polymerase under constant temperature conditions using single-stranded RNA as a template. Previously, we developed a sensor mechanism that converts sandwich-type molecular recognition into linear RNA amplification using thrombin as a model protein [17]. Herein, we designed a sensor that uses this amplified RNA as a template for the TRC-like reaction (Scheme 1).

Here, a colorimetric readout was achieved by utilizing the salt-induced non-crosslinking aggregation of AuNPs. When fully matched duplexes are formed on the AuNP surface, double-stranded (ds)DNA-AuNPs rapidly aggregate

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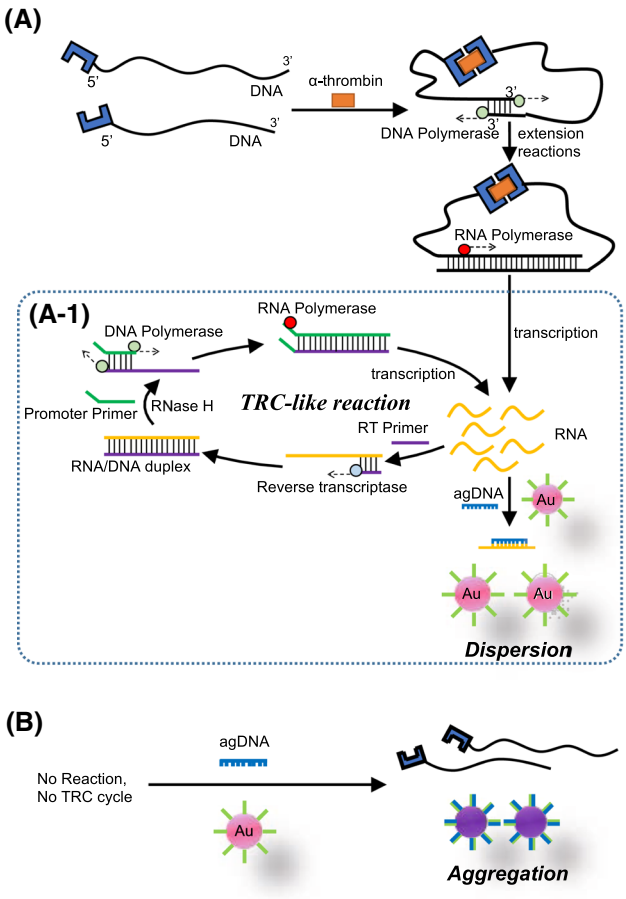

Scheme 1 Schematic illustration of the TRC-like reaction-based exponential amplified colorimetric sensor in the (**A**) presence and (**B**) absence of a target molecules

in a non-crosslinking manner at high salt concentrations, while single-stranded DNA (ssDNA)-modified AuNPs can disperse due to electrostatic and steric repulsion between particles caused by the surface-grafted ssDNA [19, 20]. It was shown that the non-crosslinking AuNP aggregation can be controlled by the concentration of the fully matched ssDNA [13, 17], which we named "aggregation-promoting DNA (agDNA)". We controlled the amount of free agDNA by capturing it with RNA synthesized in the presence of target molecules. Then, we can detect the target molecule by analyzing the dispersion and aggregation states of AuNPs. When target molecules are present, AuNPs are dispersed, and when they are absent, they aggregate.

We propose an exponential signal-amplified sandwich-type sensor based on the color difference of AuNPs. Aptamers for thrombin were used as ligands for constructing the sandwich-type assay system. First, we designed a system of TRC-like reaction-based amplified colorimetric sensors, as shown in Scheme 1. This system consisted of two DNA probes (Probe-A and Probe-B) (Fig. S1), DNA polymerase, RNA polymerase, Reverse transcriptase, RT primer, Promoter primer and ssDNA-modified AuNPs. Probe-A contains three segments: an anti-thrombin DNA aptamer (NU172) [21], T7 RNA promoter sequences, and the complementary sequence to the 3'-end segment of Probe-B. Probe-B contains two segments: an anti-thrombin DNA aptamer (HD22) [21] and a complementary sequence to the 3'-end segment of Probe-A. The complementary sequence of each probe was set to six bases because this length did not lead to the duplex formation in the absence of the target [22–24]. And even under the present experimental conditions, the DNA melting curves analysis shown in Figure S2 confirmed that each complementary strand does not form a duplex in the absence of the target. RT primer includes a part of the linker sequence that connects the segments present in Probe-B. Promoter primer contains the T7 RNA promoter sequences and is part of the sequence of Probe-A.

In the presence of thrombin (Scheme 1A), a sandwich-type interaction occurs between thrombin and the two probes (Probe-A and Probe-B), and the complementary sequences at the 3'-end of each probe form a duplex structure. DNA polymerase extends from the 3'-end of the DNA probes to form the RNA promoter duplex. Then, RNA polymerase repeats RNA synthesis due to the presence of RNA promoter sequence duplexes. This RNA synthesis triggered by the sandwich-type recognition of thrombin has been reported [17]. The synthesized RNA is used as a template for TRC-like reactions. RT primers hybridize with the RNA, and the RNA/DNA duplex is formed by the reverse transcriptase. The RNA strand is hydrolyzed by RNase H activity of the reverse transcriptase, and the Promoter primer hybridizes to the exposed DNA strand. The extension reaction by DNA polymerase forms duplex DNA containing RNA promoter sequences, and RNA is synthesized cyclically by RNA polymerase. When the synthesized RNA hybridizes with the RT primer again, the cycle of the TRC-like reaction proceeds, and exponential RNA strand synthesis takes place.

It has been reported that aggregation of ssDNA-modified AuNPs is promoted by double-strand formation of ssDNA and complementary strand at the surface [13, 19]. We have succeeded in inhibiting the aggregation of AuNPs by using the complementary strand as agDNA and trapping it with RNA [17]. As a result, no AuNP aggregation occurred in the presence of thrombin.

In contrast, in the absence of thrombin (Scheme 1B), the complementary sequences at the 3'-end of each probe dose not form a duplex structure, and little RNA was synthesized [17]. And the cycle of TRC-like reaction hardly proceeds. Then, the agDNA that was not captured by RNA hybridizes with the ssDNA modified on the surface of AuNPs, forming aggregates of AuNPs. As a result, the AuNP solution exhibited a purple color.

Next, by adding a partial sequence of RNA strands (ssRNA-70) (Fig. S1) synthesized by thrombin recognition, we confirmed that RNA strands are amplified in a TRC-like reaction and can be detected by the difference in the color of AuNPs (Scheme 1 A-1). As described above, the RNA is used as a template for TRC-like reactions, and exponential RNA strand synthesis takes place. And the RNA strand has a complementary sequence to agDNA, which inhibits the aggregation of ssDNA-AuNPs. The TRC-like reaction was incubated at 42 °C for 1 h. Figure 1A upper left photo shows the color of the AuNP solution after the addition of the reaction solution. As shown in the figure, there was a significant difference when higher concentrations of RNA were added in reaction; the solution was red when more than 25 pM RNA was added, while the color was blue upon the addition of RNA at lower concentrations. The normalized UV spectrum (530 nm) also supports a color difference with RNA concentration (Fig. 1A lower left). As the RNA concentration increased, the absorbance derived from the aggregated AuNPs decreased. The color difference due to AuNP aggregation can be evaluated by the absorbance ratio of A_{630}/A_{530} (Fig. 1A right). The difference in the ratio was evaluated by *t* test and the *p* value at 10 pM (0–10 pM) was < 0.01. This result indicates that 10 pM RNA can be detected by this system with statistical significance.

Next, to confirm that the RNA was amplified by the TRC-like reaction, the reagents necessary for the TRC reaction were removed and inhibition of the reaction was checked. Figure 1B shows the absorbance ratio of the reaction without the target, primer, and enzyme, respectively. As a positive

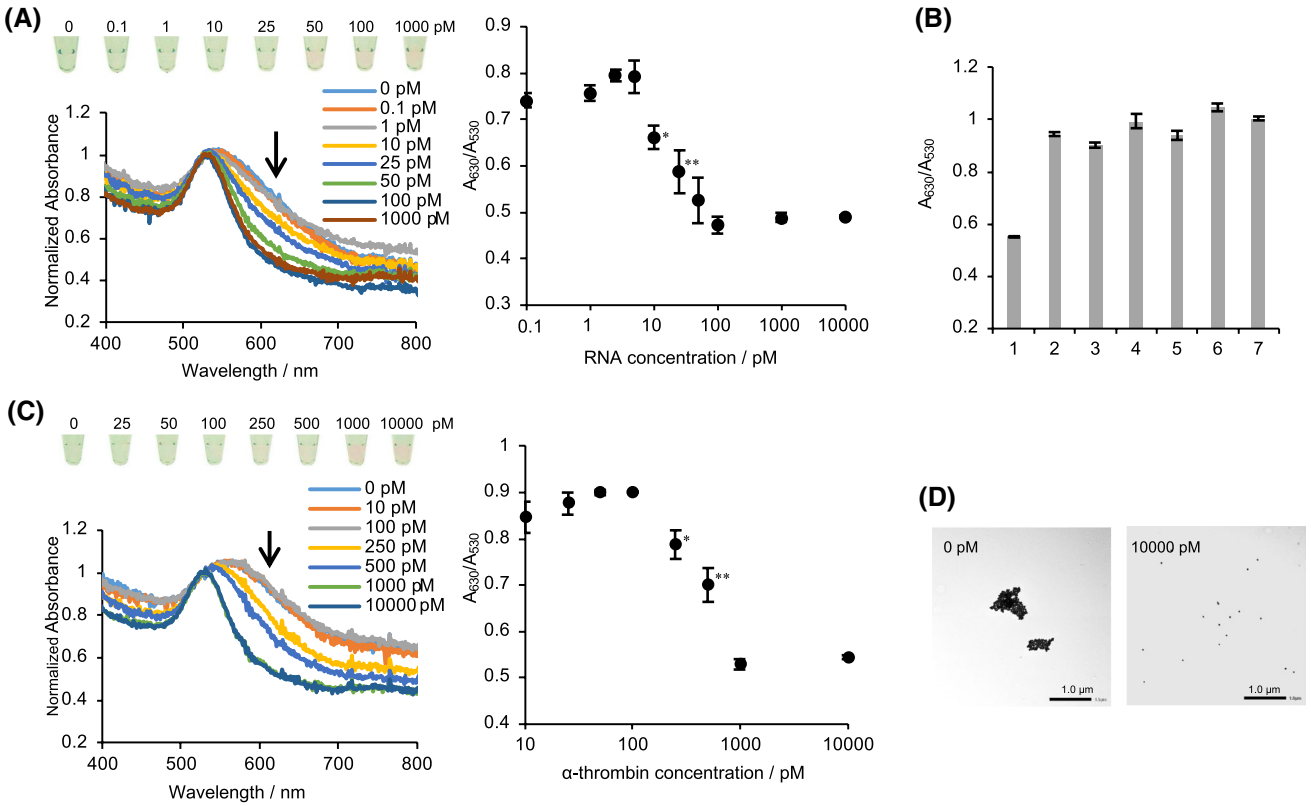

Fig. 1 Characteristics of the proposed biosensor. **A** Analytical performance of the colorimetric RNA assay using TRC-like reaction. RNA concentration indicates the concentration during the TRC-like reaction. Upper left, photographs of AuNP dispersions containing RNA at different concentrations; lower left, normalized absorption spectra (530 nm) of AuNP dispersions at various RNA concentrations; right, difference in absorbance ratio at 630 nm and 530 nm. Error bars indicate the standard deviation of the three measurements. The ratio at 0 pM was 0.773 ± 0.020. **p* value < 0.01 (0–10 pM *t* test); ***p* value < 0.005 (0–25 pM *t* test). **B** Checking the Progress of TRC-like Reaction. Ratio of the absorbance at 630 and 530 nm in the absence of various reagents. 1, positive control with target RNA and contained all reagents; 2, negative control without target RNA and contained all reagents; the following includes target RNA and does not include the reagents listed. 3, RT primer; 4, Promoter primer; 5, Reverse transcriptase; 6, RNA polymerase; and 7, DNA polymerase. Error bars indicate the standard deviation of the three measurements. **C** Analytical performance of the colorimetric thrombin assay using TRC-like reaction. Thrombin concentration indicates the concentration during the TRC-like reaction. Upper left, photographs of AuNP dispersions containing thrombin at different concentrations; lower left, normalized absorption spectra (530 nm) of AuNP dispersions at various thrombin concentrations; right, difference in absorbance ratio at 630 nm and 530 nm. Error bars indicate the standard deviation of the three measurements. The ratio at 0 pM was 0.820 ± 0.026. **p* value < 0.05 (0–250 pM *t* test); ***p* value < 0.005 (0–250 pM *t* test). **D** TEM image of AuNPs upon detection of 0 pM and 10,000 pM thrombin

control, when the reaction was carried out in the presence of 10 nM RNA, the absorbance ratio was small, and it was confirmed that AuNPs were present in a dispersed state due to RNA amplification reaction (Fig. 1B-1). As a negative control, when the reaction was carried out in the absence of RNA, the absorbance ratio became large, and it was confirmed that AuNPs were aggregated without RNA amplification reaction (Fig. 1B-2). When the reaction was performed in the presence of target RNA, except for each primer (RT primer, Promoter primer) and enzyme (reverse transcriptase, RNA polymerase, DNA polymerase), it was shown that AuNPs aggregated (Fig. 1B-3, 4, 5, 6, 7). These results indicate that the respective primers and enzymes are essential for RNA amplification, suggesting that RNA amplification is achieved by TRC-like reaction.

Because the reverse transcriptase (AMV reverse transcriptase) used in this experiment has DNA polymerase activity, we thought that the reaction would proceed even in the absence of DNA polymerase (96-7 DNA polymerase). But, in this experiment, we found that the addition of DNA polymerase was necessary to proceed the TRC-like reaction. This may be because the DNA synthesized by reverse transcription forms a secondary structure. To strip off the secondary structure, the strand-replacement type DNA polymerase used in this study is thought to be necessary.

Next, we investigated whether exponential signal amplification could be used to detect thrombin by the color difference of AuNPs. Previously, we have succeeded in converting aptamer-mediated sandwich-type thrombin recognition into RNA amplification [17]. This RNA was used as a template for a TRC-like reaction to perform exponential signal amplification for colorimetric detection of thrombin (Scheme 1).

Unfortunately, no concentration-dependent color difference of AuNPs was observed when thrombin was detected under the same conditions as RNA detection (50 nM final agDNA). The AuNP solution was red in all concentration conditions, and little change in the absorption spectrum was observed (Fig. S3A). This was thought to be due to a small amount of unwanted RNA amplification even in the absence of thrombin. Therefore, we first determined the agDNA concentration at which the AuNPs could be aggregated even if a small amount of RNA amplification was performed in the background noise (Fig. S3B). As shown in Figure S3B, the presence of agDNA at a final concentration of 100 nM caused the AuNPs to aggregate in the absence of thrombin. Thus, we decided to use agDNA with a final concentration of 100 nM for thrombin detection.

Figure 1C shows the analytical performance of the colorimetric thrombin assay. The TRC-like reaction was carried out by incubation at 42 °C for 1 h. Figure 1C upper left photo shows the color of the AuNP solution after the reaction with thrombin. As shown in the figure, the solution was red when a high concentration of thrombin was added and blue when a low concentration of thrombin was added. The normalized UV spectrum (530 nm) also supports a color difference with the thrombin concentration (Fig. 1C lower left). As the thrombin concentration increased, the absorbance derived from the aggregated AuNPs decreased. The color difference due to AuNP aggregation can be evaluated by the absorbance ratio of A_{630}/A_{530} (Fig. 1C right). The difference in ratio was evaluated by *t* test and the *p* value at 250 pM (0–250 pM) was < 0.05. This result indicates that 250 pM thrombin can be detected by this system with statistical significance. This was 10-times more sensitive than our previous detection of thrombin in the color difference of AuNPs using linear signal amplification [17]. This high sensitivity suggests that exponential signal amplification by TRC-like reaction was performed.

The AuNP aggregation caused by the double-strand formation of agDNA and the ssDNA on the AuNP surface was also confirmed by TEM (Fig. 1D). In the absence of thrombin, sufficient RNA is not synthesized to inhibit aggregation by agDNA, which results in the aggregation of AuNPs (Fig. 1D, left). On the other hand, in the presence of thrombin, sufficient RNA is synthesized to inhibit aggregation by agDNA, and the AuNPs remain in a dispersion state (Fig. 1D, right).

As a control experiment, 10 nM thrombin was added to the probe without the anti-thrombin aptamer sequence (NA and NB) (Fig. S4). In the absence of aptamer sequences, AuNPs aggregated even when thrombin was added. This result indicates that the system works by a sandwich-type interaction between the two aptamer sequences and thrombin.

In conclusion, a novel exponential signal amplified colorimetric method based on a TRC-like reaction for the detection of thrombin is presented. Exponential signal amplification was achieved by cyclic synthesis of RNA by TRC-like reaction. It was possible to improve the detection sensitivity more than tenfold compared with our previous study [17]. The advantage of this assay is that the target can be sensitively detected by the color difference of the AuNP solution. However, we also detected background noise associated with the higher sensitivity. It would be possible to reduce by optimizing the reaction conditions, such as salt concentration and probe sequence modification. In this study, we used non-crosslinking aggregation based on DNA duplex formation on the surface of AuNPs to obtain a rapid color response [19, 25]. This nanoparticle aggregation is thought to be triggered by blunt end stacking of short DNA strand duplexes [20]. It has also been reported that the formation of long DNA duplexes on the surface of AuNPs prevents aggregation due to steric repulsion [20]. Thus, it is difficult to directly induce non-crosslinking aggregation using the synthesized RNA since the RNA strand synthesized in the TRC-like reaction is long dut to the primer binding site. Therefore, we used

agDNA to convert the signal of the amplified RNA into the color difference of the AuNPs. Since this method does not require structural changes in the aptamer, it can be easily adapted to molecular recognition mechanisms other than aptamers. The results presented in this study will contribute to the construction of a versatile and highly sensitive molecular detection system using a variety of ligands.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s44211-022-00050-5.

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