Article

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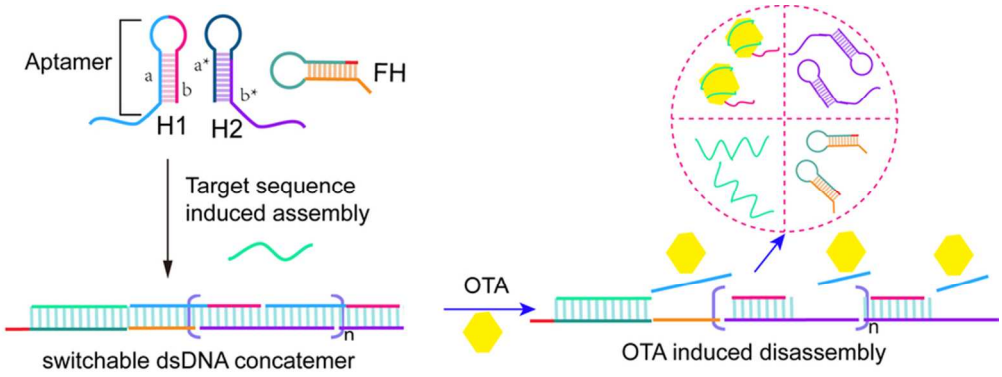

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Two-way Gold Nanoparticle Label-free Sensing of Specific Sequence and Small Molecule Targets Using Switchable Concatemers

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Abstract

A two-way colorimetric biosensor based on unmodified gold nanoparticles (GNPs) and a switchable double-stranded DNA (dsDNA) concatemer have been demonstrated. Two hairpin probes (H1&H2) were first designed that provided the fuels to assemble the dsDNA concatemers via hybridization chain reaction (HCR). A functional hairpin (FH) was rationally designed to recognize the target sequences. All the hairpins contained single-stranded DNA (ssDNA) loop and sticky-ends to prevent GNPs from salt-induced aggregation. In the presence of target sequence, the capture probe blocked in the FH recognizes the target to form a duplex DNA, which causes the release of the initiator probe by FH conformational change. This process then starts the alternate-opening of H1 and H2 through HCR, and dsDNA concatemers grow from the target sequence. As a result, unmodified GNPs undergo salt-induced aggregation because the formed dsDNA concatemers are stiffer and provide less stabilization. A light purple-to-blue color variation was observed in the bulk solution, termed as Light-Off sensing way. Furthermore, H1 ingeniously inserted an aptamer sequence to generate dsDNA concatemers with multiple small molecule binding sites. In the presence of small molecule targets, concatemers can be disassembled into mixtures with ssDNA sticky-ends. A blue-to-purple reverse color variation was observed due to the regeneration of the ssDNA, termed as Light-On way. The two-way biosensor can detect both nucleic acids and small molecule targets with one sensing device. This switchable sensing element is label-free, enzyme-free, and sophisticated instrumentation-free. The detection limits of both targets were below nM.

Keywords: Two-way sensing, Label-free, GNP, HCR, Aptamer, Switchable

The intersection between molecular and nanomaterials science offers fertile ground to advance the development of versatile biomaterials and bionanotechnology. [1]A successful example is the interaction between gold nanoparticles (GNPs) and DNA. GNPs are typical optical materials that display distance-dependent surface plasmon properties, resulting in strong color changes that rival or even exceed the most intense organic dyes. [2, 3]Nucleic acid has been used as a programmable molecule to tune the distance between GNPs. In addition to tunable properties from conventional hybridization

between one single-stranded sequence and its complementary sequence, [3] functional nucleic acids that can perform specific binding reactions with conformational changes or catalytic reactions in the presence of specific non-DNA molecules, [4, 5] or even bacteria(*6*), cells,[7, 8] or viruses[9] has been reported. [10] There are two major classes of interaction between GNPs and DNA for sensing various target molecules. The first class is GNP aggregation, which is induced by an inter-particle cross-linking mechanism that utilizes a three-strand nucleic acid sandwich hybridization assay format, [3] or a click chemistry-mediated sandwich binding assay format .[11, 12] The second class was reported by Rothberg and co-workers in 2004,who indicated that single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) have different adsorption properties toward unmodified GNPs. [13] Because ssDNA is easily uncoil and flexible, it is easily adsorbed on the surface of GNPs. As a result, high-ionic-strength induced GNP aggregation was prevented via the enhanced electrostatic repulsion between ssDNA-attached GNPs. Inversely, dsDNA is a more stiff structure and has its phosphate backbone exposed, the strong repulsion between negatively charged dsDNA and negatively charged GNPs makes nonspecific attachment negligible, which easily undergo blue GNP aggregation under high salt condition.[14] This non-cross linking sensing mechanism is more facile and is often called label-free colorimetric detection because it is free of the covalent modification of GNPs required in the sandwich hybridization assay format. Based on this strategy, Rothberg developed hybridization assays to detect specific nucleic acid sequences in genomic DNA amplified by the polymerase chain reaction (PCR) using unmodified GNPs. [15]A colorimetric sensor of potassium, adenosine triphosphate (ATP) and thrombin using the difference between the folded aptamers bound to targets and the unfolded aptamers without targets has also been demonstrated. [16-18] However, the PCR-based label-free colorimetric strategy requires enzymes and sophisticated thermal cycles to amplify the nucleic acid signals. [19, 20] In the aptamer-based assay, a single aptamer binds to a single target, which largely restricts their analytical performance.

Recently, researchers have found that hairpins can store energy to provide fuel for super double strand DNA self-assembly without enzymes at room temperature. [21-23] Dirks and Pierce[18] first reported an isothermal and enzyme-free alternative called hybridization chain reaction (HCR) for nucleic acids amplification with PCR-like sensitivity, but without the requirement of thermal cycler and protein enzyme. HCR is an initiator DNA catalyzed self-assembly process in which two metastable hairpins coexist in solution and will not switch on a fabricate reaction of long-nicked super dsDNA concatemers until an initiator DNA is added. Notably, the signal output format of HCR is generally based on the chemical modification of two hairpins with signal reporters or anchor moieties, which is costly and influences amplification efficiency. Finally, yet importantly, although there are HCR-based sensors for DNA, [24-26] proteins[27] and small targets, [28, 29] quite a few of them are capable of sensing both specific sequences and small molecules with one sensing device, let alone in an HCR-based, label-free GNP colorimetric sensing system.

Herein, we report a novel two-in-one sensing strategy by integrating target sequence-initiated

concatemers assembly and target small molecule-induced concatemers disassembly into unmodified gold nanoparticles (Figure 1). The main principle of this sensing system relies on Light-On/Light-Off, which is a smart gold particle switch by the self-assembly and disassembly of long nicked super dsDNA concatemers in bulk solution (two-way sensing).The target nucleic acids were microRNA 122b, which play critical regulatory functions in many biological processes, such as cell differentiation and cell apoptosis. [30-32]The target small molecule was Ochratoxin A (OTA), which has previously been found to induce various toxic effects in the liver and kidneys and relates to the regulation of microRNA expression. Our two-in-one sensing system provides a convenient path for integrated diagnosis of the Cause-Effect of small-molecule related disease.

Methods and Materials

Reagents and Nucleic Acids. Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and trisodium citrate were obtained from Amersco Company. Distilled water was prepared via the Millipore Milli-XQ system (Millipore). Ochratoxin A (OTA) was from our laboratory. All of nucleic acids used in this work were synthesized by Invitrogen Biotech Co., Ltd. and lyophilized nucleic acids were dissolved in distilled water before use. The detailed sequences of each nucleic acids was listed in Table 1. The HCR reaction buffer was 8 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 0.15 M NaCl, 2 mM $MgCl_2$, pH=7.4). All reagents used in this work were of analytical grade.DNA markers were purchased from Tiangen Biotechnology Co., Ltd.

Apparatus. Absorption spectra and colorimetric measurements were performed with a Thermo Scientific Varioskan Flash (Thermo Scientific) at 25°C. Typical TEM images were made on a JEM-2100 transmission electron microscope. The gel electrophoresis images were scanned by a BioDoc-It Imaging System (UVP)

Preparation of gold nanoparticles. Before preparing gold nanoparticles, the glassware was thoroughly cleaned. Gold nanoparticles (13 nm) were prepared by the citrate reduction of $HAuCl_4$. In a round-bottom flask, a 2 mL $HAuCl_4$ solution (1wt %) was added to 186 mL of ultrapure water. After the solution was boiling, 12 mL of sodium citrate was added into the boiled solution. After the color changed from pale yellow to dark to red, the reaction mixture was continuously refluxed for 10 min. Then, the solution cooled to room temperature and stored in a refrigerator at 4°C before use. The color should be cherry red, and the nanoparticle shape should be spherical under transmission electron microscopy (TEM).

Gel Electrophoresis. H1 and H2 were heated to 95°C for 5 min and then rapidly cooled to 0°C before use. Different concentrations of target sequence were incubated with 250 nM each of H1 and H2 at room temperature for 6 h. The product was analyzed by 2% agarose gel (2 g agarose was dissolved in 100 mL 1×TAE buffer (40 mM Tris-acetate, 2.0 mM Na_2EDTA, pH 8.0). 1 μL Ethidium

bromide (EB) was used as a dsDNA dye and mixed with 2% agarose gel. The gel with HCR product was run at 130 V for 20 min in 1×TAE buffer and was then taken the gel image using BioDoc-It Imaging System (CA, USA).

Characterization. In the Light-Off sensing way (target sequence assay), different concentrations of target sequence was mixed with FH (100 nM), pre-prepared H1 (250 nM) and H2 (250 nM) in an HCR reaction buffer and then was incubated at room temperature for 6 h. Then, 10μL of the reaction mixture was added into a 250 μL GNP solution. After gentle vibration and incubation for 10 min, 20 μL of 500 mM NaAc buffer (pH=5.2) was added and the solution was measured by either a microplate reader or naked eyes. During Light-On sensing (small molecule assay), 1 μL of 10 μM signal probe, 2.5 μL of 10 μM H1 and 2.5 μL of 10 μM H2 was added into 19 μL of HCR reaction buffer to catalyze the formation of aptamer-inserted super dsDNA concatemers. One microliter of various concentrations of the small molecule targets was added into 25 μL of HCR products and incubated at room temperature overnight. Then, 10 μL of the above-mentioned mixtures were added into 250 μL GNP solutions and incubated at room temperature for 10 min. Finally, 20 μL of 500 mM NaAc buffer was added into the mixtures to increase the salt concentration. The solution was also examined by visual detection and UV-vis scanning.

Results and Discussion

Fabrication of Light-Off sensing system

Initially, two fuel hairpins (H1 and H2) were designed, both having a single-strand DNA (ssDNA) sticky-end (also called a toehold) and a short loop region that ensure stabilization of unmodified GNPs. In the absence of target sequences (TS), the two fuel hairpins maintain a thermodynamic steady state and can coexist stably in solution (Supplementary Table S1).Thus, the mixture can easily absorb on the surface of unmodified GNP to protect them from high salt induced aggregation, resulting in a purple color. While in the presence of TS, it nucleates with H1 by base pairing and strand displacement to release a single-stranded segment, “a*”, mediating a branch migration that opens H2 to form the complex T-H1-H2. This complex continuously nucleates with hairpins H1,H2,H1,H2,H1…by means of the toehold-mediated base-pairing and strand displacement. In this manner, a long-nicked super dsDNA concatemers was generated by the chain-like assembly of H1 and H2 through HCR. The HCR product cannot adsorb on unmodified GNP surface due to the stiffer structure and exposed negatively charged phosphate backbone of the super dsDNA concatemers, thereby causing a red-to-blue color shift (Light-Off) under high ionic strength conditions. The detailed sequence of H1 and H2 was listed in Table 1.

(Figure 1, here)

Feasibility study of Light-Off sensing way

To illustrate the feasibility of this strategy, a proof-of-principle experiment was carried out for target sequence (signal probe, in this section) detection. We first mixed H1&H2 with 0 nM or 10 nM

target sequence (TS), then incubated the mixture with GNPs solution like the above-mentioned protocols. Figure 2a shows UV-vis absorption spectrum and the photos of GNP solutions. As shown, in the absence of TS, a surface plasmon peak of the separated GNPs was observed at 524 nm (Figure 2a, red curve), and the color of GNP solution appeared pale purple after the addition of salt buffer (Figure 2a, inset, left microwell). However, in the presence of TS, a new peak at 700 nm appeared, and the peak at 524 nm decreased sharply (Figure 2a, blue curve). Correspondingly, the color of the GNP solution shifted from pale purple to gray-blue (Figure 2a, inset, right microwell). From the UV-vis spectrum, we knew that the TS initiated a cascade of hybridization events to yield stiffer super dsDNA concatemers. Hairpin probes H1&H2 with their ssDNA sticky end and loop ssDNA bind to unmodified nanoparticles and effectively stabilize them against salt-induced aggregation, but the stiffer super dsDNA concatemers do not. Therefore, in the presence of the TS, the formed dsDNA concatemers provides negligible stabilization and GNPs undergo aggregation when ionic strength increase. As a result, the color of GNP solution turns to gray-blue due to a red shift of the surface plasmon peak. The HCR between the target sequence and the H1&H2 probes was further confirmed by HPLC (Figure S5) and gel electrophoresis (Figure 2c). Electrophoresis gel image showed that the long-nicked super dsDNA concatemers was successfully assembled by HCR in the presence of TS. Only two bands were observed in lane 2 (the water group without TS) due to the entire probes folding into the hairpin structures separately in solution, indicating there were no HCR products. As a comparison, lane 1 showed smears with ladder bands when TS was mixed with H1/H2 in the HCR reaction buffer, suggesting the long-nicked super dsDNA concatemers were successfully formed in solution. It should be mentioned that the HCR amplification continued until the fuels H1 or H2 was exhausted, and the molecular weight of HCR products was an approximate range instead of an exact numerical value. Therefore, the dsDNA concatemers generated from HCR in the gel electrophoresis images appear as smears with ladder bands. This property had also been validated by published literature.[33]

(Figure 2, here)

The hairpin-assisted and target sequence-induced aggregation of GNPs was further confirmed by TEM. As shown in Figure 2b, GNPs were well-dispersed in the absence of TS even when salt concentration was increased (Figure 2b,upper image), which resulted from the enhanced repulsion between the hairpins absorbed GNPs, while in the presence of TS, GNPs aggregated after the increase of salt concentration (Figure 2b,bottom image). The TEM results (Figure 2b) were consistent with the bathochromic shift of the UV−vis spectra as well as the color change of the GNP solutions; thus, the designed H1&H2 and unmodified GNPs solution was suitable for the detection of nucleic acid targets. We called the color change from pale purple to blue is the Light-Off sensing way. Other optimum reaction conditions were carefully chosen in subsequent experiments (Supplementary Figures S2−S3).

Design of functional hairpin for various targets

To expand the applied range, we introduced a functional hairpin to enhance the applicability of the Light-Off sensing system toward other nucleic acid targets, with micro RNA 122b selected as the

model analyte in this paper. The designed functional hairpins (FH) contained three sections: initiator probe for HCR (Ip, in blue), capture probe for micro RNA 122b (Cp, in orange) and blocking tail (Bt, in red). The 3' terminal end of Ip, which is complementary with the toehold of H1, was blocked by Bt to maintain a non-catalytic state. In the presence of micro RNA 122b, the FH opens and the Ip was exposed to catalyze the super dsDNA assembly by HCR. Notably, Bt is tunable to allow different capture probes for various nucleic acid targets alternated in this FH and background elimination. During the FH design, we found that the number of bases blocked in the FH stems significantly influenced the aggregate ratio. We set a series of FH to optimize the number of blocked bases. The detailed sequences were listed in table S2. In this section, $P/N=R_{T+}/R_{T-}$=[ratio A700/A524 (in the presence of target microRNA 122b)]/[ratio A700/A524 (in the absence of target microRNA 122b)] was chosen to monitor the optimized blocked quantity that would cause color variation upon GNP aggregation. Figure 3 shows that at least half of the Ip being blocked by the Bt from the toehold was effective to reach the maximum P (positive)/ N (Negative) ratio (FH3, P/N ratio=2.42). It should be mentioned that half of the Cp also needed to be blocked to assist the elimination of the false positives. When Bt blocked less than half the bases (FH1and FH2), HCR proceeded even without the target sequence. Thus, the resolution between target+ and target- is negligible. However, the P/N also decreased when more than half of the Ip sequence was blocked (FH5 and FH6). This decrease results from the longer blocked Ip increasing the resistance of opening FH. With this design, impressively, the Light-Off sensing system can be applied with sequence-specific selectivity (Figure S4) toward various nucleic acids targets by altering the Bt and Cp.

(Figure 3,here)

Fabrication of Light-On sensing system

Given the success of this Light-Off sensing way using micro RNA 122b induced dsDNA assembly, it would be interesting to investigate whether aptamers can be used in the same sensor in a reverse way. Alternatively, if the DNA probes contain aptamer sequences for small-molecule targets, the disassembly of the preformed DNA concatemers could be used to detect the non-nucleic acid targets, with OTA as a model target. To realize the detection of small molecules in the same sensor, we designed an improved long-nicked super dsDNA concatemers by introducing an anti-OTA aptamer sequence into the H1 probes, in which multiple OTA-binding sites were included on each super dsDNA chain. Extending the success of dsDNA assembly systems to aptamer-assisted dsDNA disassembly systems is not necessarily trivial, as the disassembly reaction requires not only binding but also peeling and releasing the aptamer from the super dsDNA concatemers. The detailed schematic of the Light-On colorimetric sensor for small molecules is shown in Figure 1. The primary sensing principle is aptamer-assisted super dsDNA disassembly. As the super dsDNA concatemers contains multiple small molecule binding sites (OTA in this paper), the super dsDNA disassembled in the presence of the target OTA through aptamer-target induced conformational change.

Feasibility study of Light-On sensing way

The feasibility of this disassembly-based sensing strategy was also characterized by a proof-of-concept experiment, with UV-vis spectroscopy used to record the plasmon peak shift of the GNP solution. The ratio of extinction at 700 and 524 nm was chosen to monitor the amount of GNP aggregation that causes the color variation. A high ratio is associated with aggregated GNPs (blue), while a lower ratio is associated with dispersed GNPs (red). Figure 4 depicts the disassembly effect of the OTA-induced disassembly by addition of unmodified GNPs. Before the Light-On sensing task, the nucleic acid-target/FH (or signal probe, noted in the parallel connection circle, Figure 1) and H1/H2 probes were used to create sufficient dsDNA concatemers in the bulk solution. A significant increase in the A700/A524 ratio (blue-shift, blue color) was therefore observed without small molecule addition (HCR, figure 4a). After the addition of OTA into the super dsDNA bulk solution, a sharp decrease in the A700/A524 extinction ratio (increase in red color) was observed (HCR-, figure 4a), which is approximately back to the extinction ratio observed without HCR products (FH+H1+H2, figure 4a). The extinction ratio recovery suggests much less GNP aggregation because of the disassembly and release of probe mixtures with ssDNA (Figure 1) that bind to GNP and prevent aggregation. Further, the disassembly was validated by HPLC (Figure S5), TEM (Figure S6) and gel electrophoresis (Figure 4b). From the gel image, a distinct reduction of the ladder band and smear was observed, which indicates the long-nicked super dsDNA concatemer was successfully digested by the small molecule target.

(Figure 4, here)

Optimization of DNA concatemers disassembly.

Two points should be noted about these improved super dsDNA concatemers. First, the 36nt aptamer sequence was reversed and then inserted into H1 from the toehold site of the hairpin that allows the aptamer binding site to be close to the nick of the super dsDNA concatemers. Second, we widened the nick (13 nt, Figure S1) between H1 and H2 in the super dsDNA concatemers to decrease the melting temperature (disassembly power of H1). This design aims to enhance the disassembly rate by increasing the power of the binding event and decreasing the resistance to probe release in the binding action. The nick width-related disassembly can be illuminated by the balance of intermolecular forces between the hydrogen bond of base pairs and the hydrophobic bond or electrostatic interactions of target-aptamer binding.[34]

The disassembly requires strict sequence design because it is negligible when a flanking sequence is added before the aptamer or reversed aptamer in the super dsDNA concatemers. This requirement may be attributed to a two-type blocking effect of the flanking sequence. First, the flanking sequence may disturb the recognition event between the target and aptamer. Second, the flanking sequence may block the release of the single stranded aptamer DNA (ssDNA) from the super dsDNA in the presence of small molecule targets by increasing the forces required to strip the aptamer (this process was confirmed by the nick optimization).To further illustrate the sequence-strict disassembly, we added a

flanking sequence before the aptamer at the 5'end of H1 (TableS3) to explore the relation between the flanking sequence and disassembly rate. Figure S7 shows that a 6nt flanking sequence decreases the disassembly rate sharply. With a series of ladder optimizations, we achieved the best disassembly ratio by eliminating the flanking sequence. Interestingly, if the aptamer sequence was not reversed, OTA cannot disassemble the super dsDNA at all even without any flanking sequence in H1. We can conclude that reversed aptamer is necessary for the disassembly of super DNA concatemers.

Analytical performance of two-way sensing system

To further characterize the detection range of this two-way assay, a series of samples containing different concentrations of microRNA 122b or OTA have been tested. After addition of microRNA 122b with concentrations ranging from 100 nM to 1 pM, the color of the solution changed purple to blue. The UV−vis spectroscopy (Figure 5a) showed that the absorbance at 524 nm gradually shifted and decreased and a new absorption (700 nm) appeared. And a good linear correlation between the A_{700}/A_{524} extinction ratio and the target concentration was obtained (Figure 5b). According to the 3σ rule, [35] the detection limits of Light-Off assay system were estimated to be around 0.5 pM and the semi-quantitative limit was around 10 pM achieved by naked eyes. Inversely, when added the small molecule target OTA, the aggregated GNPs absorption peak (700 nm) gradually shifted and decreased and the absorbance at 524 nm increased (Figure 5c). As a result, $\Delta(1\text{-}A_{700}/A_{524})$ extinction ratio and OTA concentration showed a linear calibration from 10 μM to 1 nM with a detection limit of 0.76 nM (Figure 5d).

(Figure 5,here)

The selectivity of Light-On sensing way

The red shift of spectroscopy in Light-On was attributed to the target-aptamer binding event, leading to a disassembly of the dsDNA cancatemers to probe mixture with sticky-ends, and thus recovering the salt tolerance of unmodified GNPs (The unmodified GNPs are red or pale purple in the end). Notably, this aptamer-assisted Light-On sensing way displayed higher selectivity towards OTA than to the other mycotoxins (AFB1, FumonisinB (FB), and ZEN) (Figure 6). Excellent target discrimination was also found for sensors challenged with mixed mycotoxins, in which the concentration of the competing mycotoxin was 10-fold higher than OTA. The high specificity was attributed to the high specific of aptamer in super dsDNA concatemers.

(Figure 6, here)

Conclusions

We assembled a two-way label-free colorimetric sensor that responded to both picomolar nucleic acids and nanomolar small molecules, using the modulation of super molecular DNA concatemers and the different absorption properties of unmodified GNPs between ssDNA and dsDNA. We achieved a detection limit of 0.5 pM for microRNA and 0.76 nM for OTA. Compared with conventional label-free GNP sensors, in which a single capture probe recognizes a single target, the aptamer-inserted super

dsDNA concatemers provide a switchable biology element to sense two types of target in one sensor (especially in the concatenation circle) with high sensitivity and specificity. Herein, we adopt a "FH-assisted multiple-target amplification" mechanism for the Light-Off sensing of specific sequence (decreasing the red color of the GNP solution) and a "multiple-target-binding-site" structure for the Light-on sensing of small molecules (increasing the red color of the GNP solution) to improve the analyte label-free sensing system. Thus, minor changes in the super dsDNA concatemers efficiently converted to detectable color changes in the GNP solution.

In addition, the developed sensor is suitable not only for nucleic acids but is also available for various aptamer-recognized non-nucleic acid targets. Various DNA aptamers have been selected and applied to a broad range of targets, including ions, small molecules, proteins and cells. With rational design of aptamer-inserted super dsDNA concatemers, in theory, this two-way label-free sensor could be used for many other targets and has the potential to be developed into a diagnostic kit, which could monitor hazardous molecule and key nucleic acid changes simultaneously. Furthermore, a series of functional DNA-assisted DNA machines and motors could be used to construct facile and enzyme-free GNP sensors.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge on the ACS Publications website.

1.Hairpin structures; 2, Optimized salt buffer for GNP aggregation; 3,The sequence list of the Functional Hairpins; 4, Optimized hairpin auxiliary probes H1 and H2 for GNPs solution color response; 5,Sequence specificity of the light-off biosensor; 6,Characterization of dsDNA concatemers assembly and disassembly by HPLC or TEM; 7, Optimization of sequence-strict disassembly. Additional information as noted in the text.

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Notes

The authors declare no competing financial interest.

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Figures and Figure Captions

Figure 1. Building the GNP-based two-way sensing system. Scheme for the amplified detection of a target sequence using two fuel hairpins (H1and H2) and one functional hairpin 1(FH1) that trigger the autonomous generation of dsDNA concatemers containing multiple small molecule binding sites. In this way, each target can generate a dsDNA concatemer. The formed super dsDNA concatemers are stiff and have a strong repulsion for the negatively charged GNPs, which cannot prevent salt-induced GNP aggregation. As a result, a pale purple-to-blue color variation, termed Light-Off, is observed in the GNP solution. Upon adding the small molecule targets (SMT), the aptamer inserted in the dsDNA concatemers binds with the SMT and peels off from the dsDNA; as a result, the stiff dsDNA disassembles into mixture probes with ssDNA sticky ends. These mixtures easily absorb on the GNP surfaces to protect the GNP from aggregation when increasing the salt concentration. As a result, the GNP solution remains red, termed Light-On.

Figure 2. UV-vis absorption spectra (a) and TEM images (b) of the H1/H2/GNP colorimetric detection system in the presence of 0 nM or 10nM target sequence. Inset: photograph of GNP solution in microcells.(c) Gel electrophoresis images for 0 nM or 10 nM target sequence. From left to right, lanes: (1) 250 nM H1 + 250 nM H2 + 10nM target DNA; (2) 250 nM H1 + 250 nM H2; (M) DNA ladder.

Figure 3.Optimizing the block sequence of functional Hairpin. R_{T+}/R_{T-} is the ratio of A700/A524 (in the presence of target micro RNA 122b) versus A700/A524 (in the absence of target micro RNA).

Figure 4.(a) Extinction ratio (A700/A524) and photograph showing colorimetric responses of GNP solution with assembly of super dsDNA (HCR) and disassembly of super dsDNA(HCR-).H1: H1 with salt;H2: H2 with salt; HCR: super dsDNA with salt;FH+H1+H2:FH+H1+H2 with salt; HCR-:probe mixture after OTA disassemble with salt. (b)The disassembly of super dsDNA concatemers via aptamer-OTA binding induced conformation change validated by agarose gel electrophoresis. Lane 1: H1; lane 2:H2; lane 3: FH+H1+H2; lane 4: super dsDNA concatemers assembled by HCR; lane 5: the probe mixtures after OTA disassembly.

Figure 5. UV-Vis spectra (a, c) and calibration curve (b,d) of the H1/H2/GNP label-free colorimetric sensing system with different concentrations of microRNA 122b or OTA in the solution.

Figure 6. Selectivity of the two-way system with various mycotoxins including OTA. The sensor responds only in the presence of OTA, demonstrating good selectivity.

Table 1 Sequences used in the two-way sensing system

Name	Sequence
Functional hairpin (FH)	ACCG CAA CAG CCT CGT AGC CTG TCG ATC TGT(Ip)TCA AAC ACC ATT GT CAC ACT CCA(Cp)AAT GGT GTT TGA-ACA GAT CGA CAG GCTACG(Bp)
Fuel hairpin H1	ACA GAT CGA CA[a]G GCT ACG AGG C TGT TG C GG TGGG TGT[b] GGG CTA AAC AGC CTC GTA GCC TGT
Fuel hairpin H2	CAG CCT CGT AGC CTG TCG ATC TGTACA GGC TAC GAG GCT GTT TAG CCC[a]
Micro RNA 122b	UG GAG UGU GAC AAU GGU GUU UGA
Reversed OTA aptamer	ACA GAT CGA CAG GCT ACG AGG C TG T TG C GG T GGG TGT[b]
Signal probe	ACCG CAA CAG CCT CGT AGC CTG TCG ATC TGT

a, the sticky end (toehold sequence) of H1 or H2

b, the reversed aptamer sequence of OTA, highlighted in yellow

c, the complementary section between H1 and H2 is demonstrated in Figure S2

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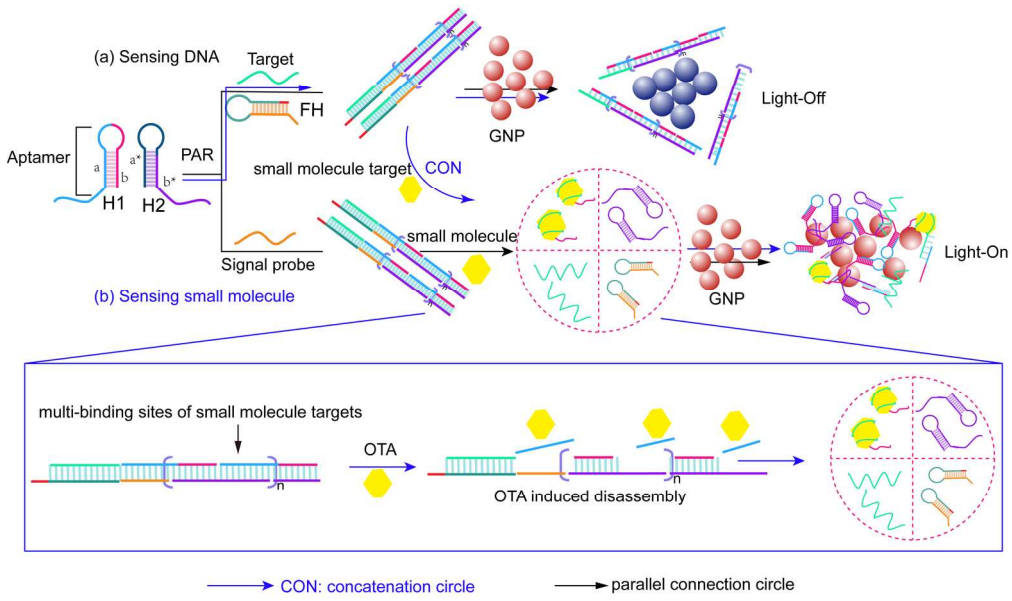

Figure 1. Building the GNP-based two-way sensing system. Scheme for the amplified detection of a target sequence using two fuel hairpins (H1and H2) and one functional hairpin 1(FH1) that trigger the autonomous generation of dsDNA concatemers containing multiple small molecule binding sites. In this way, each target can generate a dsDNA concatemer. The formed super dsDNA concatemers are stiff and have a strong repulsion for the negatively charged GNPs, which cannot prevent salt-induced GNP aggregation. As a result, a pale purple-to-blue color variation, termed Light-Off, is observed in the GNP solution. Upon adding the small molecule targets (SMT), the aptamer inserted in the dsDNA concatemers binds with the SMT and peels off from the dsDNA; as a result, the stiff dsDNA disassembles into mixture probes with ssDNA sticky ends. These mixtures easily absorb on the GNP surfaces to protect the GNP from aggregation when increasing the salt concentration. As a result, the GNP solution remains red, termed Light-On.

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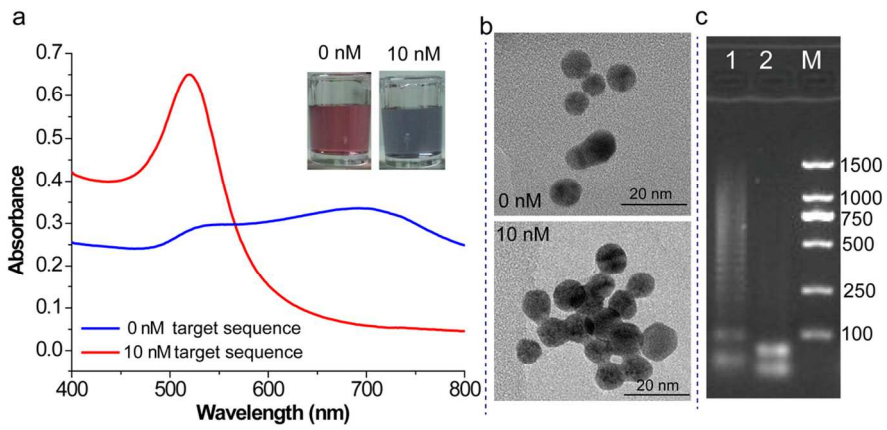

Figure 2. UV-vis absorption spectra (a) and TEM images (b) of the H1/H2/GNP colorimetric detection system in the presence of 0 nM or 10nM target sequence. Inset: photograph of GNP solution in microcells.(c) Gel electrophoresis images for 0 nM or 10 nM target sequence. From left to right, lanes: (1) 250 nM H1 + 250 nM H2 + 10nM target DNA; (2) 250 nM H1 + 250 nM H2; (M) DNA ladder.

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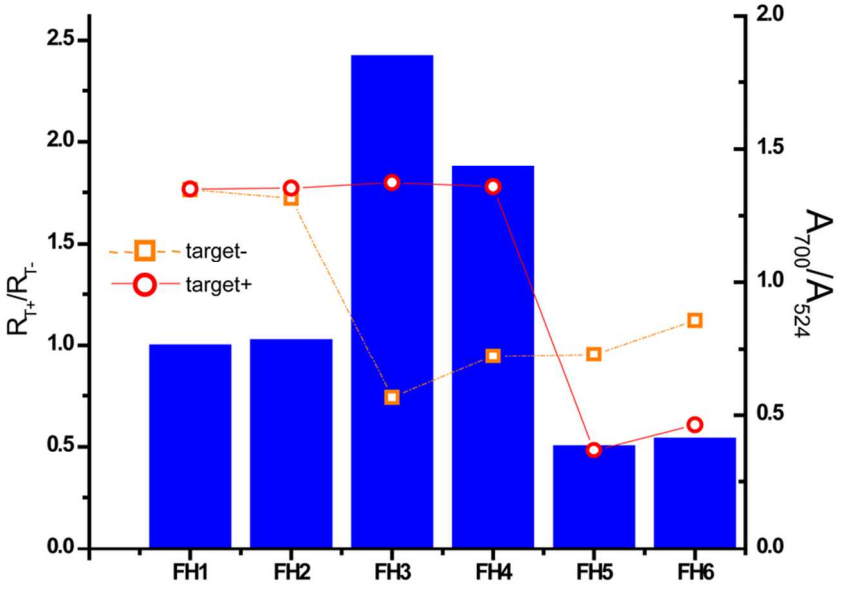

Figure 3.Optimizing the block sequence of functional Hairpin. RT+/RT- is the ratio of A700/A524 (in the presence of target micro RNA 122b) versus A700/A524 (in the absence of target micro RNA).

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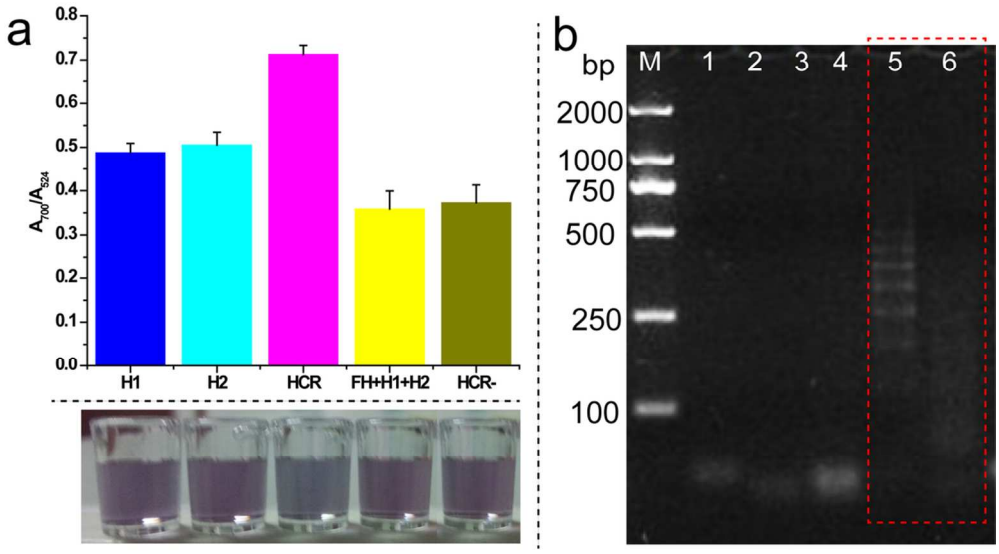

Figure 4.(a) Extinction ratio (A700/A524) and photograph showing colorimetric responses of GNP solution with assembly of super dsDNA (HCR) and disassembly of super dsDNA(HCR-).H1: H1 with salt;H2: H2 with salt; HCR: super dsDNA with salt;FH+H1+H2:FH+H1+H2 with salt; HCR-:probe mixture after OTA disassemble with salt. (b)The disassembly of super dsDNA concatemers via aptamer-OTA binding induced conformation change validated by agarose gel electrophoresis. Lane 1: H1; lane 2:H2; lane 3: FH+H1+H2; lane 4: super dsDNA concatemers assembled by HCR; lane 5: the probe mixtures after OTA disassembly.

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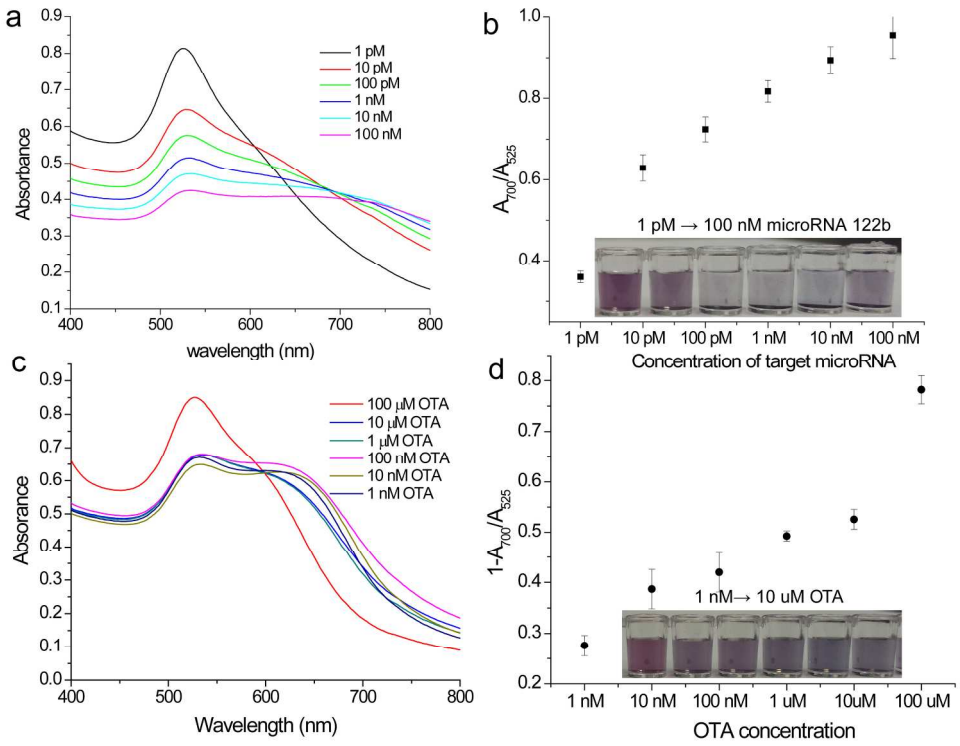

Figure 5. UV-Vis spectra (a, c) and calibration curve (b,d) of the H1/H2/GNP label-free colorimetric sensing system with different concentrations of microRNA 122b or OTA in the solution.

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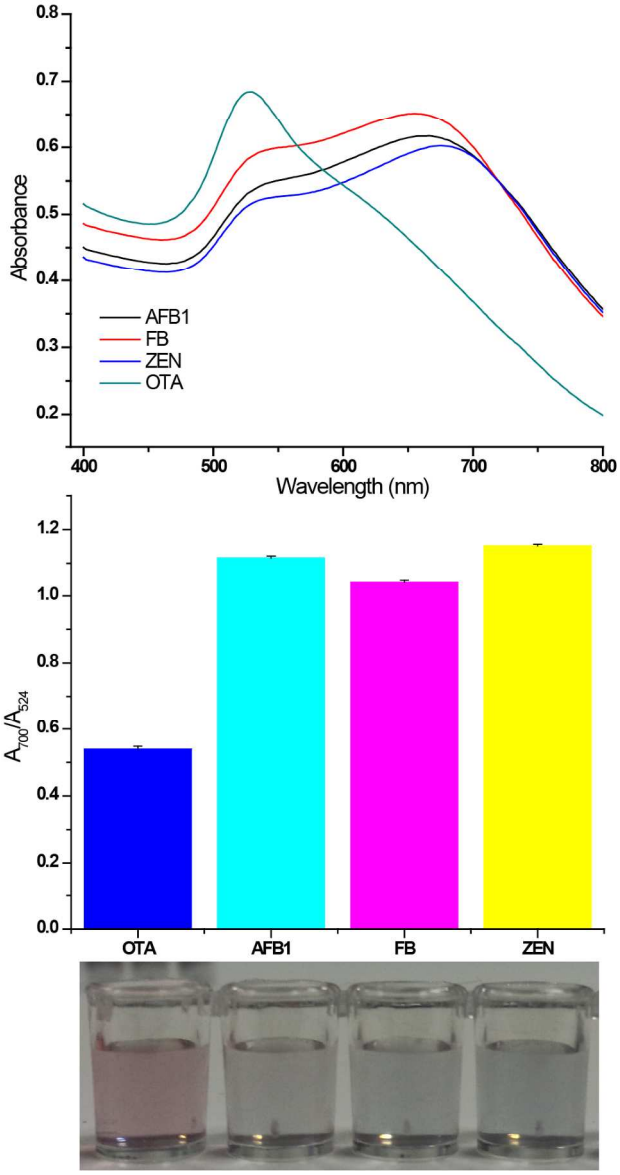

Figure 6. Selectivity of the two-way system with various mycotoxins including OTA. The sensor responds only in the presence of OTA, demonstrating good selectivity.

171x314mm (300 x 300 DPI)