Hybridization chain reaction-based colorimetric aptasensor of adenosine 5'-triphosphate on unmodified gold nanoparticles and two label-free hairpin probes

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ABSTRACT

This work designs a new label-free aptasensor for the colorimetric determination of small molecules (adenosine 5'-triphosphate, ATP) by using visible gold nanoparticles as the signal-generation tags, based on target-triggered hybridization chain reaction (HCR) between two hairpin DNA probes. The assay is carried out referring to the change in the color/absorbance by salt-induced aggregation of gold nanoparticles after the interaction with hairpins, gold nanoparticles and ATP. To construct such an assay system, two hairpin DNA probes with a short single-stranded DNA at the sticky end are utilized for interaction with gold nanoparticles. In the absence of target ATP, the hairpin DNA probes can prevent gold nanoparticles from the salt-induced aggregation through the interaction of the single-stranded DNA at the sticky end with gold nanoparticles. Upon target ATP introduction, the aptamer-based hairpin probe is opened to expose a new sticky end for the strand-displacement reaction with another complementary hairpin, thus resulting in the decreasing single-stranded DNA because of the consumption of hairpins. In this case, gold nanoparticles are uncovered owing to the formation of double-stranded DNA, which causes their aggregation upon addition of the salt, thereby leading to the change in the red-to-blue color. Under the optimal conditions, the HCR-based

colorimetric assay presents good visible color or absorbance responses for the determination of target ATP at a concentration as low as 1.0 nM. Importantly, the methodology can be further extended to quantitatively or qualitatively monitor other small molecules or biotoxins by changing the sequence of the corresponding aptamer.

Keywords: Colorimetric aptasensor, adenosine 5'-triphosphate, hybridization chain reaction, gold nanoparticles, label-free hairpin probe

1. Introduction

Aptamers are short oligonucleotide sequences by an *in vitro* selection process, which are high specificity recognition and binding affinity to their cognate targets (Xia et al., 2013; Sternberg and Pierce, 2014; Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990). Compared to conventional antibody-based assay methods, aptamers have several superior properties that make them ideal candidates for biological detection (Nagarajan et al., 2010). Firstly, aptamers directly recognize a wide range of target analytes ranging from inorganic ions (Forbes and Sisco, 2014), small molecules (MacGriff et al., 2013) to proteins (Li et al., 2012), even cells (Xie et al., 2014). Secondly, aptamers are smaller molecular weight and better chemically stable, which are prone to chemical synthesis and easy to modify functional groups (Chalker et al., 2011). Thirdly, aptamers can be obtained *in vitro*, which can choose any targets to bind academically. Also, aptamers can be developed in automated processes against various proteins or nucleic acids, and then easily modified by attaching fluorescence reporters, nanoparticle or biotin moieties, rival antibodies in high-throughput proteomics and cell separations (Nery et al., 2009). This provides high flexibility and conveniences in design, which improve the sensitivity and selectivity of aptamer-based biosensors (Kong et al., 2011). Until now, there are abundance methods that use the aptamers to detect different biomolecules based on various-signal transducer principles, *e.g.*, by using fluorescence (Ling et al., 2016), surface plasmon resonance (SPR) (Miao et al., 2016), colorimetric assay (Nguyen et al., 2016), UV-vis adsorption spectroscopy (Hizir et al., 2016), resonance light scattering technique (Chen et al., 2016), chemiluminescent detection (Khang et al., 2016) and so on (Seok et al., 2016). Among these detection strategies, aptamer-based colorimetric assay has attracted great attentions on account of its simplicity, convenience and speediness, which

53 is not necessary to use expensive instruments and is suitable for real-time analysis (Sharma et al.,
54 2015; Huang et al., 2013; Chang et al., 2013).

55 To successfully develop a good colorimetric assay, the design and use of the signal-transduction
56 tags are very crucial to achieve a highly detectable sensitivity. Gold nanoparticles (AuNPs) are
57 increasing interest in the colorimetric assay due to their high extinction coefficient and unique
58 surface plasmon resonance (SPR) properties (Zhao et al., 2015; Latorre-Sanchez and Pomposo,
59 2015). Meanwhile, gold colloids also possess specially chemical and physical properties that make
60 them play a fundamental role in the chemical biosensors (Soh et al., 2015; Ramezani et al., 2015).
61 Recent researches have witnessed the development of AuNPs-based colorimetric assay methods
62 (Zeng et al., 2015; Wu et al., 2017), depending on the distinct phenomenon that the color of
63 well-dispersed gold colloids appears as a red, whilst that of the aggregated gold colloids is blue or
64 purple (Bui et al., 2015; Wu et al., 2015). In this case, the detection could be carried out based on
65 the change in the color by using the naked eye, and without the need of sophisticated instruments.

66 Another important concern for the successful development of aptamer-based colorimetric assay is
67 to design a highly efficient target-induced DNA sensing probe (Feng et al., 2014). Liu et al.
68 designed a target-induced conformational switching of the aptamer to form a G-quadruplex
69 structure for colorimetric detection of 8-hydroxy-2'-deoxyguanosine (Liu et al., 2014a). Zhang et al.
70 also reported an AuNPs-based colorimetric aptasensor by using hairpin aptamer as the sensing
71 probe (Zhang et al., 2014). In this regard, the motivation of our work is to design new hairpin
72 aptamer-protected AuNPs for the development of colorimetric small molecular assay.

73 Adenosine 5'-triphosphate (ATP, a nucleoside triphosphate used in cells as a coenzyme) is
74 critically involved in maintaining the cell structure by facilitating assembly and disassembly of
75 elements of the cytoskeleton (Reseter and Chalovich, 1995; Grahame and Hawley, 2001; Saylor et
76 al., 1998). Recently, different methods and strategies have utilized for sensitive and selective
77 analysis of ATP, *e.g.*, by using fluorescence (Xu et al., 2016, Yang et al., 2016) and fluorescence
78 imaging (Dong and Zhao, 2016), photoelectrochemical aptasensor (Fan et al., 2016), circular
79 dichroism (Fu et al., 2016), electrogenerated chemiluminescence (Dong et al., 2016), colorimetric
80 assay (Huo et al., 2016) and electrochemical biosensor (Su et al., 2016). Despite some advances in
81 this field, there is still the request to explore simple and feasible detection schemes for quantitative
82 evaluate of ATP in biological fluids. Herein, we report on a novel colorimetric aptasensor for the

83 detection of ATP by coupling visible-colored AuNPs with two hairpin DNA probes (H1 and H2,
84 Scheme 1). The signal is amplified on the basis of hybridization chain reaction (HCR). Initially, the
85 as-synthesized AuNPs are protected through the exposed single-stranded DNA of two hairpin
86 probes at the stem ends, thereby avoiding the aggregation of AuNPs. In the presence of target ATP,
87 the analyte reacts with the 3' end of H1 (*i.e.*, the aptamer sequence) to open the hairpin probe. In
88 this case, hairpin H1 is detached from the AuNPs, and the newly exposed sticky end at the 5' end
89 nucleates at the sticky end of H2 and opens the hairpin to expose a sticky end on H2, thus
90 undergoing an unbiased strand-displacement reaction between two hairpins. In this way, a large
91 number of hairpin probes break away from the AuNPs, thus causing the aggregation of gold colloids
92 in the presence of salt to induce the blue shift of the localized surface plasmon resonance (LSPR)
93 peak of gold colloids. Meanwhile, the color of the resulting suspension also changes from red to
94 blue or purple. By monitoring the change in the color and absorbance in the LSPR peak, we can
95 qualitatively/quantitatively determine the concentration of target ATP in the sample.

97 **Scheme 1** Schematic illustration of AuNP-based colorimetric assay toward target ATP by coupling two label-free
98 hairpin probes with analyte-triggered hybridization chain reaction.

99 2. Experimental

100 2.1. Chemicals and instruments

101 All oligonucleotides designed in this study were purchased from Beijing Dingguo Biotechnol.

Co., Ltd. (Beijing, China), and purified through high-performance liquid chromatography (HPLC).
The sequences of oligonucleotides are listed as follows:

H1: 5'-TCCGCAATACTCCCCAGAATAC**CCTGGGGGAGTATTGCGGAGGAAGG**-3'

H2: 5'-GTATTCTGGGGGAGTATTGCGGA*CCTTCCTCCGCAATACTCCCCAGG*-3'

The bold letters of H1 at the 3' end are the sequence of ATP aptamer (Liao et al., 2016), which is complementary to the italic letters of H2. The underlined letters of H2 are complementary to the underlined sequences of H1. A pH 7.4 phosphate-buffer saline (PBS, 10 mM) solution concluding 10 mM Na₂HPO₄ and 10 mM NaH₂PO₄ was used for preparation of H1 and H2. Chloroauric acid (HAuCl₄·4H₂O) and trisodium citrate were purchased from Sinopharm Chem. Inc. (Shanghai, China) and Fuchen Chem. Re. Inc. (Tianjin, China), respectively. Adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), uridine 5'-triphosphate (UTP) and cytidine 5'-triphosphate (CTP) were achieved from Sigma-Aldrich (Shanghai, China). Ultrapure water obtained from a Millipore water purification system (18.2 MΩ cm⁻¹, Milli-Q, Millipore) was used in all of the experiments.

Ultraviolet-vis absorption (UV-vis) spectra were recorded on a Lab Microplate Reader (SH-1000, Corona Electric Co., Ltd., Japan). The nanomaterials were characterized by using transmission electron microscopy (TEM, Hitachi H-7650, Tokyo, Japan) and dynamic light scattering (DLS, Zetasizer Nano S90, Malvern, London, UK). Gel electrophoresis was carried out on Tanon 4100 Gel Imaging System (Shanghai, China).

2.2. Preparation of gold nanoparticles (AuNPs)

Gold nanoparticles (designated as AuNPs) with 16 nm in diameter were prepared according to our previous report (Zhang et al., 2012a). All glassware used in the following procedures was cleaned in a bath of K₂Cr₂O₇-H₂SO₄, rinsed thoroughly in double distilled water and dried in air. Briefly, 1.0 mL of 1.0 wt% HAuCl₄·4H₂O solution was initially added to 99 mL of distilled water, and then 2.5 mL of sodium citrate (1.0 wt%) was quickly dropped into the boiling solution. The mixture was boiled for about 10 min until the color became claret. The obtained gold colloids (*Note*: Gold colloids were diluted to 100 mL with ultrapure water in order to calculate the concentration of gold nanoparticles) were cooled at room temperature, and stored at 4 °C when not in use. Herein, the concentration of the AuNPs ($C_{[AuNP]}$) can be estimated to $\sim 9.95 \times 10^{-11}$ M, using the Beer's law

[$A = \epsilon lc$, where the extinction coefficient (ϵ) is calculated by the equation ($\ln \epsilon = 1.4418 \times \ln D + 18.955$), D is the diameter in nm] (Yguerabide and Yguerabide, 1998; Kanjanawarut and Su, 2009).

2.3. Colorimetric measurement

In this work, the AuNPs-based colorimetric assay was carried out in a PCR tube as follows. Before use, two hairpin DNA probes were heated to 90 °C for 5 min and allowed to cool naturally to room temperature. Following that, 5.0 μ L of target ATP standard/sample in distilled water with different concentrations was initially added into pH 7.4 PBS (10 mM) containing 20 μ L of 1.0 μ M H1 and 20 μ L of 1.0 μ M H2. Then, the mixture was incubated for 80 min at 37 °C. During this process, analyte-induced hybridization chain reaction was readily implemented between H1 and H2. Following that, 55 μ L of gold colloids ($C_{[\text{AuNP}]} = 9.95 \times 10^{-11}$ M) was injected into the resulting mixture, and reacted for 50 min at room temperature. Subsequently, 20 μ L of 1.2 M NaNO_3 was added into the resultant suspension for the colorimetric development. Finally, the suspension was transferred to a transparent microplate to monitor UV-vis absorption spectra on a Lab Microplate Reader. After the addition of salt immediately, the photographs were taken with Cannon EOS 70D digital camera. All measurements were conducted at room temperature. Analyses are always made in triplicate.

3 Results and discussion

3.1. Design and mechanism of HCR-based colorimetric assay with AuNPs

Colloidal gold, a suspension of nanometer-sized gold particles, is usually red in color because of its strong absorption toward green light at ~520 nm (corresponding to the frequency at which a plasmon resonance occurs within the gold metal). Aggregation of AuNPs inducing by addition of salt can change the color of the colloids from red to purple, and give rise to the shift in the absorption peak toward longer wavelength (Li et al., 2009). As is well known, single-stranded DNA (ssDNA) can be physically adsorbed on the AuNPs through the electrostatic interaction between its exposed nitrogen-containing bases and AuNPs to protect from salt-induced aggregation (Wang et al., 2007). In contrast, the double-stranded DNA or folded aptamers are difficultly adsorbed onto the AuNPs owing to their higher-rigidity structure. In this work, we design two new DNA hairpins

157 H1 and H2 (Scheme 1). Each hairpin has a stem of >10 base pairs enclosing a hexanucleotide loop
 158 (Note: 17 base pairs for H1 vs. 18 base pairs for H2 according to Mfolding prediction software).
 159 Importantly, each also has a relatively long sticky end at the 3' end for H1 and 5' end for H2, which
 160 is used as the single-stranded DNA to stop from the aggregation of AuNPs. The ATP aptamer lies
 161 in the 3' end (25 nt) of hairpin H1 (complementary to the 3' end of H2). Typically, the ATP aptamer
 162 consists of two highly conserved guanine-rich regions, two invariant adenine residues and two
 163 predominant Watson-Crick covariation regions. In the presence of target ATP, the 25-mer DNA
 164 aptamer can bind ATP with the dissociation constant of $6 \pm 3 \mu\text{M}$ to open the hairpin H1. The
 165 newly exposed sticky end of hairpin H1 at the 5' end nucleates with the 5' end of hairpin H2 and
 166 opens the hairpin to expose a sticky end on the H2. In this way, the exposed sticky end propagates a
 167 chain reaction of hybridization events between alternating H1 and hairpin H2 to form a nicked
 168 double-helix, thereby resulting in the detachment of H1 and H2 from the AuNPs. Although the
 169 formed nicked double-helix has the free sticky end, the whole number greatly decreases relative to
 170 the initial modification, thus causing the salt-induced aggregation and the color change from red to
 171 purple. To further explain our design for hairpin DNA probes theoretically, we used DNA Mfolding
 172 prediction software from the Web Server (<http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form>) and
 173 MFE analysis tool (<http://www.nupack.org/utilities/show/110242?token=7HPtETju5T>) simulate two DNA
 174 structures. As shown in Scheme 1, hairpin DNA probes could be formed through our method.

175
 176 **Fig. 1.** (A,B) TEM images of (A) H1 + H2 + AuNP + NaNO₃ and (B) H1 + H2 + AuNP + ATP + NaNO₃ (Insets:
 177 DLS analyses of the corresponding samples); (C) UV-vis adsorption spectra of (a) H1 + H2 + AuNP + NaNO₃
 178 and (b) H1 + H2 + AuNP + NaNO₃ + ATP, respectively (Insets: the corresponding photographs) (Note: 1.0 μM H1,
 179 1.0 μM H2, 99.5 pM AuNP, 1.2 M NaNO₃ and 0.5 μM ATP used in these cases).

180 To realize our design, one precondition for the development of HCR-based colorimetric assay
181 lies in the fact whether target ATP could induced the aggregation of AuNPs for the changing color
182 and absorbance of the detection solution in the presence of H1, H2 and NaNO₃-salt. Initially, we
183 used TEM to investigate the H1-H2-AuNP-NaNO₃ system in the absence and presence of 0.5 μM
184 ATP (used as an example). As seen from Fig. 1A, addition of 1.2 M NaNO₃ in the system did not
185 almost cause the aggregation of AuNPs in the absence of target ATP, indicating that our designed
186 H1 and H2 could be used as the protecting agents for AuNPs. In contrast, a large number of AuNPs
187 were aggregated together in the presence of ATP (Fig. 1B). Logically, a puzzling question arises to
188 whether the aggregation derived from the interaction between AuNPs and target ATP. As control
189 test, target ATP was directly added into gold colloids, which did not make AuNPs aggregated (Fig.
190 S1 in the Supporting Information). Meanwhile, we also used DLS to investigate the size of AuNPs
191 before and after reaction with target ATP. As shown from the insets from Fig. 1A-B, the average
192 size of the nanoparticles increased from 15.8 ± 0.3 nm to 376.4 ± 12.6 nm. The reason for the
193 increase in size originated from the aggregation of AuNPs.

194 Furthermore, UV-vis adsorption spectroscopy (UV-1102, TECOMP, Shanghai, China) was also
195 employed to monitor the H1-H2-AuNP-NaNO₃ system in the absence and presence of target ATP
196 (Fig. 1C). Curve 'a' gives UV-vis adsorption spectrum of the H1-H2-AuNP-NaNO₃ system without
197 target ATP, and an obvious characteristic adsorption peak at ~520 nm was obtained. The maximum
198 adsorption wavelength seemed to be more than that of 15.8 ± 0.3 nm gold colloids, which might be
199 attributed to the conjugated oligonucleotides on the surface of nanoparticles. When target ATP was
200 present in the H1-H2-AuNP-NaNO₃ system, the characteristic peak was occurred to the
201 bathochromic shift (~630 nm), while the original peak at 520 nm decreased (Fig. 1C, curve 'b').
202 Moreover, the color of the H1-H2-AuNP-NaNO₃ system changed from red to purple (Fig. 1C, inset).
203 These results preliminarily revealed that target ATP could induce the change in the absorbance or
204 color of the H1-H2-AuNP-NaNO₃ system.

205 3.2. Control tests and feasible analysis

206 In this work, the change of the detectable signal relies on the formation of the long nicked poly
207 strand between H1 and H2 with the assistance of target ATP. To realize our design, we first utilized

the gel electrophoresis to characterize their reaction (Fig. 2A). As shown from lanes 1 and 2, the base number of H1 and H2 oligonucleotides was almost the same as our design. Significantly, mixture of 1.0 μM H1 and 1.0 μM H2 did not cause their self-hybridization reaction (lane 3). When 0.5 μM ATP was added into the mixture containing 1.0 μM H1 and 1.0 μM H2, however, a strong spot was observed at lane 4 as a result of the HCR reaction between H1 and H2. Hence, target ATP could cause the hybridization reaction of DNA strands, thereby resulting in formation of long double-helix.

As mentioned above, the change in the absorbance/color derived from the addition of NaNO_3 -salt into the H1-H2-AuNP system. Maybe one may ask whether introduction of NaNO_3 -salt could really cause the aggregation of AuNPs in the simultaneous presence of H1, H2 and target ATP. To verify this concern, the H1-H2-AuNP system was used for the detection of 0.5 μM ATP, which was investigated before and after addition of NaNO_3 -salt. Fig. 2B gives typical UV-vis adsorption spectra of different components after reaction each other. Due to the difference of the baseline in the absorbance, the judgment was carried out by analyzing the ratio of the absorbance (A_{630}/A_{520}) (Fig. 2C). As shown from curve 'b' in Fig. 2B and column 'b' in Fig. 2C, the absorbance of the ATP-H1-H2-AuNP system in the absence of NaNO_3 -salt was almost the same as that of AuNPs alone (curve 'a' in Fig. 2B, column 'a' in Fig. 2C). When NaNO_3 -salt was added into this system, however, an obvious difference was achieved at the characteristic peak and A_{630}/A_{520} (curve 'f' and column 'f'). Moreover, the color of the ATP-H1-H2-AuNP system changed from red to blue (photograph 'a' vs. photograph 'f'). The comparative results suggested that the change in the color and absorbance depended on the interaction between AuNPs and NaNO_3 -salt.

Fig. 2. (A) Polyacrylamide gel electrophoreses (M: marker; lane 1: H1; lane 2: H2; lane 3: H1 + H2; Lane 4: H1 + H2 + ATP); (B) UV-vis absorption spectra of (a) AuNP, (b) AuNP + H1 + H2 + ATP, (c) AuNP + H1 + ATP +

232 NaNO₃, (d) AuNP + H2 + ATP + NaNO₃, (e) AuNP + H1 + H2 + NaNO₃ and (f) AuNP + H1 + H2 + ATP +
233 NaNO₃; and (C) The corresponding A₆₃₀/A₅₂₀ ratio showing in figure B for each sample (*Inset*: the corresponding
234 photograph).

235 Another question to be produced was whether H1 or H2 alone could also cause the significant
236 change of the detectable signal of the AuNP-ATP-NaNO₃ system without the hybridization chain
237 reaction. As shown from curves 'c-e' in Fig. 2B and columns 'c-e' in Fig. 2C, the absence of H1, H2
238 or target ATP (*i.e.*, anyone) could not cause the absorption peak of gold colloids even if NaNO₃ was
239 present. More favorably, the color in these systems was almost the same as that of colloidal gold
240 alone (Fig. 2C, inset). The results further revealed that our designed hairpin probes could protect the
241 AuNPs from the aggregation upon addition of NaNO₃. Hence, the H1-H2-AuNP-NaNO₃ system
242 should be feasible for the detection of target ATP.

243 3.3. Optimization of experimental conditions

244 To acquire an optimal analytical performance of HCR-based colorimetric assay, the experimental
245 conditions should be optimized. In this study, the assay mainly consisted of target-triggered HCR
246 reaction, reaction with AuNPs and introduction of NaNO₃. From a statistical point of view, we first
247 fixed the volume or concentration of different components in this system: 5.0 μL of target ATP, 20
248 μL of 1.0 μM H1, 20 μL of 1.0 μM H2, 55 μL of gold colloids (9.95×10^{-11} M) and 20 μL of NaNO₃.
249 As mentioned above, the reaction time of target-induced HCR directly affected the signal of this
250 system. A short HCR reaction time was unfavorable for formation of long double-helix, thus
251 resulting in a low sensitivity. As shown in Fig. 3A, the A₆₃₀/A₅₂₀ value increased with the increasing
252 of incubation time, and tended to level off after 80 min. A longer incubation time did not cause the
253 significant change in the signal. By the same token, an optimal incubation time with AuNPs could
254 also be found at 50 min (Fig. 3B), indicating that the reaction of AuNPs with H1 + H2 could reach a
255 dynamic equilibrium within 50 min. To save the assay time, 80 min and 50 min were used for the
256 target-triggered HCR reaction and AuNPs conjugation, respectively.

257 The third parameter influencing the colorimetric assay depended on the concentration of NaNO₃.
258 A low-concentration NaNO₃ could not make the unprotected AuNPs aggregated, whilst a

high-concentration NaNO_3 easily cause the false positive of the result and the denaturation of AuNPs. As indicated from Fig. 3C, the optimal concentration of using 20- μL NaNO_3 was 1.2 M. Therefore, 20 μL of 1.2 M NaNO_3 was used as the concentration of the added salt for the development of colorimetric color.

Fig. 3. Dependence of the absorbance (A_{630}/A_{520}) on (A) HCR reaction time between H1 + H2 and target ATP, (B) conjugation time with AuNPs and HCR product, and (C) NaNO_3 concentration (Note: 1.0 μM H1, 1.0 μM H2, 99.5 pM AuNP, 1.2 M NaNO_3 and 0.5 μM ATP used in these cases).

3.4. Dose-response plots of HCR-based colorimetric assay toward target ATP

Under the optimal conditions, the HCR-based colorimetric assay protocol was employed for the quantitative monitoring of target ATP on the basis of the absorbance peak ratio at 630 and 520 nm. Fig. 4A gives UV-vis adsorption spectra of the H1-H2-AuNP- NaNO_3 system toward target ATP standards with different concentrations. Upon the addition of target ATP in the system, an obvious aggregation phenomenon of AuNPs appeared with an increase of the absorbance at 630 nm and a decrease of the absorbance at 520 nm. A good linear relationship between the absorbance (A_{630}/A_{520}) and ATP level could be achieved within the dynamic range from 10 nM to 600 nM (Fig. 4B). The linear regression equation was fitted as $y = 0.1073x + 0.0015$ (nM, $R^2 = 0.9971$, $n = 11$). The limit of detection (LOD) was evaluated to 1.0 nM ATP (estimated from the expression of $3S/K$, where S is the standard deviation for 11 determinations of blank solution and K is the slope of the calibration plot). Obviously, the LOD of HCR-based colorimetric assay was partially lower than those of some previously reported works, *e.g.*, quantum dots-based electrochemiluminescence aptasensor (6.0 nM) (Huang et al., 2010), zinc(II)-protoporphyrin IX/G-quadruplex labels-based fluorescence assay (10

281 μM) (Zhang et al., 2012b), quantum dot-based chemiluminescence resonance energy transfer (185
 282 nM) (Zhou et al., 2012), “off-on” electrochemiluminescence system (7.6 nM) (Liu et al., 2014b),
 283 electrochemical aptasensor (2.1 nM) (Wei et al., 2016), electrogenerated cheemiluminescence
 284 resonance energy transfer (6.7 fM) (Dong et al., 2016), AuNPs-MoS₂-based electrochemical
 285 biosensor (0.74 nM) (Su et al., 2016), AuNPs-based colorimetric assay (50 nM) (Huo et al., 2016)
 286 and peptide-AuNPs-based colorimetric aptasensor (1.35 pM) (Li et al., 2014).

287
 288 **Fig. 4.** (A) UV-vis absorption spectra for HCR-based colorimetric assay toward ATP standards with different
 289 concentrations (Note: 10, 20, 30, 50, 80, 100, 200, 300, 400, 500 and 600 nM ATP from top to bottom) (Inset: the
 290 corresponding photographs), and (B) calibration plots for ATP concentration vs. absorbance ratio (A_{630}/A_{520}).

291 3.5. Precision, reproducibility and selectivity

292 The reproducibility and precision of HCR-based colorimetric assay was monitored by repeatedly
 293 assaying three high-middle-low ATP concentrations including 10 nM, 300 nM and 600 nM, using
 294 identical batch AuNPs. Experimental results revealed that the coefficients of variation (CVs) of the
 295 intra-assay were 7.2%, 8.30% and 6.10% ($n = 3$), while the CVs of the inter-assay with various
 296 batches were 10%, 9.80% and 11.4% ($n = 3$) toward the above-mentioned concentrations (Please
 297 see the corresponding data in Fig. S2 of the Supporting Information). Hence, the reproducibility of
 298 our system is satisfactory.

299 Further, we also examined the selectivity and specificity of HCR-based colorimetric assay for
 300 target ATP by challenging this system against other three ATP analogues (*e.g.*, CTP, GTP and UTP).
 301 The experimental results are shown in Fig. 5. It was clear that only ATP showed a new shift in the

302 SPR peak (Fig. 5A) and a significantly high absorbance ratio (A_{630}/A_{520}) (Fig. 5B). Importantly, this
 303 selectivity could be visualized with the naked eye. The system showed blue color in the presence of
 304 ATP. In the presence of three analogues, the color of the system also turned red under the same
 305 conditions (Fig. 5A, inset). No significant variations in absorbance ratio suggested that the proposed
 306 method in this work is of considerable selectivity by comparison with those analogues other than
 307 ATP, thus was important and helpful in validation of the method to meet the selective requirements
 308 of the ATP assay in complicated biological samples. The experimental results were enough to prove
 309 the specific recognition of ATP by hairpin probes.

310

311 **Fig. 5.** Specificity of HCR-based colorimetric assay system against ATP (0.5 μ M), CTP (0.5 μ M), GTP (0.5 μ M)
 312 and UTP (0.5 μ M).

313 3.6. Analysis of real sample

314 To further monitor the possibility of the new technique for testing of real samples, it was applied
 315 for the analysis of high-middle-low three ATP samples including 20 nM, 200 nM and 400 nM,
 316 which were obtained by spiking ATP standards into blank fetal calf serum. The assayed results for
 317 three samples mentioned above by using HCR-based colorimetric assay were 21 ± 1.7 nM, $189 \pm$
 318 **4.20** nM, and 422 ± 11.6 nM ATP, respectively ($n = 3$) (Please see the corresponding data in Fig. S3
 319 of the Supporting Information). The recoveries were 107%, **95.0%** and 106% for the
 320 aforementioned analytes, respectively, indicating that the aptasensor was capable of detecting target
 321 ATP in real sample. Therefore, the HCR-based colorimetric assay can be considered as an optional
 322 scheme for quantitative monitoring of ATP even in the complex system.

323 4. Conclusions

324 In summary, we demonstrated a simple and feasible colorimetric assay for label-free detection of
325 target ATP in biological fluids, coupling with AuNPs-based signal-generation tags and DNA-based
326 hybridization chain reaction. The protecting efficiency of AuNPs in the high-level salt could be
327 largely weakened through target-triggered HCR, accompanying formation of long nicked
328 double-stranded DNA. Compared with conventional aptamer-AuNP systems, introduction of two
329 hairpin DNA probes including the corresponding aptamer exhibits a relatively low limit of detection
330 because a low-concentration ATP could trigger the consumption of numerous hairpin DNA probes
331 owing to hybridization chain reaction. Also, target analyte could be (semi-)quantitatively detected
332 by the change in the color and absorbance of the detection system. Although the assay did not need
333 the sample separation and the washing step during the measurement, the whole detection time was
334 relatively long (~150 min per sample). Nevertheless, another disadvantage of using this system
335 involved in three-step reactions in the whole process. Therefore, future work should focus on the
336 improvement of the assay process and reaction time. Favorably, this methodology can be applied to
337 determine other small molecules or mycotoxins by controlling the sequence of the corresponding
338 aptamer, thus representing a versatile detection scheme.

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