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# Simple and rapid colorimetric detection of cofactors of aptazymes using noncrosslinking gold nanoparticle aggregation

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ABSTRACT

We developed a method for simply and rapidly detecting cofactors of aptazymes with high sensitivity using unique noncrosslinking gold nanoparticle aggregation. Applying this method to a theophylline-dependent aptazyme, 100 μM, 10 μM, and 1 μM theophylline were detected easily by the naked eye within 10 min, 20 min, and 65 min, respectively. This method is also applicable to other cleavase-aptazymes without altering the probe-DNA sequence on the gold nanoparticle.



Aptazymes, which are enzymatic nucleic acids (i.e., ribozymes or DNAzymes) that can be allosterically activated by their cofactor molecules, are currently attracting much attention as potential biosensors in the fields of biotechnology, diagnostics and therapy due to their usefulness in detecting their own cofactors.[1] They take active forms as ribozymes (or DNAzymes) upon changing their conformation only by binding to their cofactors. Breaker et al. identified various cleavase-aptazymes, which respond to their cofactors to change their conformations and thus cleave themselves.[2] Due to their highly optimized switching system and sensing ability, many cleavase-aptazyme-based sensors have been developed.[3–6] In these studies, various methods were used to detect the signal of ribozyme activity: labeling the ribozyme with radioisotopes[3] or fluorophores,[4] using a quartz crystal microbalance,[5] or using a cell-free translation system.[6] However, these methods are expensive and/or require technical skill, and/or their detection sensitivities are often lower than the latent abilities of aptazymes. Therefore, in this study, we aimed for simple and visible detection of the signal of the ribozyme activity with high sensitivity.

The best candidate for a simple and visible detection assay is a gold nanoparticle (AuNP) aggregation assay. Colorimetric assays with AuNP aggregation have been widely and generally used for detection of DNA.[7] Recently, some studies have reported the detection of target molecules other than DNA using their aptamers and AuNPs.[8] Lu et al. leveraged 'crosslinking' AuNP aggregation to detect the signal of DNAzyme-based aptazyme activity for sensing its cofactor.[9] However, in this detection system, only 100 μM adenosine can be detected with the detection time of 2 h. This low sensitivity and long detection time are probably caused by low activity of the DNAzyme-based aptazyme. We report here the detection of activity of ribozyme-based aptazymes, which generally have higher activity and switching efficiency than DNAzyme-based ones, using 'noncrosslinking' AuNP aggregation,[7b,10] which is distinct from the 'crosslinking' one.

DNA-tethered AuNPs are generally resistant to salt-induced colloidal aggregation due to electrostatic repulsion caused by phosphate anions in the tethered DNA and due to entropic effects of the tethered DNA. However, they aggregate rapidly (within 1 min) in the presence of certain amounts of salts by 'blunt-end' duplex formation between the tethered DNA and a complementary target DNA, presumably due to the loss of flexibility on the Au colloid surface and stacking interactions between duplex termini on the individual AuNPs (Fig. 1a).[7b,10] If this unique 'noncrosslinking' AuNP aggregation phenomenon also occurs with DNA/RNA duplexes on the surface of AuNPs, it is possible to detect the cleavage of the aptazyme simply and visibly. Figure 1b shows the detection system using noncrosslinking AuNP aggregation for sensing the aptazyme activity (i.e., the cofactor of the aptazyme) in this study. In the absence of the cofactor of the aptazyme (Fig. 1b, left), AuNP aggregation is not expected to occur even if the aptazyme hybridizes to probe-DNA on AuNPs because the surface terminal will be dangling.[7b,10] On the other hand, in the presence of the cofactor (Fig. 1b, right), the aptazyme cleaves itself to produce the cleaved RNA, which is released from the aptazyme at suitable temperature. If the cleaved RNA induces noncrosslinking AuNP aggregation with AuNPs wherein the probe-DNA completely complementary to the cleave RNA is tethered, the cofactor can be detected easily.

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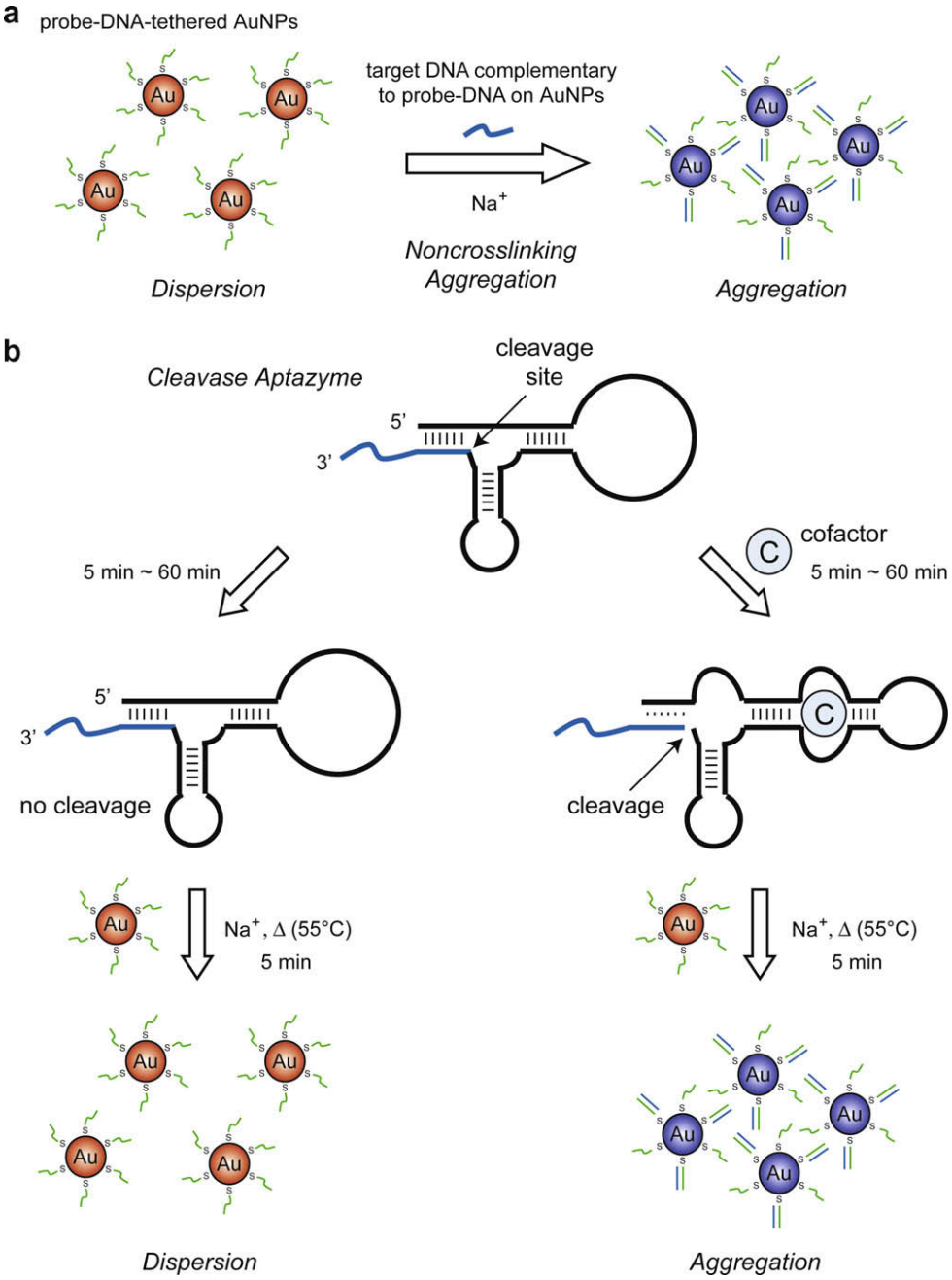


**Figure 1.** (a) Noncrosslinking AuNP aggregation.[7b,10] Although single-stranded DNA-tethered AuNPs are generally resistant to salt-induced colloidal aggregation (left), they aggregate rapidly in the presence of certain amounts of salts by addition of the target DNA complementary to probe-DNA on the Au surface (right). (b) Illustration of the detection system for the cofactor of the aptazyme using noncrosslinking AuNP aggregation. In the absence of the cofactor, neither self-cleavage of the aptazyme nor AuNP aggregation occurs (left). In the presence of the cofactor, the aptazyme cleaves itself and then the cleaved RNA (blue) induces noncrosslinking AuNP aggregation with AuNPs, wherein the probe-DNA complementary to the cleaved RNA is tethered (right).

First, we investigated whether this noncrosslinking aggregation occurs with DNA/RNA duplexes on AuNPs. Probe-DNA-tethered AuNPs were prepared as previously described,[7b,10] with the diameter of AuNP being 40 nm to maximize the sensitivity[10b] and the length of probe-DNA being 20 mer. The surface coverage was estimated as about 17 pmol/cm$^2$ (500 probe-DNA per particle), which is consistent with our previous report.[10b] While the probe-DNA-tethered AuNPs were stable at 1 M NaCl (Fig. 2a, left), when the target RNA complementary to the probe-DNA was added, noncrosslinking aggregation occurred rapidly at the same salt concentrations (Fig. 2a, right). The detection limit of the target RNA by the naked eye with 100 nM[11] probe-DNA-tethered AuNPs at 1 M NaCl was 7.5 nM, which was only 7.5% of the probe-DNA on the AuNP surface and 2-fold lower than when the target was DNA.[12]

Next, we performed noncrosslinking aggregation experiments using a theophylline-dependent aptazyme.[13,14] A 12-mer extra sequence was tethered to the 3′-terminus of the aptazyme so that the cleaved RNA hybridized to the probe-DNA on AuNPs rather than to the parent aptazyme. The transcribed theophylline-dependent aptazyme (5 μM) was exposed to 20 mM $MgCl_2$ in the presence of various concentrations of theophylline or caffeine. After incubation at 23 °C for 5 min, the reaction solution was 10-fold diluted with buffer containing probe-DNA-tethered AuNPs and NaCl, the final concentrations of which were 100 nM[11] and 1 M, respectively.

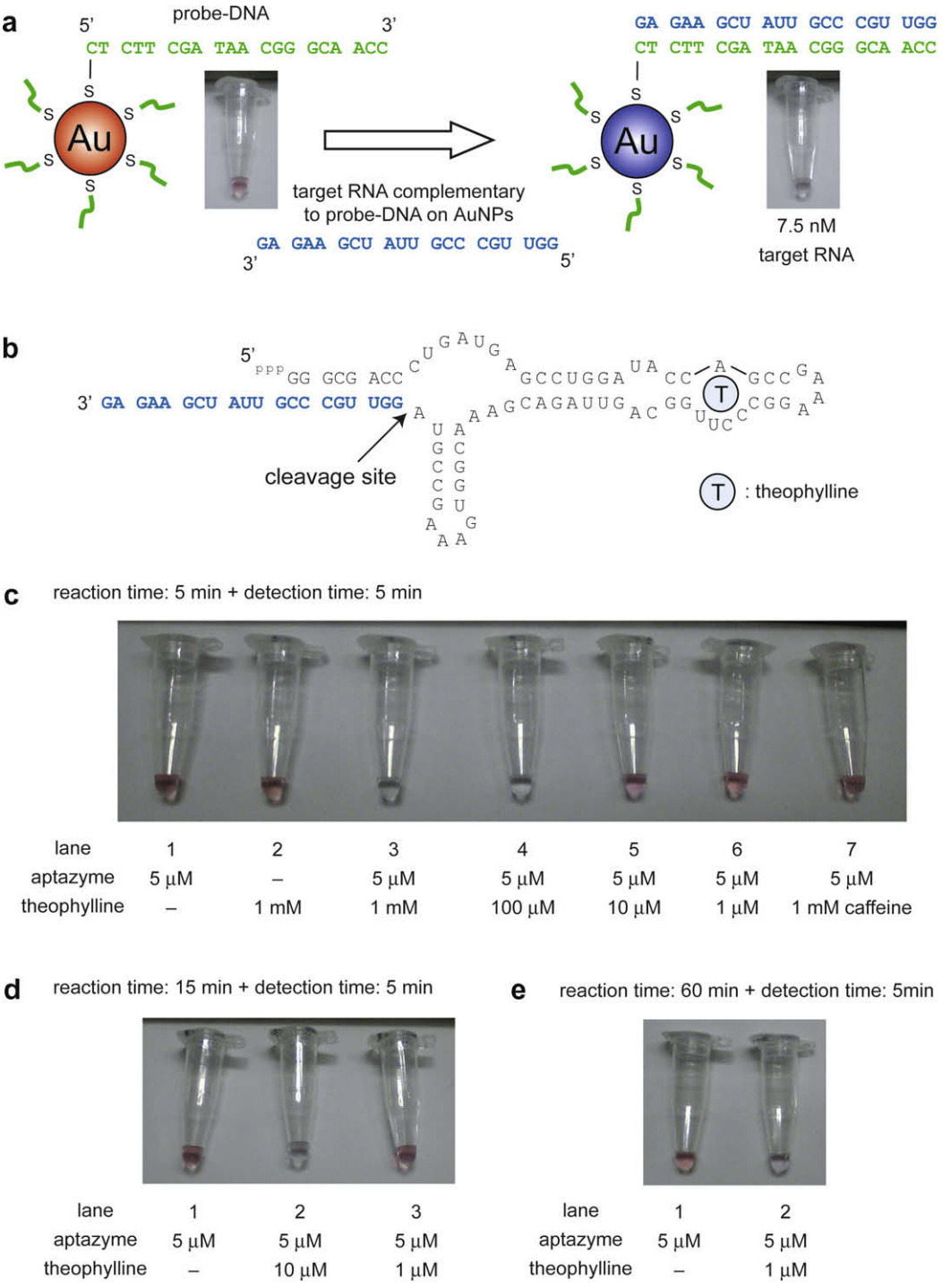


**Figure 2.** (a) Noncrosslinking AuNP aggregation as a result of hybridization between probe-DNA (green) on the Au surface and the target RNA (blue). (b) The theophylline-dependent aptazyme[13] having the target RNA sequence (blue) complementary to probe-DNA on the Au surface. (c–e) Noncrosslinking AuNP aggregation behaviors at various aptazyme-reaction times (5 min, 15 min, or 60 min) at various concentrations of theophylline or caffeine. All described concentrations are in the aptazyme-reaction solution.

At this point, detectable aggregation did not occur because the cleaved RNA still hybridized to the 5′-terminus of the parent aptazyme at 23 °C. However, when the RNA-AuNPs mixture was warmed at 55 °C for 5 min for the cleaved RNA to dehybridize from the parent aptazyme and to hybridize to the probe-DNA on AuNPs,[15] two mixtures from the reaction solution at 1 mM and 100 μM theophylline changed these colors rapidly from red to colorless as a result of the complete precipitation of AuNP aggregates (Fig. 2c, lanes 3 and 4). This result indicates that the cleaved RNA was released from the parent aptazyme and hybridized to the probe-DNA on AuNPs at this temperature. The mixture from the reaction solution at 10 μM theophylline resulted in a slight color change from red to purple (Fig. 2c, lane 5), which was difficult to detect by the naked eye but could be detected by its absorbance spectrum (see Supplementary data). When the reaction time was extended to 15 min, the color changed from red to colorless (Fig. 2d). Although the mixture from the 30-min reaction solution at 1 μM theophylline showed no change, the color changed from

red to pale purple when the reaction time was extended to 60 min (Fig. 2e). On the other hand, the mixture from the reaction solution in the absence of theophylline or in the presence of 1 mM caffeine remained unchanged (Fig. 2c–e, lane1; Fig. 2c, lane 7).[16]

In summary, we succeeded in simple and rapid detection of the activity of the theophylline-dependent aptazyme with high sensitivity using the unique noncrosslinking AuNP aggregation phenomenon to sense the cofactor, theophylline. Considering the detection limit (7.5 nM) for RNA in this method, only ~1.5% of self-cleavage of the aptazyme is theoretically required for detection under the conditions in this study.[17] Using this method, 100 μM, 10 μM, and 1 μM theophylline were detected easily by the naked eye after only 10 min, 20 min, and 65 min of examination (total time of reaction and detection), respectively. These detection limits and times are much lower and shorter than crosslinking aggregation methods using DNAzyme-based aptazymes (100 μM and 2 h, respectively).[9] Moreover, this method is applicable to other cleavase-aptazymes without altering the probe-DNA sequence, because the 5′- and 3′-terminal sequences of the aptazymes are conserved.[2] In fact, we confirmed that FMN and cGMP can be easily detected using each corresponding aptazyme[18] and the same probe-DNA-tethered AuNPs as used in this study (see Supplementary data).

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.051.

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11. Strand concentrations of total probe-DNA on the AuNPs in the RNA-AuNPs mixture.
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14. The apparent dissociation constant ($K_D$) of the aptazyme for theophylline is 40 μM.[13].
15. We optimized this cleaved RNA-releasing temperature. Although no change was detected at 40 °C, a color change from red to purple was detected at 50 °C. The color changed from red to colorless at 55 °C. This indicates that Tm between the cleaved RNA and the parent aptazyme is 50–55 °C. On the other hand, because aggregation occurred at 55 °C, Tm between the cleaved RNA and the probe-DNA on AuNPs is estimated well above 55 °C.
16. To verify that the AuNPs precipitate certainly by hybridization between the probe-DNA and the cleaved RNA, we performed the aggregation experiments using noncomplementary probe-DNA (on AuNPs)/cleaved RNA (from the aptazyme) pairs. As a result, using these noncomplementary pairs, aggregation did not occur even in the presence of cofactors and the cleaved RNA. Because the complementary but non-cleaved aptazyme or the cofactor did not also induce aggregation, it is clear that only the complementary cleaved RNA can induce aggregation by hybridizing to the probe-DNA on AuNPs.
17. In fact, the theophylline-dependent aptazyme having the target RNA sequence was only 3% cleaved in 5 min at 100 μM theophylline (Fig. 2c, lane 4), estimated from PAGE analysis (see Supplementary data). Because this detectable amount of 1/10-diluted 3% cleaved RNA (15 nM) is comparable to the detection limit of free RNA (7.5 nM), the sensitivity of the system for the cleaved RNA from the aptazyme is similar to that for the free RNA.
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