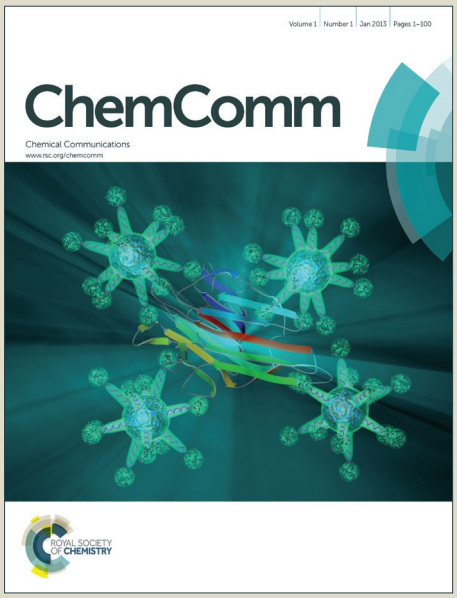
ChemComm

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: B. Wu, R. Jiang, Q. Wang, J. Huang, X. Yang, K. Wang, W. Li, N. Chen and Q. Li, *Chem. Commun.*, 2016, DOI: 10.1039/C5CC10486F.



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

View Article Online
DOI: 10.1039/C5CC10486F

Journal Name

COMMUNICATION

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

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x
www.rsc.org/

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

High affinity DNA aptamers against C-reactive protein (CRP) were obtained using microfluidic chip. The aptamers were then used for the construction of Au nanoparticle enhanced surface plasmon resonance biosensor, which was introduced for the detection of CRP at concentrations ranging from 10 pM to 100 nM in diluted human serum.

During the past two decades, the number of proposed and validated biomarkers has increased dramatically. An ability to assay such biomarkers is of obvious profound value in early and potentially lifesaving diagnosis[1]. Human C-reactive protein (CRP) is a major acute-phase reactant protein produced in the liver, and is composed of five monomeric subunits[2]. It is a general inflammatory biomarker and useful in diagnosing inflammatory responses in cancer, cardiovascular diseases (CVD) and neurological disorders[3-5]. The basal CRP levels in normal healthy subjects are below 10 mg/L (~87 nM). However, it has been found that serum CRP levels above 3 mg/L (~26 nM) are associated with an extended risk of a cardiovascular event. Levels between 1 and 3 mg/L are associated with medium risk of a cardiovascular event, whereas levels below 1 mg/L (~8.7 nM) are indicative of low risk. It was also reported that CRP levels in serum can increase more than 1000-fold in response to inflammation or injury[6].

The most commonly CRP diagnostic methods are the antibody-based assays[7-9]. Currently, some aptamer-based methods have been developed for CRP detection, such as electrochemical[10, 11], fluorescence[12, 13], chemiluminescence[14], surface plasmon resonance[15, 16] and isotachophoresis[17]. Aptamers, which were obtained by using an in vitro process named systematic evolution of ligands by exponential enrichment (SELEX)[18-20], are potential candidates to replace antibodies in therapeutics diagnostics and drug development[21-23]. There are several advantages to the development and use of aptamers. Firstly, aptamers are more stable and resistant to a harsh environment. Be different with antibodies, aptamers can survival many cycles of denaturation and regeneration. It allows aptamer-based sensing platforms to be recyclable and reusable[24]. Secondly, aptamers can be modified at specific locations in the sequence and there is no batch-to-batch variation in their quality due to efficient purification techniques, because they are produced by chemical synthesis[25]. Thirdly, since aptamers perform significant conformational changes upon target binding, aptamers offer great flexibility in design of novel biosensors[26].

Surface plasmon resonance (SPR) biosensing, which monitors the change in refractive index near the surface that occurs during complex formation or dissociation, is an attractive technology for sensitive, label-free, and real-time detection of biomolecular targets [27, 28]. SPR sensors have been employed for the detection of proteins[29], nucleic acids[30, 31] and small molecules[32, 33]. However, it is necessary to point out that SPR assays generally suffer from low signal intensity and nonspecific binding, which has impeded further application of SPR in proteomics and disease diagnostics, especially for the detection of trace targets in complex biological samples. To overcome this drawback, a variety of amplification approaches, such as gold and magnetic nanoparticles have been explored[34, 35]. For decades, the sandwich assay has been a mainstay in the fields of biodetection, clinical diagnostics, environmental monitoring, and quality control in various industries. By eliminating the requirement of labeling the molecular targets, the sandwich assays usually require the simultaneous binding of the recognition probe and signaling probe, which makes them extremely specific[36].

Herein, we present a highly sensitive and selective Au nanoparticles (AuNPs) enhanced SPR biosensor for CRP detection based on aptamer-antibody sandwich assay (Fig. 1). The novel aptamers against CRP were selected using microfluidic chip, and were immobilized on Au surface to construct SPR biosensor. Next, CRP was added, and the immobilized aptamers can capture CRP on the Au film by specific binding. Then, signal enhanced after the addition of anti-CRP coated AuNPs. Furthermore, three CRP measurement strategies, direct measurement, aptamer-antibody sandwich measurement and AuNPs enhanced aptamer-antibody sandwich measurement were compared. Significantly, this sensitive SPR biosensor also be applied to the specific detection of CRP in diluted human serum.

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. Tel/Fax: +86 731 88821566.
† B. Wu and R. Jiang contributed equally to this work.
* E-mail: yangxiaohai@hnu.edu.cn, kmwang@hnu.edu.cn.
† Electronics supplementary information (ESI) available.

View Article Online
DOI: 10.1039/C5CC10486F

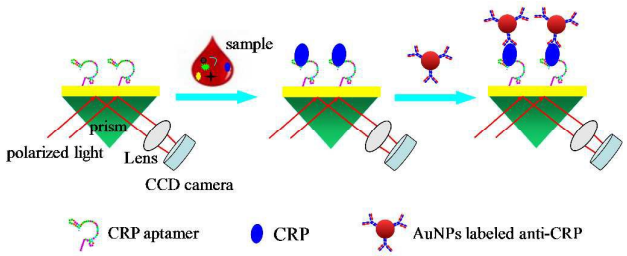


Fig. 1 Schematic illustration of the AuNPs enhanced SPR biosensor with an aptamer-antibody sandwich assay.

We selected high affinity and specificity aptamers against CRP. After 10 rounds of selection, eight aptamers named 6th-18, 6th-62, 6th-125, 8th-91, 8th-93, 10th-16, 10th-30 and 10th-33 were chosen for further binding affinity studies based on the repeated sequences and similarity of secondary structure. After the affinity and specificity determination, the aptamer named 6th-62 with high SPR signal and specificity was selected and the dissociation constants (K_d) was 22.1 nM. In consideration of blocking the primer-binding sites in the process of selection, 6th-62 was optimized by cutting nucleotides of the two constant regions. Therefore, a shorter aptamer with K_d 16.2 nM was obtained, which contained only 40 nt and named as 6th-62-40. See details in ESI.

Aptamers are usually immobilized on the surface of supports during the construction of biosensors. In the aptamer-target-antibody configuration, the immobilization of aptamer will not affect its interaction with the epitope on the target. Additionally, using an aptamer to capture the target offers the advantage of reusability, as the aptamer can be reused to capture a subsequent target. Aptamer immobilized SPR chips have been previously reported to exhibit better resistance to nonspecific adsorption than antibody-coated surfaces[37, 38]. Thus, we investigated the ability of the immobilized aptamers to bind CRP using SPR analysis. Four biotinylated aptamers 6th-62-5', 6th-62-3', 6th-62-40-5' and 6th-62-40-3' (sequences see Table S4, in ESI) were designed for this examination. The results indicated that the immobilized 6th-62 and 6th-62-40 still preserved the affinity to CRP. Meanwhile, 6th-62-40-3' immobilized on the Au film showed most significant change of resonance angle (Fig. 2).

Five control proteins including human immunoglobulin G (IgG), human serum albumin (HSA), hemoglobin (Hb), transferrin (TRF) and myoglobin (Myo) were chosen to selectivity investigation, which based on the possible interference to CRP in practical usage. It is clear that the immobilized aptamers 6th-62 and 6th-62-40 specifically binds to CRP against control proteins (Fig. S5, ESI†). Therefore, the aptamers can serve as candidate recognition element for biosensor applications.

According to the SPR analysis result, 3'-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 chosen to be immobilized on a bare gold chip to construct biosensor. The AuNPs enhanced SPR biosensor measurements were performed with EC-SPR1010 device (Changchun Dingcheng Technology, China). Firstly, the aptamer (6th-62-40) was immobilized on SRP chip (Au film). Next, CRP was added, and the immobilized aptamer can capture CRP on the Au film by specific binding. Then, signal enhanced after the addition of the anti-CRP coated AuNPs. The result showed in Fig. 3 demonstrated the feasibility of our desired biosensor for CRP detection. In addition, the concentration of anti-CRP coated AuNPs was optimized as 200 nM (Fig. S6, ESI†). There are two major advantages through Au-S bond immobilization method. First, it is a simple and low cost immobilization method, which just need SH-DNA and 6-mercapto-1-hexanol (MCH) in all two process. Moreover, this method offers the advantage of reusability by robust Au-S bond.

In order to detect CRP target at human serum levels using SPR biosensor, it is necessary to obtain correspondingly low detection limit. Herein, the detection limit of three SPR biosensor measurement strategies, direct measurement, aptamer-antibody sandwich measurement and AuNPs enhanced aptamer-antibody sandwich measurement were compared. Different concentrations of CRP solution were reacted with aptamer which was modified on Au film, then the Au film was washed with binding buffer repeatedly and the resonance angle was recorded. Then, anti-CRP coated AuNPs were added, washed with binding buffer repeatedly and the resonance angle was recorded. The relationship between Δθ and CRP concentrations have obtained. The result showed that AuNPs enhanced aptamer-antibody sandwich strategies have a largest measurement range and lowest detection limit (Fig. 4).

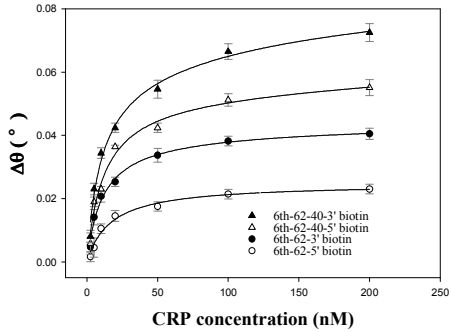


Fig. 2 The change of resonance angle of the four immobilized aptamers at various concentrations of CRP. Error bars showed the standard deviation of measurement taken from three experiments.

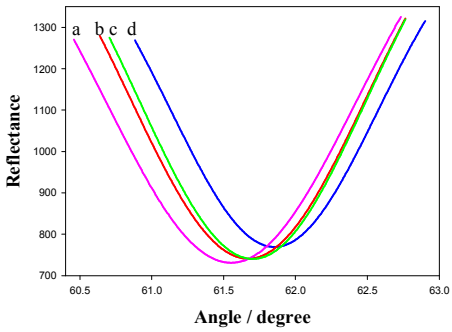


Fig. 3 SPR spectra of binding buffer (a), SH-aptamer modified Au film (b), blocking Au film by MCH in order to reduce nonspecific binding (c), the modified aptamer binding 50 nM CRP in the present of 200 nM anti-CRP coated AuNPs (d).

View Article Online
DOI: 10.1039/C5CC10486F

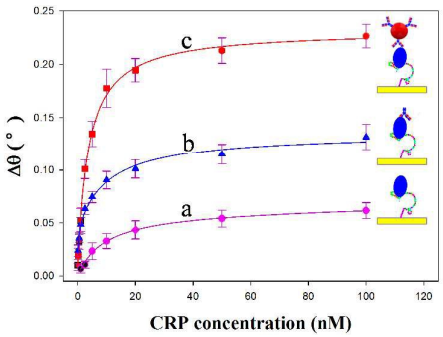


Fig. 4 The relationship between Δθ and CRP concentrations by using different sensing strategies. Direct measurement (a), aptamer-antibody sandwich measurement (b), AuNPs enhanced aptamer-antibody sandwich measurement (c).

The detection limit of above three strategies was 1 nM, 0.1 nM and 10 pM, respectively. Therefore, the AuNPs enhanced SPR biosensor was suitable for the determination of CRP in diluted human serum. As presented in Table S5†, CRP detection performance of our strategy was comparable to most of previous aptamer-based works.

Besides sensitivity, the selectivity of this aptamer-based SPR biosensor was also investigated. Five proteins, IgG, HSA, Hb, TRF and Myo were used as contrast. The results in Fig. 5 implied that this sandwich biosensor showed high selectivity for CRP detection.

Aptamer immobilized sandwich SPR chips have advantage of regeneration, therefore, the regeneration of the biosensor chip was also investigated. After a sample was detected, the Au film surface could be regenerated using 0.1 % sodium dodecyl sulfate (SDS) / 10 mM NaOH for the next round of analysis. When this SPR 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 ESI†, implying that this SPR biosensor exhibited appreciable regeneration.

This biosensor was also used to 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 1:100 diluted serum samples. As shown in Fig. S8 ESI†, the signal caused by CRP in human serum was similar to that in buffer, suggesting that the other components of the serum did not interfere significantly in this assay.

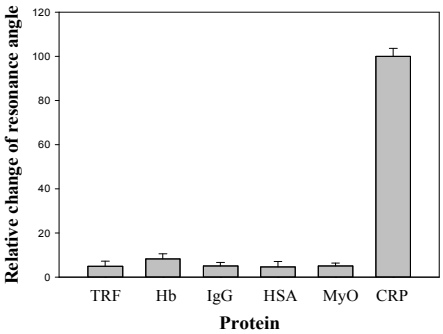


Fig. 5 The specificity investigation of the SPR biosensor. The change of resonance angle was obtained with 50 nM control protein and are shown relative with the CRP results, in the present of 200 nM anti-CRP coated AuNPs.

In conclusion, we present a highly sensitive and selective AuNPs enhanced SPR biosensor for CRP detection with an aptamer-antibody sandwich assay. The new aptamers exhibited good affinity and specificity toward CRP were selected by ourselves, and SPR analysis results showed that 6th-62-40-3' immobilized preferable to construct aptamer-based sensors for CRP detection. Then, we developed an AuNPs enhanced SPR biosensor with an aptamer-antibody sandwich, which obtained target concentrations ranging from 100 nM down to 10 pM. In addition, this nanoparticle enhanced SPR biosensor could also be applied to the specific detection of CRP in diluted human serum. The excellent result of our method for CRP detection in human serum demonstrated its potentials for practical applications in disease diagnostics.

This work was supported by National Natural Science Foundation of China (21190040, 21175035, 21375034), and International Science & Technology Cooperation Program of China (2010DFB30300).

Notes and references

1 X. Luo, Q. Xu, T. James and J. J. Davis, *Anal. Chem.*, 2014, **86**, 5553-5558.
2 M. B. Pepys and G. M. Hirschfield, *J. Clin. Invest.*, 2003, **111**, 1805-1812.
3 T. Hong, A. Liu, D. Cai, Y. Zhang, D. Hua, X. Hang and X. Wu, *Cancer Invest.*, 2013, **31**, 279-285.
4 P. M. Ridker, *Circulation*, 2003, **107**, 363-369.
5 G. Hergenroeder, J. B. Redell, A. N. Moore, W. P. Dubinsky, R. T. Funk, J. Crommett, G. L. Clifton, R. Levine, A. Valadka and P. K. Dash, *J. Neurotrauma*, 2008, **25**, 79-93.
6 W. Ansar and S. Ghosh, *Immunol. Res.*, 2013, **56**, 131-142.
7 R. Matsuura, K. Tawa and Y. Kitayama, *Chem. Commun.*, 2016, DOI: 10.1039/C5CC07868G.
8 S. K. Vashist, G. Czilwik, T. van Oordt, F. von Stetten, R. Zengerle, E. M. Schneider and J. H. Luong, *Anal. Biochem.,* 2014, **456**, 32-37.
9 A. Scholten, B. Menges, M. Juebner, M. A. Rothschild and K. Bender, *Analyst*, 2013, **138**, 1705-1712.
10 S. Centi, L. Bonel Sanmartin, S. Tombelli, I. Palchetti and M. Mascini, *Electroanalysis,* 2009, **21**, 1309-1315.
11 A. Qureshi, Y. Gurbuz, S. Kallempudi and J. H. Niazi, *Phys. Chem. Chem. Phys.*, 2010, **12**, 9176-9182.
12 E. D. Bernard, K. C. Nguyen, M. C. DeRosa, A. F. Tayabali and R. Aranda-Rodriguez, *Anal. Biochem.,* 2015, **472**, 67-74.
13 J. Pultar, U. Sauer, P. Domnanich and C. Preininger, *Biosens. Bioelectron.*, 2009, **24**, 1456-1461.
14 Y. N. Yang, H. I. Lin, J. H. Wang, S. C. Shiesh and G. B. Lee, *Biosens. Bioelectron.*, 2009, **24**, 3091-3096.
15 A. Bini, S. Centi, S. Tombelli, M. Minunni and M. Mascini, *Anal. Bioanal. Chem.,* 2008, **390**, 1077-1086.
16 S. A. Vance and M. G. Sandros, *Sci. Rep.*, 2014, **4**, 5129.
17 C. Eid, J. W. Palko, E. Katilius and J. G. Santiago, *Anal. Chem.*, 2015, **87**, 6736-6743.
18 C. Tuerk and L. Gold, *Science*, 1990, **249**, 505-510.
19 X. Yang, Y. Wang, K. Wang, Q. Wang, P. Wang, M. Lin, N. Chen and Y. Tan, *RSC Adv.*, 2014, **4**, 30934-30937.
20 Z. Zhu, Y. Song, C. Li, Y. Zou, L. Zhu, Y. An and C. J. Yang, *Anal. Chem.*, 2014, **86**, 5881-5888.
21 S. Eissa, A. Ng, M. Siaj, A. C. Tavares and M. Zourob, *Anal. Chem.*, 2013, **85**, 11794-11801.

View Article Online
DOI: 10.1039/C5CC10486F

22 S, Radom, P. Jurek, M. Mazurek, J. Otlewski and F. Jeleń, *Biotechnol. Adv.*, 2013, **31**, 1260-1274.
23 W. Tan, M. J. Donovan and J. Jiang, *Chem. Rev.,* 2013, **113**, 2842-2862.
24 L. Wang, C. Zhu, L. Han, L. Jin, M. Zhou and S. Dong, *Chem. Commun.*, 2011, **47**, 7794-7796.
25 X. Lin, L. Cui, Y. Huang, Y. Lin, Y. Xie, Z. Zhu, B. Yin, X. Chen and C. J. Yang, *Chem. Commun.*, 2014, **50**, 7646-7648.
26 Y. Wang, D. Xu and H. Y. Chen, *Lab Chip*, 2012, **12**, 3184-3189.
27 S. Zeng, D. Baillargeat, H. P. Ho and K. Yong, *Chem. Soc. Rev.*, 2014, **43**, 3426-3452.
28 H. H. Nguyen, J. Park, S. Kang and M. Kim, *Sensors*, 2015, **15**, 10481-10510.
29 M. J. Kwon, J. Lee, A. W. Wark and H. J. Lee, *Anal. Chem.*, 2012, **84**, 1702-1707.
30 Q. Wang, R. Liu, X. Yang, K. Wang, J. Zhu, L. He and Q. Li, *Sens. Actuators B.*, 2016, **223**, 613-620.
31 Q. Wang, Q. Li, X. Yang, K. Wang, S. Du, H. Zhang and Y. Nie, *Biosens. Bioelectron.,* 2016, **77**, 1001-1007.
32 X. Li, Y. Wang, L. Wang and Q. Wei, *Chem. Commun.*, 2014, **50**, 5049-5052.
33 E. Golub, G. Pelossof, R. Freeman, H. Zhang and I. Willner, *Anal. Chem.*, 2009, **81**, 9291-9298.
34 Y. Liu and Q. Cheng, *Anal. Chem*., 2012, **84**, 3179-3186.
35 S. Krishnan, V. Mani, D. Wasalathanthri, C. V. Kumar and J. F. Rusling, *Angew. Chem. Int. Ed*., 2011, **50**, 1175-1178.
36 J. Shen, Y. Li, H. Gu, F. Xia and X. Zuo, *Chem. Rev.*, 2014, **114**, 7631-7677.
37 S. Kim, J. Lee, S. J. Lee and H. J. Lee, *Talanta*, 2010, **81**, 1755-1759.
38 S. Kim and H. J. Lee, *Anal. Chem.*, 2015, **87**, 7235-7240.