Electronic Supplementary Information

A Simple Strategy Based on Combinatorial Gold Nanoparticle Sizes for Enhanced Sensitivity in Colorimetric Bioassay

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Supporting Experimental Details

Size distribution. AuNPs particles of four sizes were measured at first. This solution was prepared by dispersing 30 μL AuNPs of different sizes in 1 mL Millipore water, and then processed by sonication for 1 min prior to the measurement with Zetasizer Nano ZS.

Absorption spectrum of normalized A520. Four sizes of AuNPs were purchased with an optical density of 1 OD. The maximal absorption peak of 10 nm, 20 nm, 30 nm and 40 nm AuNPs are measured at 519 nm, 522 nm, 524 nm and 528 nm, respectively. The absorbance of AuNPs of four sizes needed to be controlled at an identical initial value at 520 nm (A520). Therefore, a normalization process was performed by H_2O dilution to ensure an identical initial value of A520. These diluted AuNPs served as normalized solutions for absorption spectrum acquisition (UV/VIS-1750). After absorbance normalization of 10 nm, 20 nm, 30 nm, 40 nm AuNPs, according to Lambert Beer Law A = εbc, with corresponding ε of $1.01*10^8$ M^{-1} cm^{-1}, $9.21*10^8$ M^{-1} cm^{-1}, $3.36*10^8$ M^{-1} cm^{-1}, $8.42*10^8$ M^{-1} cm^{-1} provided by product supplier, their concentration is calculated to be about 4.1 nM, 0.50 nM, 0.14 nM, 0.06 nM, respectively. Thus, the combinatorial AuNPs mixed with equal volume is 22.5 μL*(4.1 nM + 0.50 nM + 0.14 nM + 0.06 nM)/4 = 1.2 nM. The final concentration of 10 nm, 20 nm, 30 nm, 40 nm and combinatorial AuNPs in the detection system is about 1.85 nM, 0.23 nM, 0.06 nM, 0.03 nM and 0.54 nM, respectively.

Aptamer adsorption amount and adsorption kinetics. To study the DNA adsorption amounts, the DNA (15-mer thrombin aptamer) and AuNP mixtures were prepared by incubating 90 μL 1.2 nM AuNPs of four sizes with 2 μM 10 μL DNA for overnight at room temperature. The DNA was dissolved with 50 mM sodium citrate buffer (pH 6.0, 500 mM Na^+). The free DNAs was removed by 3 rounds of centrifugation (8000 rpm, 8 min). The absorbance of both the removed supernatant DNAs and total DNA were measured at 260 nm. Thus, the amount of adsorbed DNA on the AuNP surface was calculated according to the formula, $A_{total}/C_{total} = A_{supernatant}/C_{supernatant}$.

As regard to the adsorption kinetics, the fluorescence change after adding combinatorial AuNPs was monitored. 10 μL of 200 nM FAM-DNA (15-mer thrombin aptamer) was dissolved in 90 μL of 5 mM citrate buffer (pH 6.0, 50 mM Na^+). The fluorescence emission intensity of DNA was first monitored for 1 min under the kinetic mode using a plate reader, then quickly adding and mixing with 20 μL of 1 nM AuNPs for another 4 min. Final AuNP concentration = 0.3 nM.[1]

Zeta potential measurement. The zeta potential of AuNPs (20 nm, 1 nM) and AuNPs/Aptamers (15-mer) were measured. 10 μL of 50 μM aptamer was introduced to 90 μL AuNPs and incubation for 30 min. The free DNAs was removed by 3 rounds of centrifugation (8000 rpm, 8 min) and the

remaining AuNPs/Aptamer conjugate was resuspended with 100 μL 1 mM citrate buffer (pH 6.0, 10 mM Na^+). 50 μL of such resuspended AuNPs/Aptamer was dissolved in 600 μL H_2O and under 1 min of sonication before zeta potential measurement. In contrast, zeta potential of bare AuNPs was measured in a similar way, briefly, 50 μL AuNPs absent of aptamer was adding into 600 μL H_2O, and such 650 μL of AuNPs sample contained 0.76 mM Na^+ was used for correction of the change induced by NaCl.

Evaluation of salt-responsive effect on analytical behavior. To investigate the size-dependent behavior in response to various salt concentrations, a sequence of sodium citrate (5 mM, pH 6.0) buffer containing 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 120 mM and 140 mM Na^+ were prepared, respectively. Briefly, 90 μL AuNPs was mixed with 10 μL 15-mer aptamer and incubated at room temperature for 2 min, then 6 nM thrombin target was added in the five systems respectively and incubated for another 5 min, and finally followed by addition of 95 μL above buffer. Equivalent BSA was utilized for background correction in N.C. group. The absorbance was measured at 520 nm and 650 nm with a Microplate Reader (H1M) from Biotek (United States). Each error bar was calculated from triplicate analyses.

Optimization of length of thrombin aptamer in cNCLs system. Two thrombin aptamers of 15-mer and 29-mer were used for evaluation of the analytical performance. Aptamer was diluted with 10 mM sodium citrate buffer (pH 6.0, 500 mM Na^+). Typically, 90 μL combinatorial AuNPs (final concentration was 0.54 nM) was mixed with 10 μL of 2 μM aptamer and incubated for 2 min at room temperature, then 5 μL of thrombin was separately added to make a final concentration of 0.5 pM and 5 pM, and incubated for another 5 min, and finally followed by addition of 95 μL 5 mM sodium citrate buffer containing 70 mM Na^+. Equivalent BSA instead of pure buffer solution was utilized for background correction in the negative control (N.C.). The absorbance at 520 nm and 650 nm, respectively, was measured with a Microplate Reader (H1M) from Biotek (United States). The value of ΔS (650/520), a difference between the intensity of positive control and negative control, indicated target-induced signal increase after correction of background. Each error bar was calculated from triplicate analyses.

Single nucleotide polymorphism assay. The differentiated capability from full-matched DNA target to a single-mismatch mutant (SNP1) was tested. DNA was diluted with 10 mM sodium citrate buffer (pH 6.0, 500 mM Na^+). Typically, 90 μL combinatorial AuNPs was incubated with 10 μL ssDNA probe for 2 min at room temperature, then 50 nM SNP1 and full matched target was added, respectively, and mixed for 5 min, and finally followed by addition of 95 μL 5 mM sodium citrate buffer containing 70 mM Na^+. Equivalent correction DNA was utilized for background correction

in the negative control (N.C.). Each error bar was calculated from triplicate analyses. Photographs were taken with an iphone 7.

Specificity test and performance of thrombin detection in diluted human serum. To test the specificity, thrombin aptamer recognition to four interferents was introduced to test the response to platelet derived growth factor BB (PDGF-BB), human Immunoglobulin G (lgG), interleukin 6 (IL-6) and adenosine triphosphate (ATP). Briefly, 90 μL combinatorial AuNPs was incubated with 10 μL 15-mer aptamer for 2 min at room temperature before 250 nM above non-targets and 5 nM thrombin target were added, respectively, then mixed for another 5 min, and finally followed by addition of 95 μL 5 mM sodium citrate buffer containing 70 mM Na^+. Equivalent BSA was utilized for background correction in N.C. group. Then the absorbance was measured at 520 nm and 650 nm, respectively, with a Microplate Reader (H1M) from Biotek (United States). Each error bar was calculated from triplicate analyses.

Furthermore, the performance of thrombin detection in diluted serum was tested. Typically, 90 μL combinatorial AuNPs was mixed with 10 μL 15-mer aptamer and incubated at room temperature for 2 min. Various concentrations of thrombin were mixed with 5% human serum (centrifugation at 3000 rpm, 4℃, for 2 min, and diluted with 1xPBS buffer) before addition of the above mixture and followed by a final addition of 95 μL 5 mM sodium citrate buffer containing 70 mM Na^+. Equivalent BSA was utilized for background correction in N.C. group. Then they were pipetted into the 96-well microplates for absorbance measurement at 520 nm and 650 nm at room temperature. Each error bar was calculated from triplicate analyses. Photographs were taken with an iphone 7.

Table S1. DNA sequences.

Name		5’ → 3’
Thrombin aptamer	**FAM-15-mer**	GGT TGG TGT GGT TGG-FAM
	15-mer	GGT TGG TGT GGT TGG
	29-mer	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
PDGF-BB aptamer		ACA GGC TAC GGC ACG TAG AGC ATC ACC ATG ATC CTG
DNA probe		TAC GAG TTG AGA ATC CTG AAT GCG
DNA target		CGC ATT CAG GAT TCT CAA CTC GTA
Noncomplementary target (correction DNA)		TAG CTA TGG AAT TCC TCG TAG GCA
Single-mismatch mutant (SNP1)		CGC ATT CAG GCT TCT CAA CTC GTA

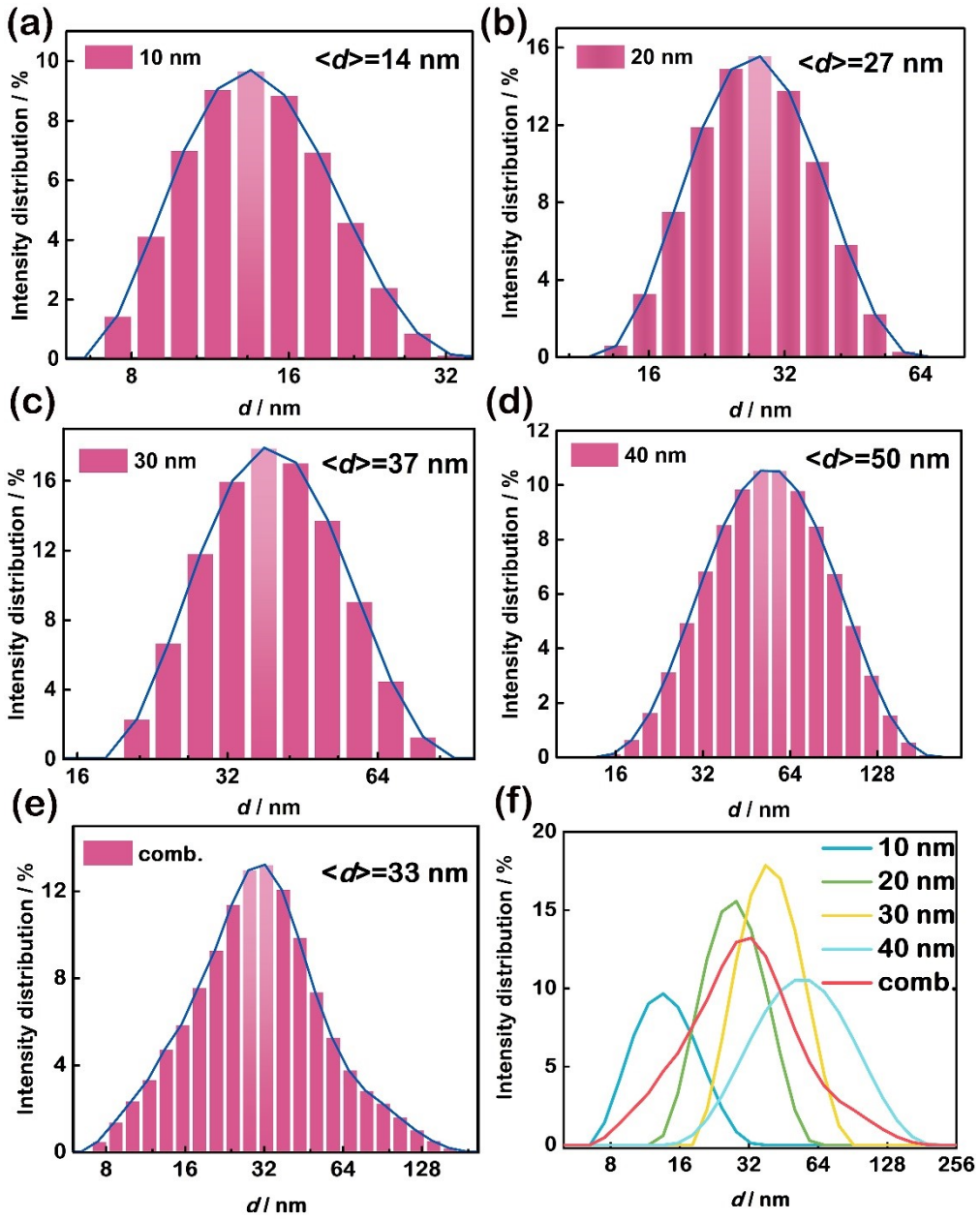

Figure S1. Particle size distribution of 10 nm, 20 nm, 30 nm and 40 nm AuNPs (a, b, c, d) and (e) combinatorial AuNPs mixed with four sizes at the volume ratio of 1: 1: 1: 1. (f) Comparison of size distribution among five AuNPs systems. 30 μL AuNPs (1 OD) was dispersed in 1 mL H_2O, and then followed by sonication procession for 1 min prior to the measurement with a Zetasizer Nano ZS.

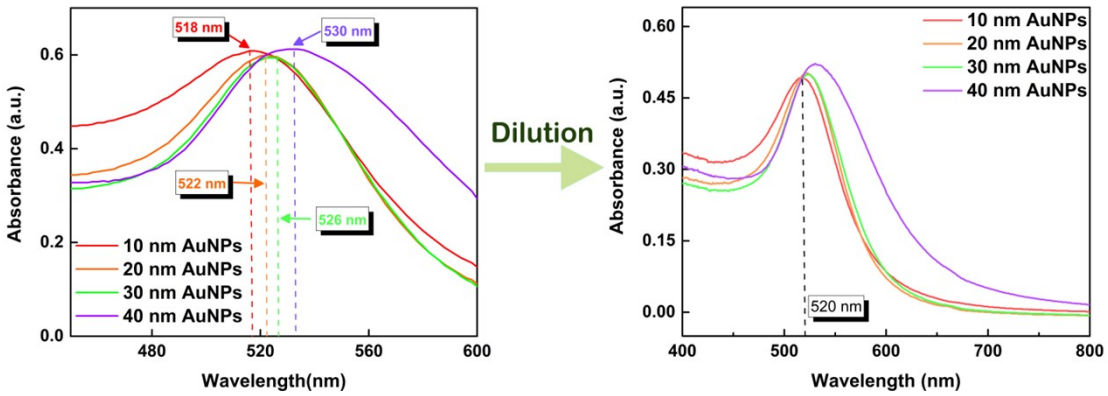

Figure S2. Absorbance normalization of four sizes of AuNPs into an identical A520 by H_2O dilution. The peaks of four AuNPs before normalization were 518 nm, 522 nm, 526 nm and 530 nm, and then the four particles were diluted to give consistent absorption intensity at 520 nm. The absorption spectra were measured with a UV/VIS-1750.

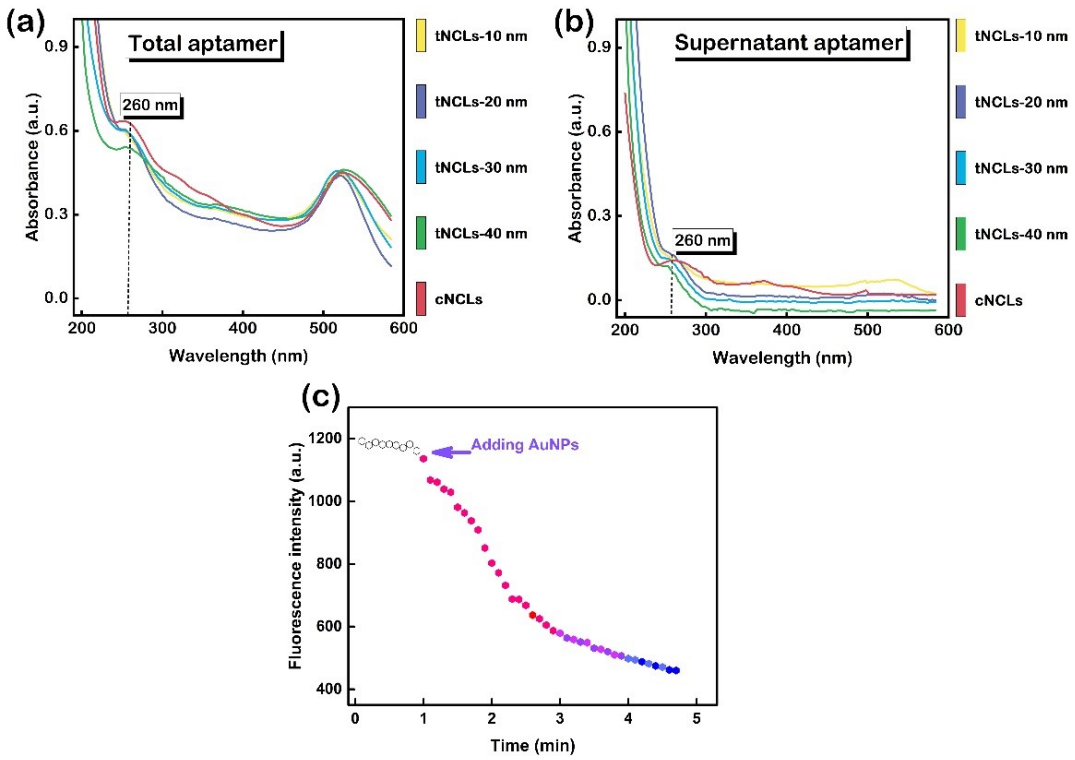

Figure S3. Aptamer adsorption amount on AuNPs (a, b) and its adsorption kinetics (c). (a) and (b) 15-mer thrombin aptamer was employed to investigate AuNPs adsorption capacity. Aptamer (200 nM) was first incubated with AuNPs (1.2 nM) of different sizes overnight, then the free aptamer was removed by centrifugation (8000 rpm, 8 min). The amounts of aptamer were measured by UV/VIS-1750 at 260 nm. Take the cNCLs system for example. The absorbance of 200 nM aptamer A_{total} after double dilution was 0.631. The absorbance of supernatant aptamer $A_{supernatant}$ after quadruple dilution was 0.156. According to the formula $A_{total} / C_{total} = A_{supernatant} / C_{supernatant}$, the calculated $C_{supernatant}$ was nearly 99 nM. Thus, aptamer adsorption amounts on AuNPs was about 100 nM calculated by C_{total} subtracting $C_{supernatant}$. (c) The adsorption kinetics was determined by monitoring fluorescence changes of FAM-labelled aptamer after adding combinatorial AuNPs. The fluorescence intensity of FAM- aptamer (15-mer, 17 nM, 50 mM Na^+) was monitored for 1 min prior to a quick addition and mixing with 20 μL of 1 nM combinatorial AuNPs. The excitation and the emission wavelengths were 480 nm and 520 nm, respectively. As can be seen, the adsorption process can be accomplished within 3 min.

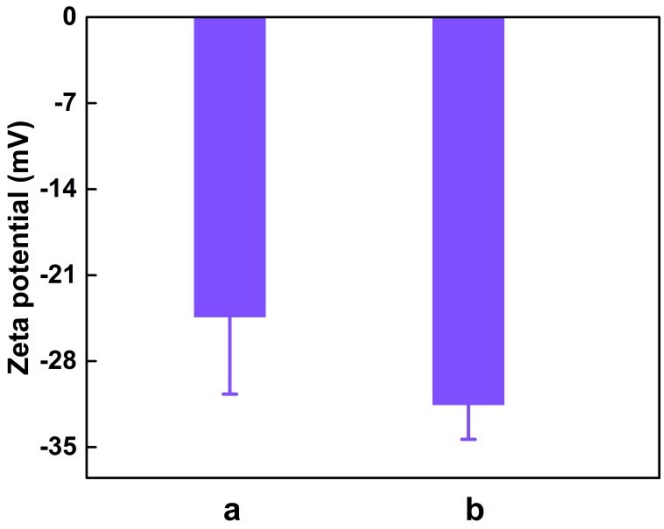

Figure S4. Zeta potential measurement: (a) 0.09 nM combinatorial AuNPs (mixed with four sizes) containing 0.76 mM Na^+; (b) 0.09 nM combinatorial AuNPs/Aptamer after the removal of free aptamer, with the final Na^+ concentration of 0.76 mM. When the aptamer was attached on the surface of AuNPs, the zeta potential value would be shifted to negative direction due to the negative charge of DNA strands. Each error bar represents one standard deviation from triplicated analyses.

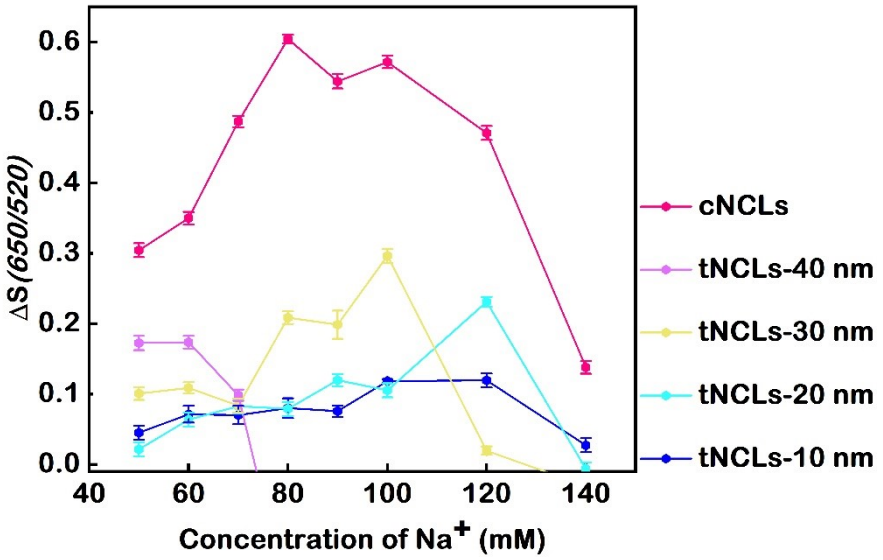

Figure S5 Evaluations of salt-responsive effect on analytical behavior between tNCLs and cNCLs systems. Briefly, 100 nM 15-mer aptamer was first incubated with 90 μL AuNPs, followed by addition of 6 nM thrombin target, and finally sodium citrate buffer containing 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 120 mM, 140 mM Na^+ were added, respectively, to investigate their response to salt. The A520 and A650 were measured with a Microplate Reader (H1M) from Biotek (United States). ΔS (650/520) represents the value of (P.C. (A650/A520)-N.C.(A650/A520)). N.C. represents negative control containing 6 nM BSA, while P.C. represents positive control containing 6 nM thrombin target. Each error bar was calculated from triplicate analyses.

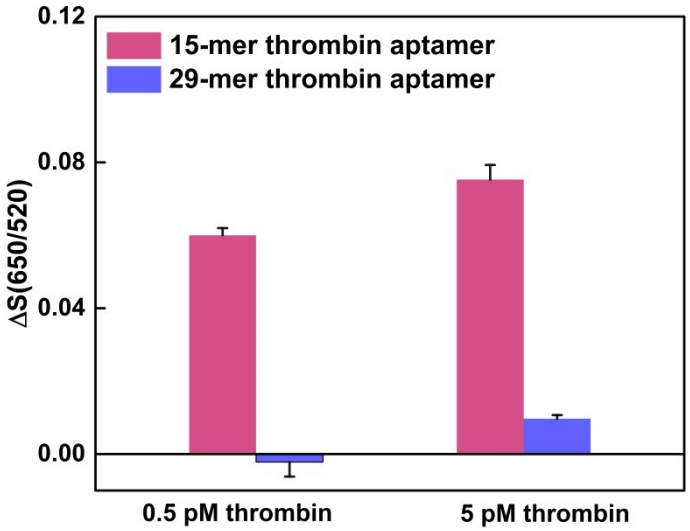

Figure S6. Optimization of length of thrombin aptamer in cNCLs system. 29-mer aptamer presents stronger binding affinity to thrombin [2] while a weaker desorption than 15-mer aptamer, [3] thus an optimal aptamer length was carried out to test their response to thrombin. Aptamer of two lengths (100 nM) were mixed with 0.54 nM combinatorial AuNPs, then 0.5 pM and 5 pM thrombin were added respectively, and finally followed by addition of 95 μL 5 mM sodium citrate buffer (70 mM Na^+). Equivalent BSA was utilized for background correction in the negative control (N.C.). Then the absorbance was measured at 520 nm and 650 nm with a Microplate Reader (H1M) from Biotek (United States). ΔS (650/520) represents the value of (P.C. (A650/A520)-N.C.(A650/A520)). Each error bar was calculated from triplicate analyses.

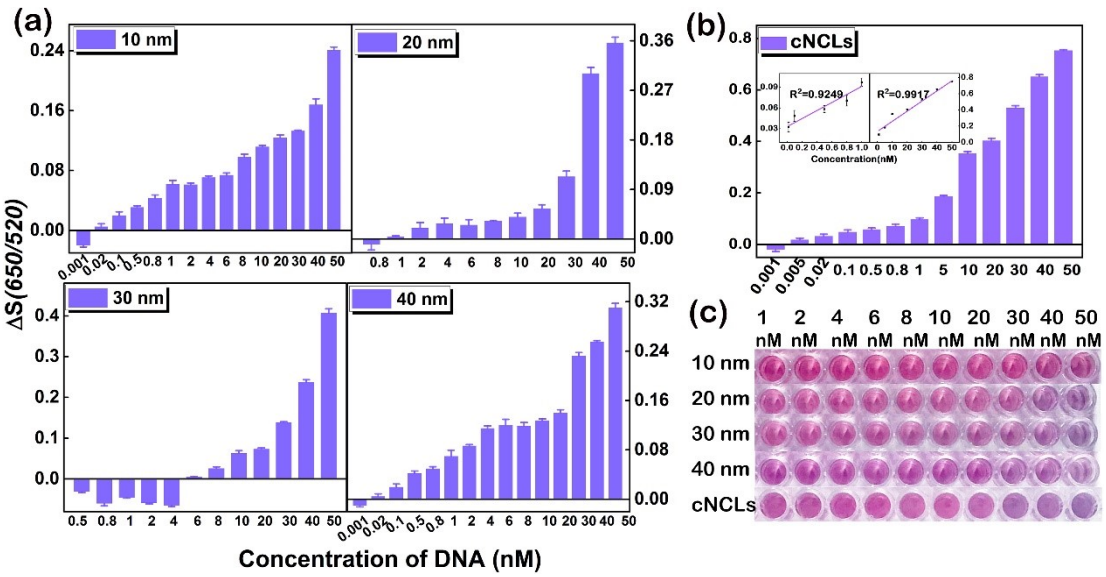

Figure S7 AuNPs -based colorimetric assay for DNA. (a) tNCLs-based assays including tNCLs-10 nm, tNCLs-20 nm, tNCLs-30 nm, tNCLs-40 nm AuNPs systems; (b) cNCLs-based colorimetric assay; and (c) visual readout. 100 nM DNA probe was mixed with AuNPs, then various concentrations of DNA targets were added, respectively, then followed by addition of 95 μL 5 mM sodium citrate buffer (70 mM Na^+). Equivalent correction DNA was utilized for background correction in the negative control (N.C.). Then the absorbance was measured at 520 nm and 650 nm with a Microplate Reader (H1M) from Biotek (United States). ΔS (650/520) represents the value of (P.C. (A650/A520)-N.C.(A650/A520)). N.C. group contained equal correction DNA in contrast to the DNA target in P.C. group. Each error bar was calculated from triplicate analyses.

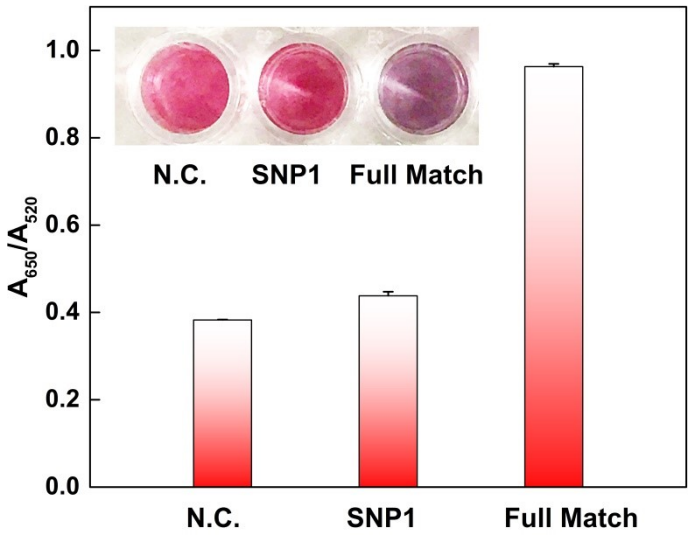

Figure S8. Single nucleotide polymorphism assay. The mutation site of SNP1 is highlighted in Table S1. The concentration of full match DNA and SNP1 was 50 nM. Equivalent correction DNA was utilized for background correction in the negative control (N.C.). [combinatorial AuNPs] = 0.54 nM. To coincide with protein analysis, the assay for DNA was performed by a pre-mixing procedure, in which ssDNA probe was attached to AuNPs first, and then added with DNA target and sodium citrate buffer (70 mM Na^+) to induce detectable signal. Each error bar was calculated from triplicate analyses.

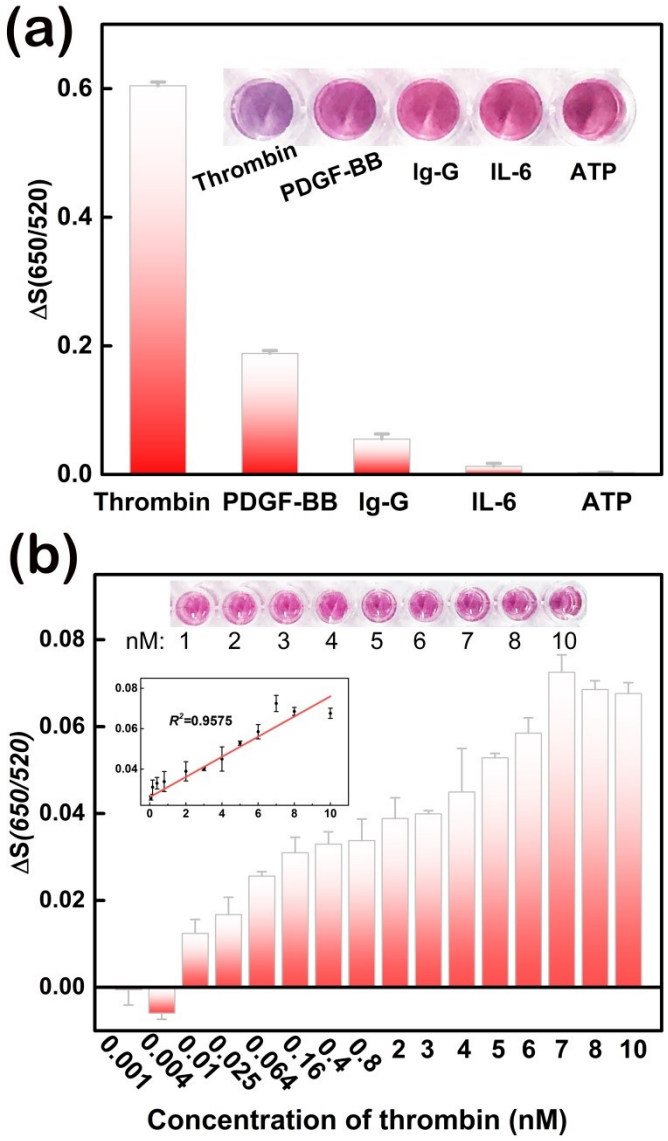

Figure S9. (a) Specificity test. Thrombin target and interfernts (PDGF-BB, lg-G, IL-6, ATP) employed in the test were 5 nM and 250 nM, respectively; and (b) the detection of thrombin in 5% human serum and its linear fitting between the absorbance ratio and thrombin concentration. 100 nM 15-mer aptamer was first incubated with combinatorial AuNPs (0.54 nM) in 5% serum, and added with thrombin target and sodium citrate buffer (70 mM Na^+) to induce detectable signal. Equivalent BSA was utilized for background correction in N.C. group. ΔS (650/520) indicates the difference between P.C. (650/520) and N.C. (650/520). Each error bar was calculated from triplicate analyses.

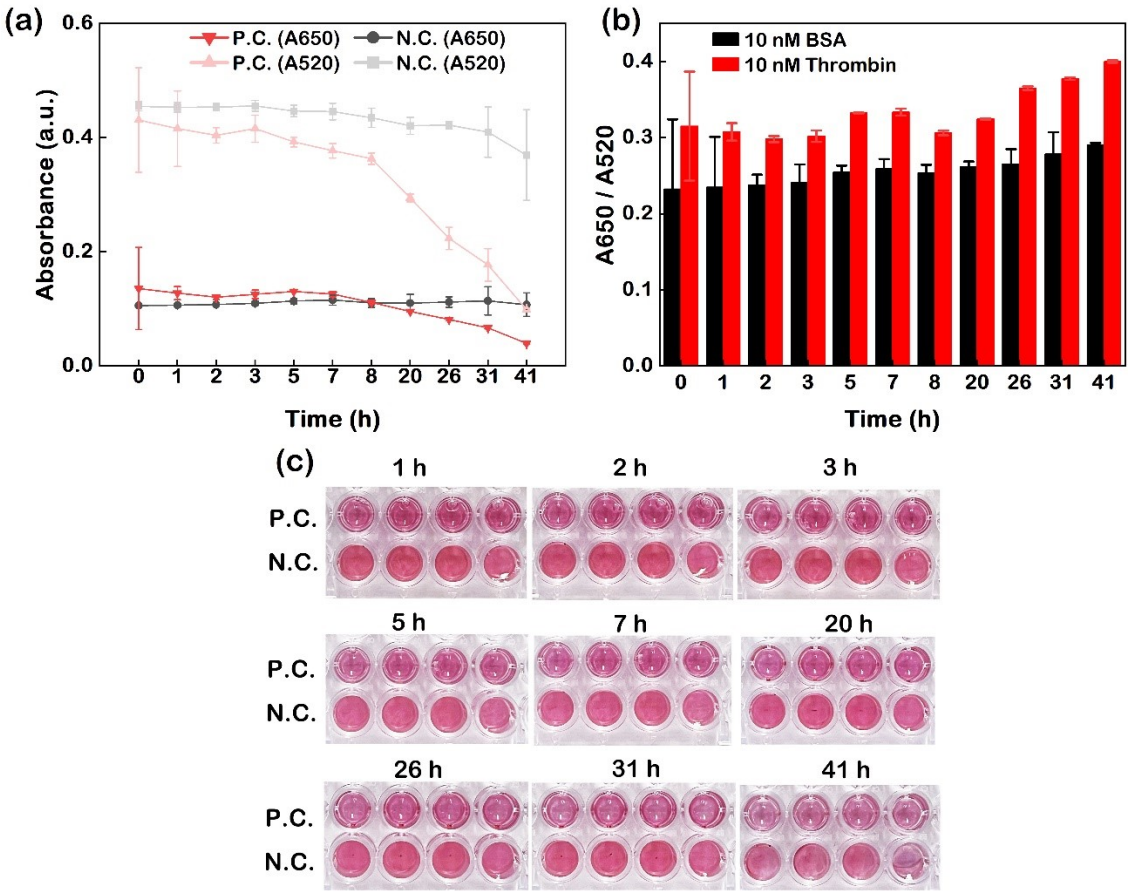

Figure S10 Stability test. (a) Absorbance change of cNCLs system over different time. (b) Intensity of A650/A520 ratio of P.C. group and N.C. group over different time. (c) Visual readout. Equivalent BSA was utilized for background correction in the negative control (N.C.). Then the absorbance was measured at 520 nm and 650 nm with a Microplate Reader (H1M) from Biotek (United States). N.C. group contained 10 nM BSA in contrast to the 10 nM thrombin in P.C. group. Each error bar was calculated from four trials.

Table S2 Unmodified AuNPs-based colorimetric assay for proteins and small molecules.

Target	Sensing probe	LOD	Time	Advantages	Limitations	Ref.
dopamine	dopamine-induced AuNPs growth	2.5 μM	Not mentioned	Rapid	Poor sensitivity, No applicable to practical samples	[4]
caspase-3	an unlabelled DEVD-containing peptide-AuNP probe	0.01 μg/mL	At least 30 min	simple, fast, applied for Jurkat cells detection	Cumbersome cell pretreatment	[5]
thrombin	aptamer-AuNPs probe	0.83 nM, visual LOD of 83 nM	35 min	Simple, sensitive, modification-free	Great demand for AuNPs (200 μL) per test.	[6]
Lysozyme	unmodified AuNPs probe	100 pM	Not-mentioned	Rapid and ideal for DNA analysis in food matrix	Large demand for AuNPs (900 μL) per test.	[7]
thrombin	aptamer-AuNPs probe & cationic conjugated polyelectrolytes	10 nM	Not-mentioned	Proposal of a novel conjugated polyelectrolytes that specifically binds ssDNA and induces AuNPs aggregation	The assay follows a 'post-mixing' procedure which suffer from slow binding kinetics	[8]
coralyne	ploy A-AuNPs probes	91 nM	22 min	Simple, free of modification and separation	'post-mixing' stratrgy, slow kinetics	[9]
antibiotics	multifunctional aptamer-AuNPs probe with multiple detection ability	32.9 nM for tetracycline, 7 nM for chloramphenicol	At least 16 min	Multiplex detection capability, simple, reliable	Need sample extraction and matrices process	[10]
Dopamine	Dopamine-AuNPs probe	4 μM	45 min	Unveiling new phenomenon that protein-AuNPs binding event dominate aptamer-based sensing	Low sensitive	[11]
kanamycin	Kanamycin-AuNPs probe	90 nM	1.3 h	Unveiling a new mechanism in visual assays	Low sensitive	[12]
cysteine	S-adenosyl-l-methionine (SAM)-AuNPs	21.7 nM	5 min	Simple, rapid, modification-free, separation-free	Low sensitive	[13]

	probe					
thrombin	aptamer-AuNPs probe & size effect	0.4 pM, visual LOD of 0.4 nM	7 min	Simple, quick, sensitive, modification-free and separation-free	Not applicable for serum of high concentration	This work

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