Electronic Supplementary Information (ESI)

Detection of C-reactive protein using nanoparticle-enhanced surface plasmon resonance with an aptamer-antibody sandwich assay

Bin Wu, † Rui Jiang,† Qing Wang, Jin Huang, Xiaohai Yang,* Kemin Wang,* Wenshan Li, Nandi Chen, Qing Li.

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Key Laboratory for Bio-Nanotechnology and Molecule Engineering of Hunan Province, Hunan University, Changsha, China 410082.
E-mail: yangxiaohai@hnu.edu.cn, kmwang@hnu.edu.cn; Tel/Fax: +86 731 88821566.

Experimental details

S-1. Materials and reagents

C-reactive protein (CRP) was purchased from Biovision (USA). Myoglobin protein (from human heart tissue) was purchased from Abcam (USA). Human hemoglobin (Hb) was purchased from Fitzgerald (USA). Anti-CRP and anti-CRP coated Au nanoparticles were purchased from Beijing Biosynthesis Biotechnology Co., Ltd (China). Transferrin (TRF), bovine serum albumin (BSA), human serum albumin (HSA), and human immunoglobulin G (IgG) were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (China). PCR Mix was purchased from Fermentas (Lithuania). Polybead microspheres (25 μm) were purchased from Polysciences (USA). SYBR Gold was purchased from Invitrogen (USA). Hepes and guanidine isothiocyanate were purchased from Amresco (USA). All of the chemical reagents were of analytical grade or higher. All of the DNA used in the experiment was synthesized from Sangon Biotechnology (Shanghai) Co., Ltd. (China). Ultrapure water (18.2 MΩ·cm) was used throughout.

S-2. Design and fabrication of microfluidic chip

The microfluidic device was fabricated on 1.6 mm-thick borosilicate glass substrates using photolithographic and wet chemical etching techniques. It consisted of a main straight channel (200 μm wide, 70 μm deep), which included a pinch (1 mm long, 30 μm wide, 15 μm deep) (Fig S1). The pinch blocked target coated microbeads. Type SG3006 glass substrate with 145 nm-thick chromium and 570 nm-thick S-1805 photoresist coating and glass cover plates of the same material were obtained from Shaoguang Microeletronics Corp (Changsha, China). Production of the photomask and procedures for fabrication of microchannels referred to our previous work[S1].

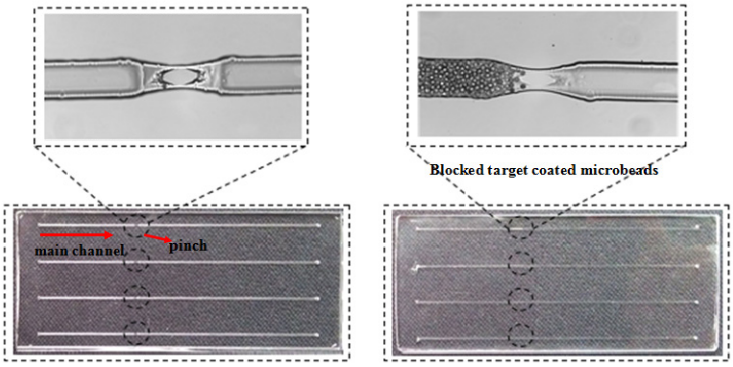


Fig. S1 Structure of microfluidic device

S-3. Design of a DNA library

40-base randomized domain was placed in the middle of a FAM-labeled DNA pool, then, 16-nt constant regions were placed at both ends of pool. The forward primer (FP) and biotin-labeled reverse primer (RP) were used for PCR amplification. Forward primer and reverse primer were used for cloning and sequencing. DNA sequences used in this selection were shown in Table S1.

Table S1. Sequences of DNA used in this selection

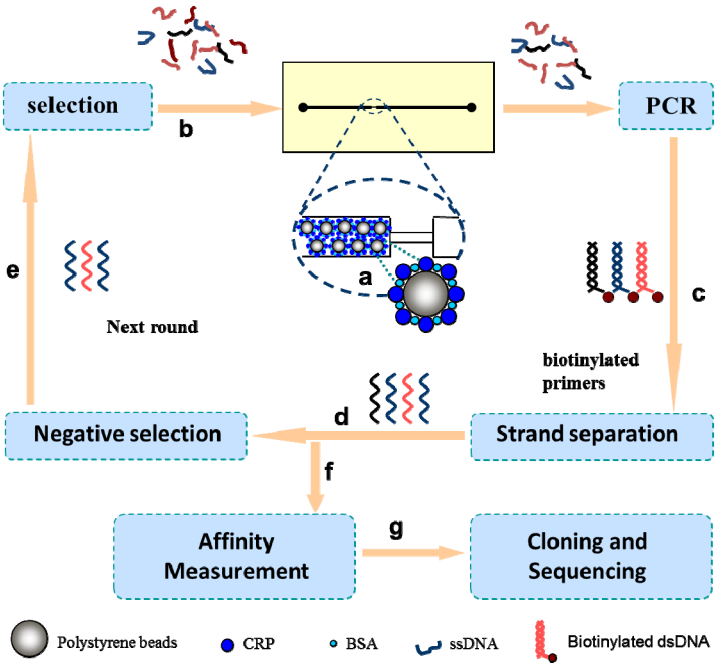
Name	Sequence
ssDNA pool	5'-GGC AGG AAG ACA AAC A-N_{40}-TGG TCT GTG GTG CTG T-3
FP	5'-GGC AGG AAG ACA AAC A -3'
RP	5'-ACA GCA CCA CAG ACC A-3'
RP-Biotin	5'-biotin-ACA GCA CCA CAG ACC A-3'

*FP: forward primer; RP: reverse primer

S-4. Selection of aptamers against CRP

(1) Selection of aptamers

The working principle of our microfluidic chip for aptamer screening was schematically demonstrated in Scheme S1. The target protein (CRP) was first incubated with polystyrene microbeads. The protein can be easily coupled to polystyrene microbeads via passive adsorption according to the operational manual of polybead microspheres. Since the concentrations of proteins in solution can be determined by UV-visible spectrophotometry, we can estimate the effectiveness of this coating procedure by monitoring the change of absorbance before and after the immobilization of proteins on microbeads. Then, protein coated beads were blocked for 1 h with 0.1 mM random short ssDNA (20 nt). Next, the prepared beads were inhaled into the channel using the pump and blocked before the pinch. A random ssDNA library (~10^{14} random molecules) was used which consisted of a central random region of 40 nucleotides flanked by two fixed regions of 16 nucleotides sequences at the 5′ and 3′ ends. Then, the ssDNAs of library (200 pmol) was added to binding buffer (20 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1mM $MgCl_2$, pH 7.3) and carried out at 95 °C for 10 min, and then, it was gradually cooled to room temperature. Next, the prepared library was injected into the channel at 0.5 μL/min from the inlet. 120 μL of washing buffer (binding buffer, 0.05% Tween 20) and eluting buffer (20 mM Tris-HCl, 4 M guanidine isothiocyanate, 1 mM DTT, pH 8.3) were pumped into the channel at 2 μL/min from inlet in turn. Finally, the eluted ssDNA was collected from the outlet.



Scheme S1. Schematic illustration of the steps involved in the microfluidic chip procedure of CRP aptamer screening.

(2) PCR amplification

Collected products of each round selection were amplified immediately. 5 μL of the collected products as added into a 50 μL PCR mixture with 25 μL of 2× Taq Master Mix, 1 μL of 25 μM FAM-labeled FP, and 1 μL of 25 μM biotinylated RP. The thermal cycling conditions were as follows: 94 °C for 5 min, 10 cycles of three-step PCR (94 °C for 45 s, 62 °C for 30 s, and 72 °C for 30 s), terminated by an extra extension at 72 °C for 7 min. Then, the amplified PCR products were used as template and diluted to 500 μL in the same PCR mixture. These mixtures were amplified again. During the extension step of each two cycle, 10 μL of PCR mixture was collected and resolved on 3% agarose gel to find the optimal PCR amplification cycle number. Next, the remaining collected products were all amplified at the optimized cycle number.

(3) ssDNA generation

The biotinylated, double-stranded PCR products were incubated with 200 μL of GE Streptavidin agarose microbeads for 0.5 h at room temperature. Then, ssDNAs were generated by adding 200 mM NaOH and incubating for 10 min at room temperature, after which the supernatant was collected, desalted and characterized by UV-visible measurement at 260 nm.

(4) Counter selection

After the 2rd round, a counter selection was carried out. The purified ssDNA pool (120 pmol) of 2rd round was incubated with the 1% BSA immobilized microwell plate for 2 h, the unbound ssDNAs were collected. Then the collected DNA products used for the next round selection.

S-5. Binding affinity evaluation for selected products of each cycle

As shown in Fig. S2A, for monitoring the enrichment of aptamers, binding affinity of each round selected pool was determined using a homemade surface plasmon resonance (SPR) instrument[S2]. In brief, 200 μg/mL CRP was first immobilized on uncoated Au film. After being blocked by 1% BSA, an aliquot of ssDNA library from each round was added and incubated for 30 min at 25 °C, Au film surface was washed repeatedly with binding buffer. The wavelength shift of SPR for each sample was recorded and analyzed as shown in Fig. S2B. All oligonucleotides were heated at 95 °C for 5 min and instantly cooled in ice for 5 min, and then, it was gradually cooled to room temperature.

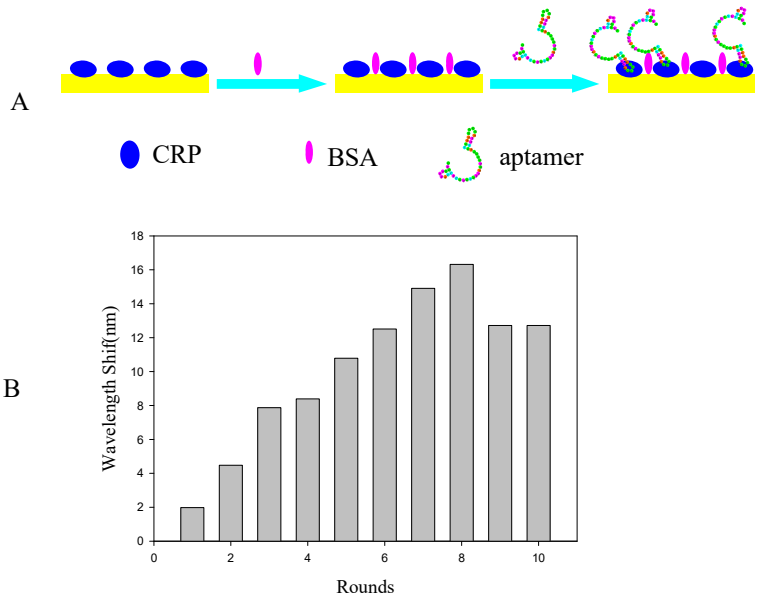


Fig. S2 Schematic mechanism of monitoring the progress of screening aptamers by SPR (A), results of the evolution of binding affinity between CRP and every round selection library (B).

S-6. Cloning and sequencing of selected aptamers

After 10 rounds of selection, the sixth, eighth, and tenth selected products were PCR amplified with unlabeled primers, respectively. The PCR products of each round were sent to Sangon Biotechnology Co., Ltd. (Shanghai, China) for cloning. A hundred colonies of every round were randomly picked and sequenced at the Sangon (Shanghai, China). The secondary structure analysis of selected aptamers was performed with the Internet tool Mfold, http://frontend.Bioinfo.Rpi.edu/applications/mfold/). The sequence of picked aptamers were showed in Table. S2.

Table. S2 the sequence of picked aptamer

Name	Sequence
6th-18/25	5'-CCAGGAGGCGAGAATAGATGTTAGACTAGGGGCCGGCGTA-3'
6th-62/76/85	5'-CGAAGGGGATTCGAGGGGTGATTGCGTGCTCCATTTGGTG-3'
6th-125	5'-CCAGGAGGCGGCTTAAGGGATACGGGCAAATAGGGGGCGT-3'
8th-91/121	5'-CGGAGAGCAGGAAGTAGTGGAGAGAATGATCGTACTGGCC-3'
8th-93/112	5'-CGCAGAAGGATCAGGATGAATAGCTTAGGGGGGCCTTGAT-3'
10th-16/94	5'-CGCGGACGGGGAACGGTCCAGTGCCACAGGAACGGGCTCG3'
10th-30/38	5'-CGGGAGCAGTGCGGGGAAGTATCAGCAGGCGAATGGGAGGA-3'
10th-33/45	5'-CGGCAACGGTGGGTCAGGGATAGAAAAGCGCAGTAGG-3'

S-7. K_d determination and selectivity test of potential aptamers

The dissociation constants (K_d) of individual ssDNA aptamers were measured using the BIAcore X system (GE Healthcare). Au film was first modified using 200 μg/mL CRP, and then the uncovered sites on the Au surface were blocked by injecting 1% BSA. Different concentrations of candidate aptamers (from 0 nM to 200 nM) were added and incubated for 30 min at 25 °C, Au film surface was washed repeatedly with binding buffer. The change of signal (i.e., response unit (RU)) was recorded and analyzed (Fig. S3). For investigating the specificity of selected aptamers to HSA, and BSA were chosen as controls. 200 nM HSA and BSA was introduced at a constant flow rate of 5 μL/min, respectively, and the change of signal (i.e., response unit (RU)) was recorded (Fig. S4). The K_d of individual ssDNA aptamers were showed in Table S3.

Table S3. Result of K_d determination test of potential aptamers

Name	K_d /nM
6th-18	2.8
6th-62	22.1
6th-125	3.6
8th-91	44.5
8th-93	22.5
10th-16	56.2
10th-30	16.2
10th-33	23.8

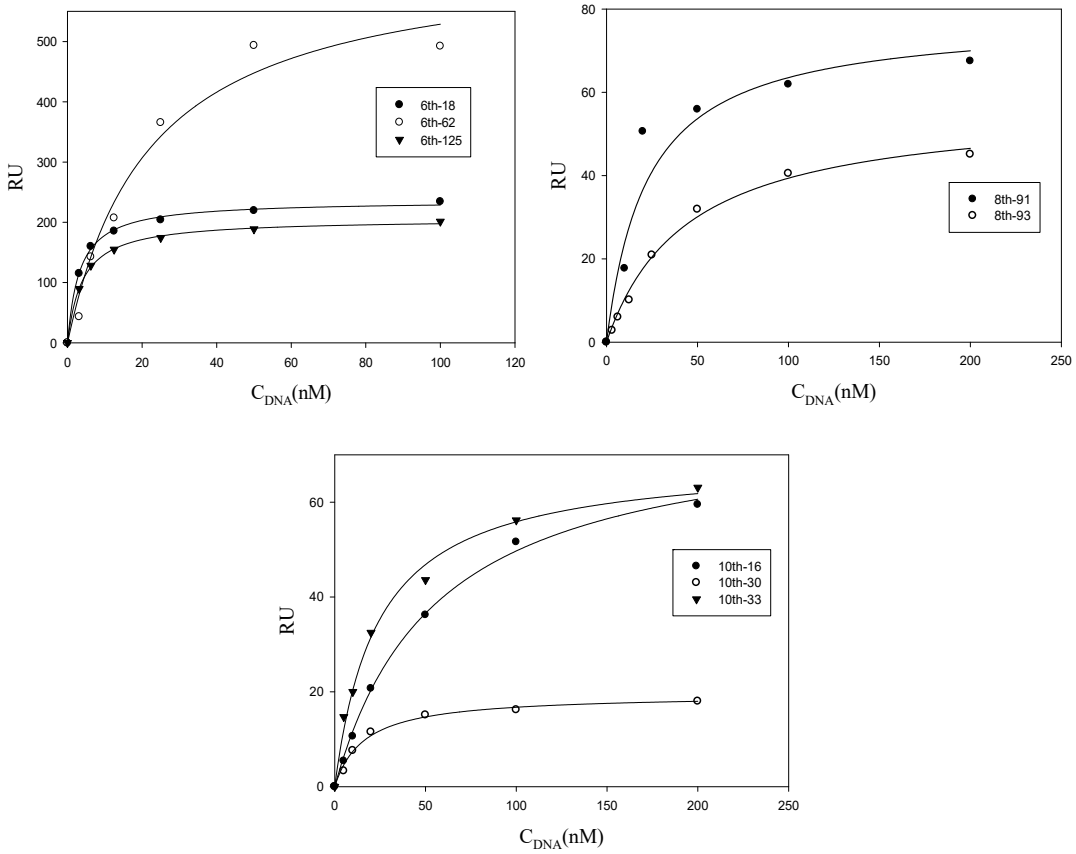


Fig. S3 The change of signal (RU) of the selected aptamers at various concentrations of CRP.

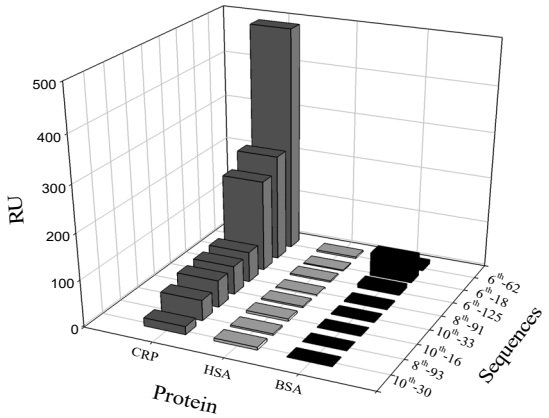


Fig. S4 The specificity of selected aptamers investigation

S-8. Ability of immobilized aptamer to bind CRP using aptamer-based SPR analysis

Aptamers are usually immobilized on the surface of supports during the construction of biosensors. Thus, we investigated the ability of the immobilized aptamers to bind CRP using SPR analysis. After the affinity and specificity determination, the aptamers named 6th-62 with high SPR signal and specificity was selected. Four biotinylated aptamers 6th-62-5', 6th-62-3', 6th-62-40-5' and 6th-62-40-3' were designed for this examination. Aptamer-immobilized Surface Plasmon Resonance measurements were conducted on an EC-SPR1010 facility (Changchun Dingcheng technology, China). Au film was first modified using 1 mg/mL biotin-BSA and avidin, respectively. Then 1 μM biotin functionalized aptamer was immobilized by biotin-avidin interaction. After different concentrations of CRP (from 0 nM to 200 nM) were added and incubated for 30 min at 25 °C, Au surface was washed repeatedly with binding buffer. The change of resonance angle was recorded and analyzed. Five control proteins including human immunoglobulin G (IgG), human serum albumin (HSA), hemoglobin (Hb), transferrin (TRF) and myoglobin (Myo) were chosen to selectivity investigation (Fig. S5).

Table S4. The sequences of used in this part

Name	Sequence
6th-62-5'	5'-biotin-GGCAGGAAGACAAACACGAAGGGGATTCGAGGGGTGATTGC GTGCTCCATTTGGTG TGGTCTGTGGTGCTGT-3'
6th-62-3'	5'-GGCAGGAAGACAAACACGAAGGGGATTCGAGGGGTGATTGCGTGC TCCATTTGGTG TGGTCTGTGGTGCTGT-biotin-3'
6th-62-40-5'	5'-biotin-TTTTTTTTTTTT CGAAGGGGATTCGAGGGGTGATTG CGTGCTCCATTTGGTG -3'
6th-62-40-3'	5'-CGAAGGGGATTCGAGGGGTGATTGCGTGCTCCATTTGGTG TTTTTTTTTTTT-biotin-3'

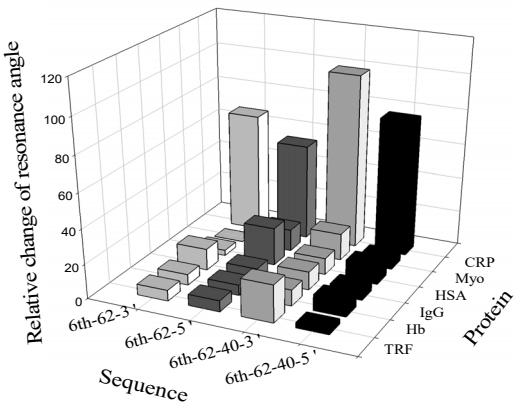


Fig. S5 The specificity of selected aptamers investigation in aptamer immobilized SPR analysis. The change of resonance angle was obtained with 100 nM control protein and are shown relative with the CRP results.

S-9. AuNPs enhanced SPR biosensor with an aptamer-antibody sandwich

Aptamer-immobilized surface plasmon resonance measurements were conducted on an EC-SPR1010 facility (Changchun Dingcheng technology, China). Thiol-modified 6th-62-40 (5'-CGA AGG GGA TTC GAG GGG TGA TTG CGT GCT CCA TTT GGT GTT TTT TTT TTT T-SH-3') was immobilized on a bare gold chip by injecting 60 μL of 1 μM thiol-modified 6th-62-40 in binding buffer (20 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1mM $MgCl_2$, pH 7.3), then washing the un-immobilized aptamer. The blocking step consisted of injecting 60 μL of 10 mM 6-mercapto-1-hexanol (MCH) in the same buffer. Au film was passivated with MCH for 30 min at room temperature in order to reduce nonspecific binding on the Au surface and this method is a classical strategy to reduce nonspecific adsorption. [S3] This prepared biosensor chip using for the next experiment. Next, CRP was added, and the immobilized aptamer can capture CRP on the Au film by specific binding. Then, signal enhancement after the addition of 200 nM anti-CRP coated AuNPs and the resonance angle was recorded. Furthermore, the injected AuNPs labeled anti-CRP concentration was optimized as 200 nM in the present of 20 nM CRP (Fig. S6).

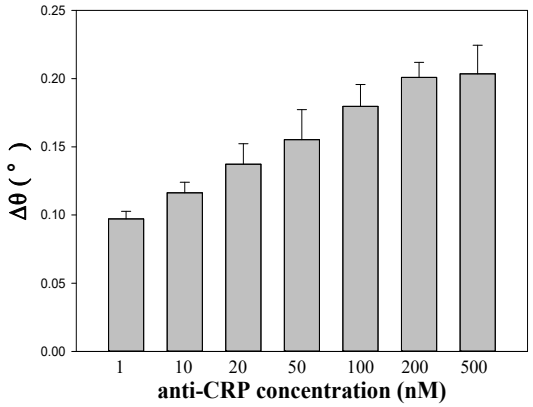


Fig. S6 Optimization of anti-CRP coated AuNPs in the present of 20 nM CRP.

S-10. Reproducibility investigation of the SPR biosensor

The regeneration of the biosensor chip was investigated. After a sample was detected, the Au film surface could be regenerated by 0.1 % sodium dodecyl sulfate (SDS) / 10 mM NaOH for the next round of analysis. When this biosensor was used to detect 50 nM CRP in the present of 200 nM anti-CRP coated AuNPs, only slightly signal degradation was observed during the 5 cycles shown in Fig. S7.

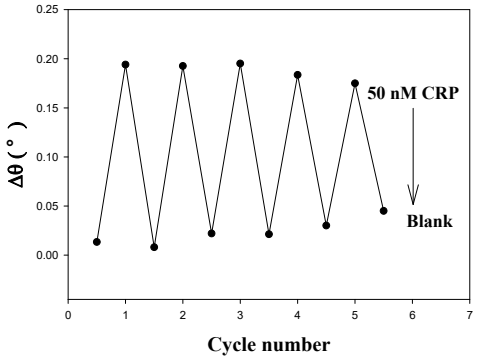


Fig. S7 Reproducibility investigation of the SPR biosensor. After a sample was detected, the biosensor chip was regenerated using 0.1% SDS/10 mM NaOH for 5min. Δθ was the shift of resonance angle after subtracting the response of the blank solution.

S-11. Detect CRP in human serum

The human serum was diluted 100 times using binding buffer. Next, the CRP-spiked serum samples were prepared by adding CRP in diluted serum samples and then analyzed. As shown in Fig. S7, the signal caused by CRP in human serum was similar to that in buffer.

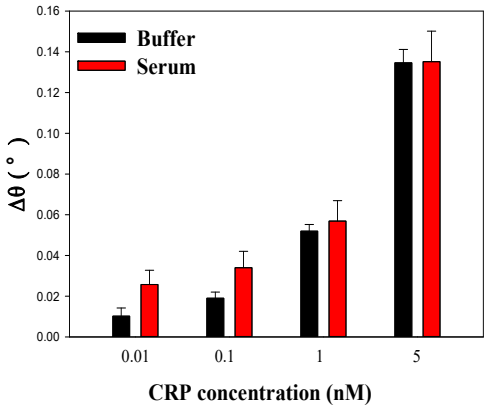


Fig. S8 Detection of CRP in buffer and human serum. Δθ was the shift of resonance angle after subtracting the response of the blank solution.

Table S5 CRP detection performance comparison of our strategy with other aptamer-based methods.

Methods	Aptamer type	Detection limit	Reference
Electrochemical	RNA	1.75 nM	*Electroanalysis,* 2009, 21, 1309-1315.
	RNA	0.87 nM	*Phys. Chem. Chem. Phys.*, 2010, 12, 9176-9182.
Fluorescence	RNA	3.5 nM	*Anal. Biochem.*, 2015, 472, 67-74.
	RNA	87 pM	*Biosens. Bioelectron.,* 2009, 24, 1456-1461.
Chemiluminescence	DNA	1.1 nM	*Biosens. Bioelectron.,* 2009, 24, 3091-3096.
Isotachophoresis	DNA	2 nM	*Anal. Chem.*, 2015, 87, 6736-6743.
SPR	RNA	43.9 pM	*Anal. Bioanal. Chem.,* 2008, 390, 1077-1086.
	DNA	7 zM	*Sci. Rep.,* 2014, 4, 5129.
	DNA	10 pM	This work

S1 Q. Wang, W. Liu, Y. Xing, X. Yang, K. Wang, R. Jiang, P. Wang and Q. Zhao, *Anal. Chem.*, 2014, **86**, 6572-6579.

S2 X. Yang, Q. Wang, K. Wang, W. Tan, J. Yao and H. Li, *Langmuir*, 2006, **22**, 5654-5659.

S3 T. Herne and M. Tarlov, *J. Am. Chem. Soc.*, 1997, **119**, 8916-8920.