A facile scanometric strategy for ultrasensitive detection of protein using aptamer-initiated rolling circle amplification†

Wei Cheng,[ab] Lin Ding,[a] Yunlong Chen,[a] Feng Yan,[c] Huangxian Ju*[ab] and Yibing Yin*[b]

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A pragmatic and simple strategy was developed for ultrasensitive detection of protein with good specificity and low matrix effect, which combined aptamer-initiated rolling circle amplification with a Au nanoparticle probe and convenient scanometric readout.

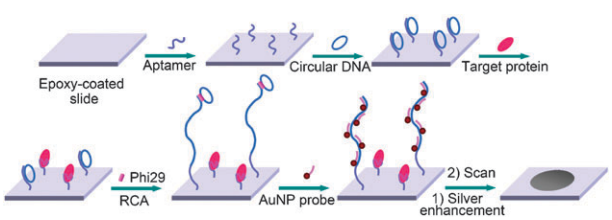
Many important protein biomarkers are present at ultra-low levels in body fluids or tissues, especially during the early stages of diseases.[1] Great effort has been made by integrating different amplification techniques or nanotechnologies with protein recognition to develop quantitative analytical methods for ultrasensitive detection of protein biomarkers due to their importance in early diagnosis of diseases, drug screening and monitoring of therapeutic treatments. However, a simple, pragmatic and specific method for this purpose is still an urgent demand.

Recently, aptamers have attracted much interest as new protein recognition elements due to their competitive advantages over antibodies.[2] Many aptamer-based technologies for highly sensitive protein detection have been developed by combining with polymerase chain reaction (PCR),[3a] electrochemistry,[3b] capillary electrophoresis,[3c] mass spectrometry,[3d] and quartz crystal microbalance.[3e] The majority of these technologies are performed in laboratories equipped with expensive instruments or staffed with highly qualified personnel. Although several label-free or colorimetric strategies based on aptamers have been developed for simple and rapid detection of proteins,[4] the limited sensitivity of these methods impedes their further application in different fields. Aiming at the detection need of ultra-low abundant proteins, this work designed a novel methodology, an aptamer-initiated rolling circle amplification (RCA) technique, for improving the sensitivity of colorimetric detection by combining a Au nanoparticle (AuNP) probe with simple scanometric readout. The scanometric readout could be conveniently performed with a computer-controlled scanner after a silver enhanced step on an aptamer chip.

RCA can replicate a circular oligonucleotide template with linear kinetics under isothermal conditions,[5] which has gained considerable attention as a novel tool for signal amplification.[1,6] Different from the reported aptamer-based RCA technique,[7] which needs a capture antibody or aptamer and an aptamer–primer complex to form a sandwich complex of protein analyte, the proposed aptamer-initiated RCA method used only one aptamer to serve as both the primer to form aptamer–circular DNA duplex for RCA and the recognition element to sense target protein. Such a technique avoided the further modification of aptamer with the primer and could use higher-affinity aptamer for the recognition of analyte because the former need two recognition elements with different binding sites to recognise the analyte. This is an advantage for improving the detection sensitivity. Moreover, the signal readout was also much simpler and low-cost.

The designed strategy is conceptually depicted in Scheme 1. Firstly, the amino-modified aptamer for human vascular endothelial growth factor 165 (VEGF)[8] was immobilized on an epoxy-coated glass slide. The resulting aptamer chip was then hybridized with a complementary circular DNA template to form a duplex structure (see ESI†). In presence of target protein, the bound circular DNA could be substituted by a target-induced strand release process due to the higher affinity of the selected aptamer to its protein than to the complementary DNA strand.[9] The remaining aptamer–circular DNA duplex, whose amount depended on the quantity of target protein, could initiate RCA catalyzed by phi29 DNA polymerase. The long DNA strand generated by the RCA step contained hundreds of tandem-repeat complementary sequences of the circular DNA and could be hybridized with complementary AuNP probes. After silver enhancement, the simple scanometric readout was finally performed with a scanner for detection of target protein.

3.5 nm AuNPs were used in this system,[10] which were protected with bis(*p*-sulfonatophenyl) phenylphosphine (BSPP) to minimize the salt effect on the particle stability.[11] The AuNPs were modified with a thiol-DNA probe to obtain a AuNP probe (see ESI†). The UV-vis absorption spectrum of the AuNP probe showed both the absorption peak of oligonucleotides at 260 nm and the surface plasmon absorption of AuNPs at 510 nm (Fig. S1 in ESI†). The transmission electron



Scheme 1 Schematic representation of the designed strategy for protein detection.

[a] *Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing 210093, P.R. China. E-mail: hxju@nju.edu.cn; Fax: 86 25 83593593; Tel: 86 25 83593593*
[b] *Laboratory of Laboratory Medical Diagnostics (Ministry of Education of China), Department of Key Laboratory Medicine, Chongqing Medical University, Chongqing 400016, P.R. China*
[c] *Jiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, P.R. China*

† Electronic supplementary information (ESI) available: Experimental details, absorption spectrum of AuNP probe, TEM image of AuNPs labeled on RCA products. See DOI: 10.1039/c002078h

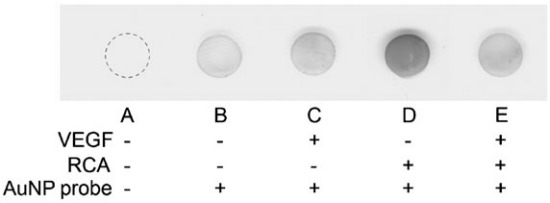


Fig. 1 Scanometric image of aptamer chip after being hybridized with circular DNA and treated with VEGF, RCA and/or AuNP probe, followed by identical silver enhancing step. The concentration of VEGF was 1 nM.

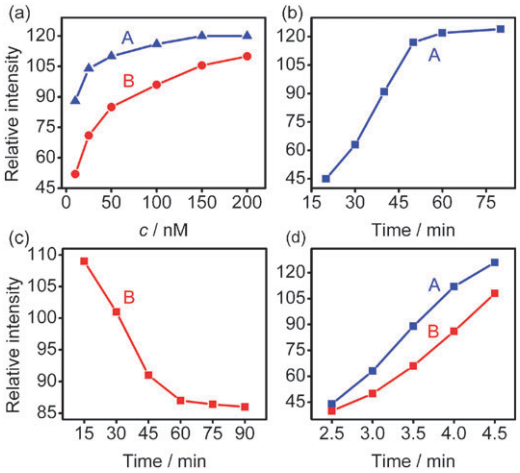


Fig. 2 Dependence of relative intensity of greyscale on aptamer concentration for aptamer chip preparation (a), incubation times with circular DNA (b) and VEGF (c), and reaction time of silver enhancement (d) at 0 (A) and 1 pM VEGF (B). When one parameter changes the others are under their optimal conditions except the aptamer concentration of 150 nM used in (b) for chip preparation.

microscopy (TEM) image showed that the diameter of the AuNPs was approximately 3.5 nm (Fig. S2 in ESI†). The uniform and small size of the AuNPs ensured the easy tagging of the AuNP probe on the linearly concatenated RCA product. As shown in the TEM image, a large number of AuNPs regularly aligned on the random coil of RCA products (Fig. S2 in ESI†), which confirmed the successful RCA initiated by the aptamer and tagging of AuNP probes on the RCA product.

The aptamer chip was prepared by dropping 5 μL of amido-modified aptamer solution on an epoxy-coated glass slide at a defined location and allowing reaction at room temperature for 3 h in a humidified chamber. A five-spot aptamer chip was used to examine the designed strategy (Fig. 1). After the chip was hybridized with circular DNA, it was treated with VEGF, RCA and/or AuNP probe, respectively. Compared with spot A, the treatment with the AuNP probe produced grey spots (spots B and C), which was attributed to little nonspecific adsorption of the AuNP probe on these spots. The grey spots demonstrated the catalyzed deposition of silver by AuNPs. The RCA-treated spot showed significantly deeper black than that without RCA (spots D and B), indicating that aptamer-initiated RCA generated a long DNA strand for tagging numerous AuNP probes for signal enhancement. After the bound circular DNA was treated with VEGF, the spot became much weaker than that without treatment (spots E and D), indicating the substitution of circular DNA by target protein. The effective displacement resulted from the high affinity of the surface-confined aptamer to VEGF,[9a] which has a dissociation constant of 1.9 nM in solution,[8] and its low affinity to complementary DNA due to the particular stem–loop structure of the aptamer[8,12a] and the low conformational flexibility of circular complementary DNA.

The greyscale values of the spots could be obtained with Adobe Photoshop software. The relative intensity herein was defined as the greyscale of the background subtracted from that of a given spot, which could eliminate the random variations of different scans and was related to the amount of target protein used for the substitution step, leading to a sensitive and simple method for quantitation of the target protein. The alternative mode in which the spots were incubated with VEGF and then circular DNA showed worse analytical performance.

To obtain high analytical performance, some important detection parameters were optimized (Fig. 2). With the increasing concentration of aptamer, both relative intensities of the greyscale at 0 and 1 pM VEGF increased, but their difference tended to diminution. Considering the detection limit and dynamic range, 50 nM was chosen as the optimal concentration. A long hybridization time of excess circular DNA could generate more aptamer–circular DNA duplex for RCA, thus the relative intensity increased with the increasing incubation time. At 60 min the hybridization reached saturation, which led to a constant intensity. Thus 60 min was used for the hybridization process. Similarly, the substitution reaction reached equilibrium at an incubation time of 75 min. For sufficient binding of target protein, 90 min was chosen as the optimal incubation time. The long silver enhancing time could produce high intensity at both 0 and 1 pM VEGF. However, the difference of their relative intensities reached a maximal value at 4 min. Thus the silver enhancement was performed with a time of 4 min.

Under the optimal conditions, the relative intensity of the greyscale was proportional to the logarithmic value of VEGF concentration over a 5-decade range from 1 nM to 10 fM with a linear correlation coefficient of 0.992 (Fig. 3). At high target concentrations the relative intensity showed greater standard deviations due to the low relative intensity and the effect of background. In presence of 10 fM VEGF, the change of the relative intensity was obvious due to the substitution of circular DNA by VEGF. Although the amount of substituted circular DNA was very small, the highly efficient RCA and following hybridization with AuNP probes and silver enhancement resulted in detectable difference from the blank sample (spots B and A in Fig. 3). The difference was statistically significant. The detectable VEGF concentration of 10 fM was remarkably lower than the detection limit of the label-free and colorimetric strategy based on aptamers,[4] also lower than those of 10 pM PDGF-BB for microbead-based RCA by using two recognition elements,[7b] and 0.76 pM thrombin for electrochemical analysis based on a dual-functional aptamer,[12b] and comparable with those of 20 fM thrombin for electrochemiluminescent aptasensor,[13] and 10 fM PDGF-BB for electrochemical aptamer–RCA immunosensor based on

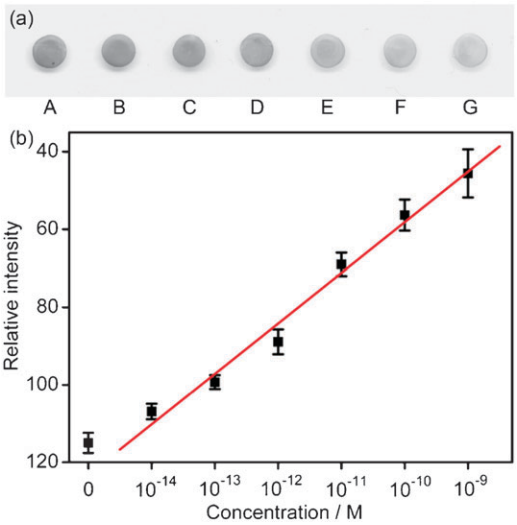


Fig. 3 (a) Scanometric image of the spots responding to 0, 10^{-14}, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10} and 10^{-9} M VEGF (A–G), and (b) quantitative dynamic range of the designed strategy. The error bars represent the standard deviations calculated from three different spots.

enzymatic silver deposition.[7a] The high sensitivity of the designed method was attributed to the following reasons: (i) high affinity of the aptamer to its target protein; (ii) excellent signal amplification capability of RCA and silver enhancement; (iii) obvious steric effect of the bound target protein on RCA; (iv) low random variation of the signal. More importantly, such a performance was obtained without the need for any sophisticated analytical instrument.

The specificity and matrix effect were further examined. The relative intensities responding to 1 μM IgG, 1 nM AFP and CEA were similar to that of blank Tris-HCl buffer (Fig. 4), demonstrating that these nonspecific proteins had no significant influence on aptamer-initiated rolling circle amplification and the proposed strategy was specific for the target protein. The relative intensities obtained after treatment with 10 pM VEGF in Tris-HCl buffer, 50% cell culture fluid, 50% human serum and 5% BSA did not show significant difference, indicating very little interference of complex matrices on the designed strategy. The high specificity and low matrix effect could further ensure the practicality of the proposed strategy.

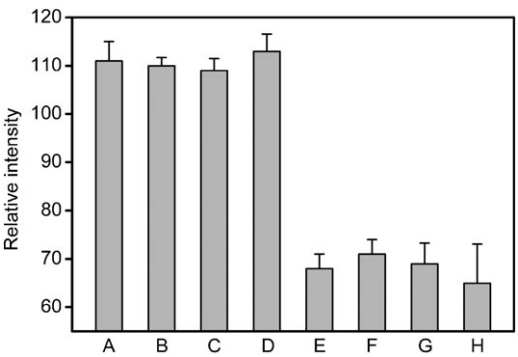


Fig. 4 Relative intensities responding to 1 μM IgG (A), 1 nM AFP (B), 1 nM CEA (C), and 0 (D) and 10 pM VEGF (E) in Tris-HCl buffer, and 10 pM VEGF in 50% cell culture fluid (F), 50% human serum (G) and 5% BSA (H).

In summary, a versatile strategy was developed to implement ultrasensitive protein detection by combining aptamer-initiated rolling circle amplification with a AuNP probe and simple scanometric readout. The designed strategy showed a broad dynamic range, ultra-high sensitivity, excellent specificity and a low matrix effect. By immobilizing multiplex aptamers on one slide, the strategy had the potential for multiple and high-throughput detection of proteins. By integrating multiple molecular biotechnologies, nanotechnology, bioconjugate chemistry and scanometric detection, this primary research opens new horizons for integrating different disciplines to develop pragmatic and simple technology with significant analytical performance. The proposed strategy would become a powerful tool for proteomics research and clinical diagnostics.

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Notes and references

1 H. Q. Zhang, Q. Zhao, X. F. Li and X. C. Le, *Analyst*, 2007, **132**, 724–737.
2 (*a*) A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818–822; (*b*) T. Hermann and D. J. Patel, *Science*, 2000, **287**, 820–825; (*c*) J. Liu, Z. Cao and Y. Lu, *Chem. Rev.*, 2009, **109**, 1948–1998.
3 (*a*) J. S. Lin and K. P. McNatty, *Clin. Chem.*, 2009, **55**, 1686–1693; (*b*) C. Deng, J. Chen, L. Nie, Z. Nie and S. Yao, *Anal. Chem.*, 2009, **81**, 9972–9978; (*c*) H. Zhang, Z. Wang, X. F. Li and X. C. Le, *Angew. Chem., Int. Ed.*, 2006, **45**, 1576–1580; (*d*) T. M. A. Gronewold, A. Baumgartner, J. Hierer, S. Sierra, M. Blind, F. Schäfer, J. Blümer, T. Tillmann, A. Kiwitz, R. Kaiser, M. Zabe-Kühn, E. Quandt and M. Famulok, *J. Proteome Res.*, 2009, **8**, 3568–3577; (*e*) C. Yao, Y. Qi, Y. Zhao, Y. Xiang, Q. Chen and W. Fu, *Biosens. Bioelectron.*, 2009, **24**, 2499–2503.
4 (*a*) F. Pu, Z. Huang, D. Hu, J. Ren, S. Wang and X. Qu, *Chem. Commun.*, 2009, 7357–7359; (*b*) W. Zhao, W. Chiuman, J. C. F. Lam, S. A. McManus, W. Chen, Y. Cui, R. Pelton, M. A. Brook and Y. Li, *J. Am. Chem. Soc.*, 2008, **130**, 3610–3618; (*c*) Y. Wang, D. Li, W. Ren, Z. Liu, S. Dong and E. Wang, *Chem. Commun.*, 2008, 2520–2522.
5 P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, *Nat. Genet.*, 1998, **19**, 225–232.
6 W. Zhao, M. M. Ali, M. A. Brook and Y. F. Li, *Angew. Chem., Int. Ed.*, 2008, **47**, 6330–6337.
7 (*a*) L. Zhou, L. Ou, X. Chu, G. Shen and R. Yu, *Anal. Chem.*, 2007, **79**, 7492–7500; (*b*) J. Lee, K. Icoz, A. Roberts, A. D. Ellington and C. A. Savran, *Anal. Chem.*, 2010, **82**, 197–202.
8 A. S. R. Potty, K. Kourentzi, H. Fang, G. W. Jackson, X. Zhang, G. B. Legge and R. C. Willson, *Biopolymers*, 2009, **91**, 145–156.
9 (*a*) X. Zuo, S. Song, J. Zhang, D. Pan, L. Wang and C. Fan, *J. Am. Chem. Soc.*, 2007, **129**, 1042–1043; (*b*) Y. Lu, X. Li, L. Zhang, P. Yu, L. Su and L. Mao, *Anal. Chem.*, 2008, **80**, 1883–1890; (*c*) J. Yoshizumi, S. Kumamoto, M. Nakamura and K. Yamana, *Analyst*, 2008, **133**, 323–325.
10 A. Gole and C. J. Murphy, *Chem. Mater.*, 2004, **16**, 3633–3640.
11 Z. X. Deng, Y. Tian, S. H. Lee, A. E. Ribbe and C. D. Mao, *Angew. Chem., Int. Ed.*, 2005, **44**, 3582–3585.
12 (*a*) H. J. Lee, A. W. Wark and R. M. Corn, *Analyst*, 2008, **133**, 975–983; (*b*) X. Li, J. Liu and S. Zhang, *Chem. Commun.*, 2010, **46**, 595–597.
13 X. Yin, Y. Xin and Y. Zhao, *Anal. Chem.*, 2009, **81**, 9299–9305.

Electronic Supplementary Information (ESI) for Chemical Communications

A facile scanometric strategy for ultrasensitive detection of protein using aptamer-initiated rolling circle amplification

Wei Cheng, Lin Ding, Yunlong Chen, Feng Yan, Huangxian Ju* and Yibing Yin*

Key Laboratory of Analytical Chemistry for Life Science (Education Ministry of China), Department of Chemistry, Nanjing University, Nanjing 210093; Key Laboratory of Laboratory Medical Diagnostics (Ministry of Education of China), Department of Laboratory Medicine, Chongqing Medical University, Chongqing 400016; Jiangsu Institute of Cancer Prevention and Cure, Nanjing 210009, P. R. China

Experimental details

Materials. Recombinant human vascular endothelial growth factor 165 (VEGF) was purchased from Bio Basic Inc. (Canada). Phi29 DNA polymerase, T4 DNA ligase, Exonuclease III, *E.coli* (ExoIII), Exonuclease I, *E.coli* (ExoI) and dNTP mixture were obtained from Fermentas (Lithuania). Salmon sperm DNA, bovine serum albumin (BSA), 3-glycidoxypropyltrimethoxysilane (GPTMS), bis(p-sulfonatophenyl) phenylphosphine dihydrate dipotassium salt (BSPP), and silver enhancer solutions A and B were purchased from Sigma-Aldrich (USA). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and trisodium citrate were obtained from Shanghai Reagent Company (Shanghai, China). Sodium borohydride ($NaBH_4$) was obtained from Sinopharm Chemical Reagent Co. Ltd (China). All other reagents were of analytical grade. All aqueous solutions were prepared using ultra-pure water (≥ 18 MΩ, Milli-Q, Millipore).

The oligonucleotides with the following sequences were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China):

Aptamer for VEGF: 5'-NH_2-$(CH_2)_6$-AAA AAA AAA CCG TCT TCC AGA CAA GAG TGC AGG G-3'

Circular template: 5'-p-TCT GGA AGA CGG ACA ACA TGA AGA TTG TAG GTC AGA ACT CAC CTG TTA GAA ACT GTG AAG ATC GCT TCC CTG CAC TCT TG-3'

thiolated DNA probe: 5'-ACT GTG AAG ATC GCT -$(CH_2)_3$-thiol-3'

Apparatus. The UV-vis absorption spectrum was recorded with an UV-3600 UV-vis-NIR spectrophotometer (Shimadzu, Japan). The TEM image was observed under a JEM-2100 transmission electron microscope (JEOL Ltd, Japan). Scanometric images were obtained with a flatbed scanner (HP scanjet 2400, Hewlett-Packard).

Preparation of Au nanoparticle probe. 3.5 nm Au nanoparticles (AuNPs) were synthesized according to the method reported previously.[1] Typically, 0.6 mL of ice-cold 0.1 M $NaBH_4$ was added into 20 mL aqueous solution containing 0.25 mM $HAuCl_4$ and 0.25 mM trisodium citrate with stirring. The orange-red solution was stirred for 1 h to obtain AuNPs solution, which was stored at 4 °C.

1 mg BSPP was added to the AuNPs solution (4 mL), and the mixture was stirred overnight. The AuNPs were then subjected to ultrafiltration using Vivaspin concentrator (Sartorius, 10,000 MW) at 10,000 g for 10 min to remove excessive BSPP. The upper phase was washed twice with pH 7.4 PBS, and dissolved in 500 μL PBS to obtain a solution of BSPP-protected AuNPs. 100 μL of 10 μM thiolated DNA probe was then added to the solution and left overnight under shaking. The resulting mixture was ultrafiltrated using Vivaspin concentrator (10,000 MW) at 10,000 g for 10 min at 4 °C to remove the non-conjugated DNA. The upper phase was washed thrice with PBS by ultrafiltration. The obtained AuNP probe was dissolved in 500 μL PBS and kept at 4 °C. Prior to use, the AuNP probe was blocked with 0.5% BSA, 50 μg mL^{-1} salmon sperm DNA and 0.025% Tween 20 at 4 °C for 1 h.

Preparation of circular DNA. 10 μM of circular template oligonucleotide and 10 μM of aptamer oligonucleotide were mixed in 100 μL of ligation buffer (50 mM pH 7.5 Tris-HCl buffer, 10 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM ATP), and incubated at 37 °C for 30 min. Then 160 units of T4 DNA ligase was added and incubated at 22 °C for 1 h. After ligation, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. Then the exonucleases, 400 U ExoI and 2000 U ExoIII, were

applied to digest the residue linear DNA at 37 °C for 50 min.[2] The circular DNA was extracted with phenol/chloroform (1:1) and chloroform, respectively, and the product was precipitated with ethanol, which was finally dissolved in 100 μL double distilled H_2O and stored at -20 °C.[3]

Preparation of aptamer chip. A glass slide (25 mm × 75 mm × 1 mm) was firstly treated with piranha solution (30% hydrogen peroxide and 70% sulfuric acid) for 24 h, and silylanized by dipping it in toluene solution of 1% GPTMS for 24 h at room temperature to obtain epoxy-coated slide.[4]

5 μL of 50 nM aptamer solution was dropped on the epoxy-coated slide at a defined location, respectively, to form a droplet array of aptamer, which was then incubated at room temperature for 3 h in a humidified chamber. After the slide was thrice washed with ultrapure water and the remaining active epoxy groups were reduced with sodium borohydride (1 mg in 4 mL 25% ethanol), the resulting aptamer chip was twice rinsed with ultrapure water and used for following operation.

Protein detection protocol. Prior to use, the aptamer chip was washed in boiling water for 30 s. 5 μL of 100 nM circular DNA in pH 7.4 PBS containing 1% BSA and 100 μg mL^{-1} salmon sperm DNA was carefully added onto each aptamer spot and incubated at 37 °C for 1 h in a humidified chamber. The resulting slide was washed thrice with washing buffer I (50 mM pH 7.5 Tris-HCl buffer and 0.05% Tween 20), and twice rinsed with washing buffer II (50 mM pH 7.5 Tris-HCl buffer). Then 5 μL of VEGF solutions at different concentrations were added on the defined spots and incubated for 1.5 h at 37 °C in a humidified chamber to displace the circular DNA. After the slide was washed thrice with washing buffer I, and twice rinsed with washing buffer II, rolling circle amplification was initiated by dropping 5 μL reaction buffer containing 0.5 units of phi29 DNA polymerase, 50 mM pH 7.5 Tris-HCl buffer, 10 mM magnesium acetate, 33 mM potassium acetate, 1 mM dithiothreitol and 10 mM dNTP on each defined spot, and continued for 1 h at 37 °C in a humidified chamber. After the washing steps, 5 μL of AuNP probe was added to each spot and hybridized at 37 °C for 30 min in a humidified chamber. The slide was carefully washed thrice with washing buffer I, twice rinsed with washing buffer II to remove the non-tagged AuNP probe, and dried under a stream of nitrogen. Finally, the sliver enhancement was preformed on each spot by reaction with 5 μL 1:1 mixture of silver enhancer solutions A and B for 4 min.

After rinsed with ultrapure water and dried under a stream of nitrogen, the resulting slide was scanned with a flatbed scanner.

Absorption spectrum of AuNP probe

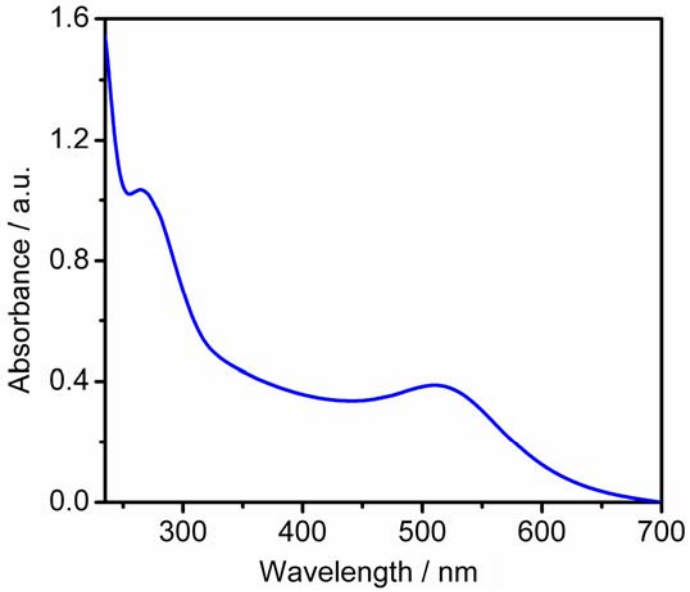

Fig. S1. *UV-vis absorption spectrum of AuNP probes*

TEM image of AuNPs labeled on RCA products

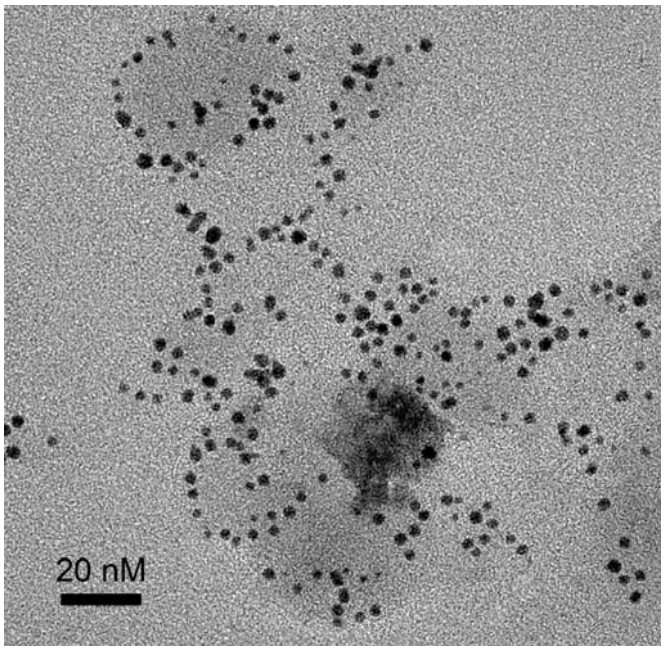

Fig. S2*. TEM image of AuNPs labeled on RCA products.*

References

1. A. Gole and C. J. Murphy, *Chem. Mater*., 2004, **16**, 3633-3640.

2. N. Li, C. Jablonowski, H. Jin and W. Zhong, *Anal. Chem.*, 2009, **81**, 4906-4913.

3. Y. Y. Huang, H. Y. Hsu and C. C. Huang, *Biosens. Bioelectron*., 2007, **22**, 980-985.

4. Z. J. Yang, H. Liu, C. Zong, F. Yan and H. X. Ju, *Anal. Chem.*, 2009, **81**, 5484-5489.