Binding-Induced Fluorescence Turn-On Assay Using Aptamer-Functionalized Silver Nanocluster DNA Probes

ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2012

Impact Factor: 5.64 · DOI: 10.1021/ac3006268 · Source: PubMed

5 AUTHORS, INCLUDING:

Hongquan Zhang
University of Alberta
35 PUBLICATIONS **1,192** CITATIONS

SEE PROFILE

X. Chris Le
University of Alberta
248 PUBLICATIONS **7,757** CITATIONS

SEE PROFILE

analytical chemistry

Letter

pubs.acs.org/ac

Binding-Induced Fluorescence Turn-On Assay Using Aptamer-Functionalized Silver Nanocluster DNA Probes

Jingjing Li,[†,‡] Xiaoqin Zhong,[†] Hongquan Zhang,[‡] X. Chris Le,*[,‡,§] and Jun-Jie Zhu*[,†]

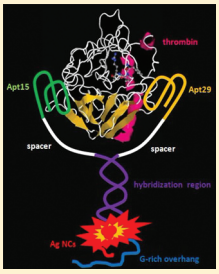
[†]State Key Lab of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, People's Republic of China

[‡]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada, T6G 2G3

[§]Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada

Supporting Information

ABSTRACT: We present here a binding-induced fluorescence turn-on assay for protein detection. Key features of this assay include affinity binding-induced DNA hybridization and fluorescence enhancement of silver nanoclusters (Ag NCs) using guanine-rich DNA sequences. In an example of an assay for human α-thrombin, two aptamers (Apt15 and Apt29) were used and were modified by including additional sequence elements. A 12-nucleotide (nt) sequence was used to link the first aptamer with a nanocluster nucleation sequence at the 5′-end. The second aptamer was linked through a complementary sequence (12-nt) to a G-rich overhang at the 3′-end. Binding of the two aptamer probes to the target protein initiates hybridization between the complementary linker sequences attached to each aptamer and thereby bring the end of the G-rich overhang to close proximity to Ag NCs, resulting in a significant fluorescence enhancement. With this approach, a detection limit of 1 nM and a linear dynamic range of 5 nM−2 μM were achieved for human α-thrombin. This fluorescence assay is performed in a single tube, and it does not require washing or separation steps. The principle of the binding-induced DNA hybridization and fluorescence enhancement of Ag NCs can be extended to other homogeneous assay applications provided that two appropriate probes are available to bind with the same target molecule.



Molecular clusters of pure metals, such as gold and silver, are a new class of fluorophores and have great potential for applications in biomedicine.[1] Silver nanoclusters own an appealing set of features that complements the properties of organic dyes and quantum dots.[2−5] They have desirable photophysical properties and low toxicity suitable for biological applications.[6,7] In recent years, oligonucleotide-templated silver nanoclusters have attracted special attention due to their facile synthesis, tunable fluorescence emission, and high photostability.[8,9] They have been used to detect thiol compounds,[10] metal ions,[11−13] protein,[14] and a single-nucleotide mutation.[15] However, multiple schemes were proposed based on the quenching mode, which is not preferred in practice because there are a variety of ligands or solvents that may interfere with quenching, leading to "false positive".[16] Werner et al. reported an interesting phenomenon that the red fluorescence of DNA/Ag NCs could be enhanced by 500-fold when nanoclusters were in proximity to guanine-rich DNA sequences. On the basis of this, they successfully developed a "turn-on" nanocluster beacon for DNA detection.[17] More "turn on" homogeneous assays would be desirable for a broader class of targets. However, the difficult bioconjugation limited wider application of Ag NCs.[18,19] Although DNA-Ag NCs have been applied to transfection of HeLa cells,[20] live cell surface labeling,[21] and intracellular staining,[22] methods to functionalize these nanoclusters with affinity ligands for cell labeling and imaging are still necessary.

Aptamers are functional nucleic acid or peptide molecules with high specificity and affinity which are comparable to antibodies. Their targets include small molecules, proteins, and even cells.[23−26] Aptamer can be easily incorporated into a DNA-based system for various targets detection.[27] Since their discovery in the 1990s, many aptamer assays employing a DNA scaffold-aptamer assembly have been documented.[28−32] Most of these assemblies require time-consuming labeling and conjugation steps. An alternative approach is to extend the aptamer sequences, thereby providing the additional sequence to stabilize fluorescent Ag NCs while retaining the aptamer's function. Recently aptamer-templated fluorescence Ag NCs have been synthesized without compromising the aptamer's specificity and affinity to its target.[14,33,34]

We described here a label-free, binding-induced fluorescence turn-on assay for protein detection, combining affinity recognition, binding-induced DNA hybridization, and the fluorescence enhancement of Ag NCs by guanine-rich DNA sequences.[17,35,36] As a proof of principle, human α-thrombin and its two aptamers, Apt29 and Apt15,[37] were used in this study. Human α-thrombin is a coagulation protein in the bloodstream, which converts soluble fibrinogen into insoluble strands of fibrin as well as catalyzing many other coagulation-related reactions. The 15-mer Apt15 binds to the fibrinogen-

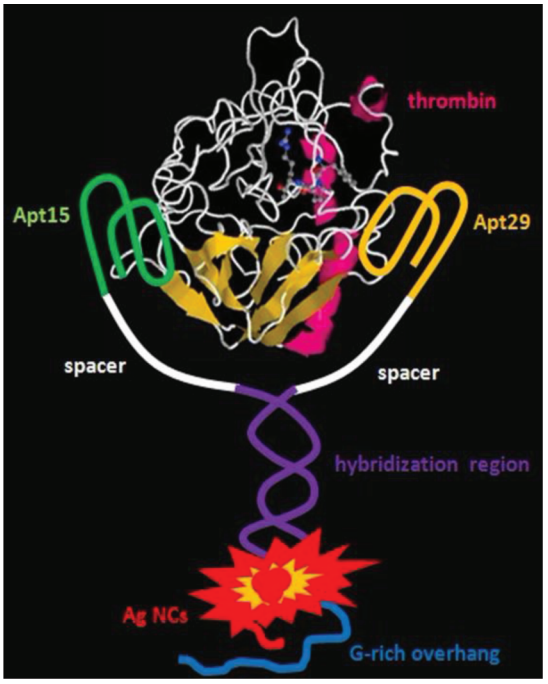
Received: March 3, 2012
Accepted: May 15, 2012
Published: May 15, 2012

   dx.doi.org/10.1021/ac3006268 | *Anal. Chem.* 2012, 84, 5170−5174

binding site ($K_d \sim 100$ nM) and the 29-mer Apt29 binds to the heparin-binding site ($K_d \sim 0.5$ nM) of human α-thrombin.[38−40] These two binding sites are spatially separated and localized at opposite sides of the thrombin molecule.[41]

In this assay, Apt15 was modified by adding a stem sequence (6−30 nt) and an Ag NC nucleation sequence (12 nt) at the 5′-end, and Apt29 was modified by adding a complementary stem sequence (6−30 nt) and a G-rich overhang (18 nt) at the 3′-end (Scheme 1 and Table S1 in the Supporting Information). Binding of the two aptamers to the same target molecule promotes hybridization between the complementary stem sequences attached to the two aptamers. The hybridization brings the G-rich overhang to be close to Ag NCs, enhancing the fluorescence of Ag NCs. These complementary stem sequences are designed not to form a stable hybridization when there is no target binding.

Scheme 1. Schematic Diagram Showing the Principle of Binding-Induced Fluorescence Turn-On Assay for Protein Detection



The configuration of the present sensing system involves the following elements: (a) The Apt15-NC probe is composed of Apt15 (green), poly T spacer (white), stem sequence for hybridization (purple), and Ag NC nucleation sequence (red). (b) The Apt29-G rich probe comprises of Apt 29 (yellow), poly T spacer (white), complementary stem sequence (purple), and G-rich overhang (blue). After Apt15 and Apt29 bind to human α-thrombin, the effective T_m of the stem sequences increases which promotes duplex formation. Such hybridization brings the G-rich overhang to get close proximity to Ag NCs. The interaction of G-rich sequence with Ag NCs greatly enhances the fluorescence signal of Ag NCs. As a result, this assay is capable of effectively detecting target protein.

The design principle is guided by T_m differences between the hairpin loop structure (ternary complex) and the free hybrids of the two strands. It is necessary to minimize the hybridization between the two complementary sequences in the absence of the target. Key to the success of our design is the binding-induced hybridization, i.e., the hybridization is triggered by the binding of the two probes to the target molecule. For example, when a pair of nonoptimized probes with a 30-nt complementary sequence, Apt15-NC Probe-30 and Apt29-G rich Probe-30 (Table S1 in the Supporting Information), was tested initially, only weak green fluorescence was observed with 465 nm excitation (Figure S1 in the Supporting Information). There was no red fluorescence emission from Ag NCs stabilized by Apt15-NC Probe-30 when excited at 570 nm. These results are consistent with a previous report.[31] When the two probes were added into the binding buffer with a final volume of 500 μL and incubated at 37 °C for 30 min, a strong red fluorescence emission was observed even in the absence of the target molecule. In this case, the chimeric conjugation with aptamers did not interfere with the fluorescence enhancement of Ag NCs by the G-rich overhang. However, the target-independent hybridization between the two long complementary sequences (30 nt) leads to the problem of high background.

To make the assay useful for target detection, the length of hybridization elements needs to be optimized to obtain the best signal to background ratio (Table S1 in the Supporting Information), i.e., higher fluorescence enhancement in the presence of the target and lower fluorescence intensity in the absence of the target.

We used the melting temperature (T_m) (calculated using OligoAnalyzer 3.1 software from IDT) to compare the design of the stem sequence, as previously reported.[35] Generally, the sensitivity of the assay would be higher with a larger difference between the T_m of the binding-induced hybrids and the free hybrids. As shown in Figure 1, the 12-nt hybridization sequence produced the best signal-to-background (F/F_0).

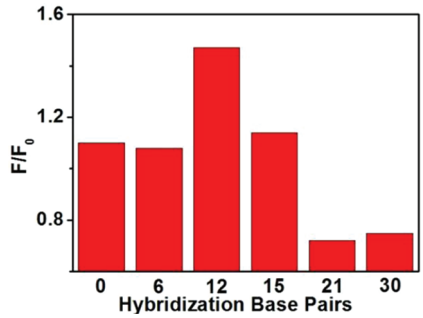


Figure 1. Effect of the length of stem sequences on the signal-to-background ratio for detecting human α-thrombin. The concentration of human α-thrombin was 50 nM. Excitation wavelength: 581 nm.

We also performed polyacrylamide gel electrophoresis to determine whether such design would alter the binding affinity of aptamers (Figure S2 in the Supporting Information). The Apt29-thrombin complex could be observed in the gel (lane 7), while the Apt15-thrombin complex band was difficult to identify (lanes 3 and 5). This might be due to the lower affinity of Apt15. However, the band of Apt15 after incubation with thrombin was weaker with (lane 3) or without Ag NCs formation (lane 5) compared with Apt15 only (lanes 2 and 4). This indirectly confirmed the formation of a complex of Apt15 and thrombin. In addition, Apt15-thrombin-Apt29 complex (lane 8) showed slower migration than the Apt29-thrombin complex (lane 7). Binding of aptamer to human α-thrombin was not affected by the presence of Ag NCs (lane 10).

dx.doi.org/10.1021/ac3006268 | Anal. Chem. 2012, 84, 5170−5174

Therefore, aptamers with an additional sequence retained the affinity for human α-thrombin even in the presence of Ag NCs.

After confirming the probe sequences, we further optimized the concentration and proportion of these two probes (Figures 2 and 3). The best signal to background ratio was obtained

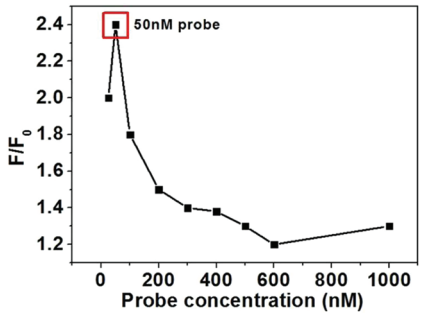


Figure 2. Optimization of probe concentration. The concentration of human α-thrombin was 50 nM.

using 50 nM of both probes (Figure 2). Then we fixed the Apt15-NC Probe-12 stabilized Ag NCs concentration at 50 nM and varied the concentration of Apt29-G rich Probe-12 to reach a ratio from 0.1 to 10. Probe ratio of 5:1 (Apt15/Apt29) showed the highest signal-to-background (F/F_0) (Figure 3). The need for higher concentration of Apt15 than Apt29 is probably due to the different K_d of Apt15 (~100 nM) and Apt29 (~0.5 nM) to human α-thrombin.

Next, the concentrations of K^+, Mg^{2+}, Na^+, and Ca^{2+} in the binding buffer were also optimized (details are shown in Figure S3 in the Supporting Information). The optimized conditions for the following analysis were 50 nM Apt15-NC Probe-12 stabilized Ag NCs and 10 nM Apt29-G rich Probe-12 incubated at 37 °C for 30 min with thrombin in a binding buffer containing 100 mM NaCl, 20 mM Tris-HCl, 1 mM $MgCl_2$, 5 mM KCl, 5 mM $CaCl_2$, and 0.02% Tween 20, pH 7.4. With the optimized parameters, a dynamic range from 5 nM to 4 μM, covering 3 orders of magnitude, was achieved. The red fluorescence intensity of DNA stabilized Ag NCs increased linearly with the concentration of human α-thrombin (Figure 4). The detection limit was 1 nM based on a signal to background ratio of 3.

The specificity of this assay was also investigated by examining whether other common proteins would interfere with the assay for human α-thrombin. Four proteins, trypsin, hemoglobin, cytochrome c, and lysozyme, were chosen to be tested under the same experimental conditions as those for human α-thrombin (Figure 5). The fluorescence intensities remained low for these proteins even though their concentrations were 10 times higher than human α-thrombin. These results suggest good selectivity of the assay for human α-thrombin. The good selectivity probably came from the specific and high affinity binding of the probes to the target and the subsequent hybridization induced by the binding events. At a low probe concentration, only a small amount of nonspecific proteins would be expected to have bound to one proximity probe, and even a lower chance for them to bind with both probes to produce background (false positive) signal.[42]

We finally challenged the assay by analyzing samples of complicated matrix to evaluate the applicability of the assay to real samples. Three concentrations of human α-thrombin (50, 100, and 250 nM) were spiked into 20-fold diluted human serum (obtained from the local hospital). The recovery values were determined and were acceptable (Table 1). Therefore, our proposed binding-induced fluorescence turn-on assay could potentially be applied to realistic biological samples, such as diluted human serum.

In summary, we have developed a binding-induced fluorescence turn-on assay for protein detection based on the binding-induced DNA hybridization and the fluorescence enhancement of DNA-stabilized Ag NCs by guanine-rich DNA sequences. DNA scaffold for fluorescence Ag NCs synthesis was successfully coupled with aptamers. The design did not affect the synthesis of fluorescence Ag NCs or the binding affinity of aptamers. This assay would broaden the application of fluorescence Ag NCs in specific target detection. Such incorporation of target recognition and signal transduction could potentially overcome the problem of difficult bioconjugation for Ag NCs.

This method requires the binding of two affinity probes to the target protein. Although two binding events could enhance specificity, the requirement for two affinity probes limits the targets that can be detected using this method. The present work demonstrated the use of two aptamers as affinity probes to bind to thrombin. However, the number of targets for which multiple, distinct aptamers have been generated to date is small.[43] As an alternative, a single aptamer, such as an aptamer for ATP and an aptamer for cocaine, was split into two fragments and used in a "sandwich assay" format.[44,45] Additionally, several techniques have been reported to obtain pairs of aptamers recognizing nonoverlapping epitopes of the protein, for example, by a two-step sequential SELEX technique.[46,47] Other affinity probes, such as antibodies, could be linked with appropriate oligonucleotide sequences, and these modified probes could be adapted in our reported assay format.

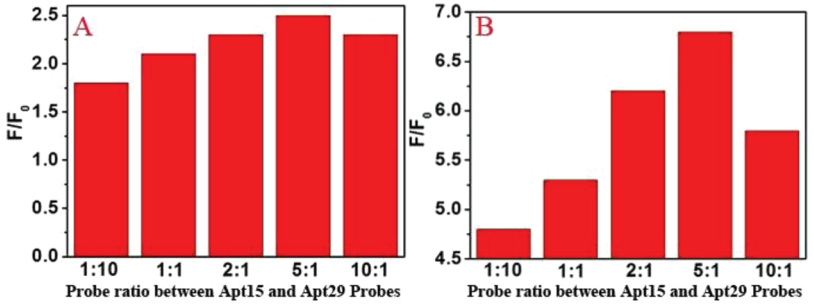


Figure 3. Optimization of the ratio between the Apt15-NC Probe-12 and Apt29-G rich Probe-12. The Apt15-NC Probe-12 concentration was kept at 50 nM and the Apt29-G rich Probe-12 concentration varied. The concentrations of human α-thrombin were 50 (A) and 250 nM (B).

dx.doi.org/10.1021/ac3006268 | Anal. Chem. 2012, 84, 5170−5174

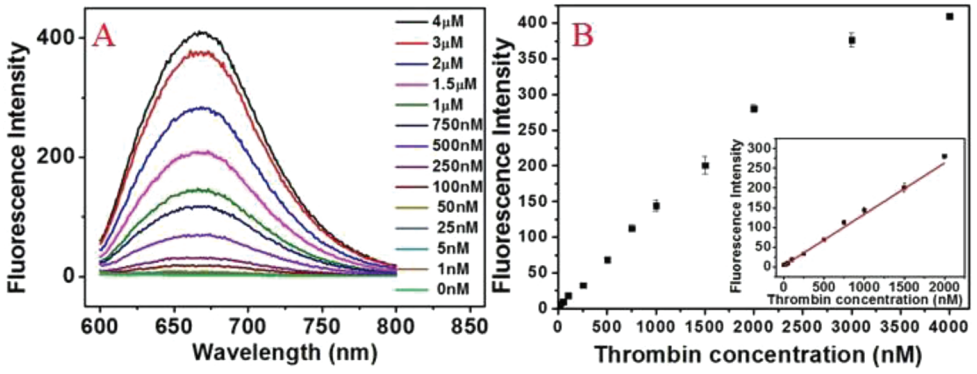


Figure 4. (A) Typical fluorescence spectra from the assay for human α-thrombin (0–4 μM). (B) Intensity of fluorescence (emission at 669 nm) increases with the concentration of human α-thrombin. Inset shows the linear range from 5 nM to 2 μM.

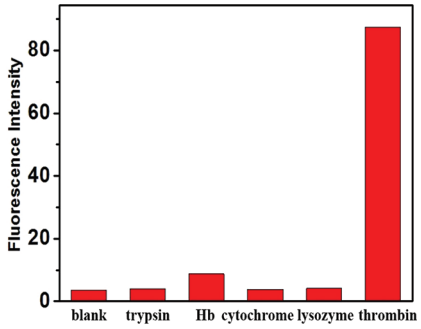


Figure 5. Specificity test, comparing signals from the target human α-thrombin and other nontarget proteins (trypsin, hemoglobin, cytochrome c, and lysozyme). The experimental conditions were the same as those for human α-thrombin. Concentration for human α-thrombin was 0.5 μM and for the other proteins were 5 μM.

Table 1. Recovery of Human α-Thrombin in 5% Human Serum

samples	thrombin added (nM)	found (nM)	recovery (%)
1	50	46.2	92.4 ± 3.0
2	100	95.1	95.1 ± 1.25
3	250	259.9	104 ± 2.15

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jjzhu@nju.edu.cn (J.-J.Z.); xc.le@ualberta.ca (X.C.L.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was financially supported by the National Basic Research Program of China (Grant 2011CB933502), National Natural Science Foundation of China (Grants 50972058 and 21121091), Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs Program, Alberta Health and Wellness, and the China Scholarship Council.

■ REFERENCES

(1) Wang, H. H.; Lin, C. A. J.; Lee, C. H.; Lin, Y. C.; Tseng, Y. M.; Hsieh, C. L.; Chen, C. H.; Tsai, C. H.; Hsieh, C. T.; Shen, J. L.; Chan, W. H.; Chang, W. H.; Yeh, H. I. *ACS Nano* **2011**, *5* (6), 4337–4344.
(2) Isabel, D.; Robin, H. A. R. *Nanoscale* **2011**, *3*, 1963–1970.
(3) Kuno, M.; Fromm, D. P.; Hamann, H. F.; Gallagher, A.; Nesbitt, D. J. *J. Chem. Phys.* **2000**, *112*, 3117–3120.
(4) Derfus, A. M.; Chan, W. C. W.; Bhatia, S. N. *Nano Lett.* **2004**, *4*, 11–18.
(5) Lee, S. F.; Osborne, M. A. *J. Am. Chem. Soc.* **2007**, *129*, 8936–8937.
(6) Vosch, T.; Antoku, Y.; Hsiang, J. C.; Richards, C. I.; Gonzalez, J. I.; Dickson, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12616–12621.
(7) de Souza, N. *Nat. Methods* **2007**, *4*, 540–540.
(8) Richards, C. I.; Choi, S.; Hsiang, J. C.; Antoku, Y. *J. Am. Chem. Soc.* **2008**, *130*, 5038–5039.
(9) Sharma, J.; Yeh, H. C.; Yoo, H.; Werner, J. H.; Martinez, J. S. *Chem. Commun.* **2010**, *46*, 3280–3282.
(10) Huang, Z. Z.; Pu, F.; Lin, Y. H.; Ren, J. S.; Qu, X. G. *Chem. Commun.* **2011**, *47*, 3487–3489.
(11) Guo, W. W.; Yuan, J. P.; Wang, E. K. *Chem. Commun.* **2009**, *23*, 3395–3397.
(12) Su, Y. T.; Lan, G. Y.; Chen, W. Y.; Chang, H. T. *Anal. Chem.* **2010**, *82*, 8566–8572.
(13) Lan, G. Y.; Huang, C. C.; Chang, H. T. *Chem. Commun.* **2010**, *46*, 1257–1259.
(14) Sharma, J.; Yeh, H. C.; Yoo, H.; Werner, J. H.; Martinez, J. S. *Chem. Commun.* **2011**, *47*, 2294–2296.
(15) Guo, W. W.; Yuan, J. P.; Dong, Q. Z.; Wang, E. K. *J. Am. Chem. Soc.* **2010**, *132*, 932–934.
(16) Cho, E. J.; Lee, J. W.; Ellington, A. D. *Annu. Rev. Anal. Chem.* **2009**, *2*, 241–264.
(17) Yeh, H. C.; Sharma, J.; Han, J. J.; Martinez, J. S.; Werner, J. H. *Nano Lett.* **2010**, *10*, 3106–3110.
(18) Petty, J. T.; Zheng, J.; Hud, N. V.; Dickson, R. M. *J. Am. Chem. Soc.* **2004**, *126*, 5207–5212.
(19) Zhou, Z. X.; Du, Y.; Dong, S. J. *Anal. Chem.* **2011**, *83*, 5122–5127.
(20) Antoku, Y.; Hotta, J. I.; Mizuno, H.; Dickson, R. M.; Hofkens, J.; Vosch. *J. Photochem. Photobiol. Sci.* **2010**, *9*, 716–721.
(21) Yu, J. H.; Choi, S.; Richards, C. I.; Antoku, Y.; Dickson, R. M. *Photochem. Photobiol. Sci.* **2008**, *84* (6), 1435–1439.
(22) Choi, S.; Yu, J. H.; Patel, S. A.; Tzeng, Y. L.; Dickson, R. M. *Photochem. Photobiol. Sci.* **2011**, *10*, 109–115.
(23) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818–822.
(24) Ellington, A. D.; Szostak, J. W. *Nature* **1992**, *355*, 850–852.
(25) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505–510.
(26) Hamula, C. L. A.; Zhang, H. Q.; Guan, L. L.; Li, X. F.; Le, X. C. *Anal. Chem.* **2008**, *80*, 7812–7819.

(27) Shlyahovsky, B.; Li, D.; Weizmann, Y.; Nowarski, R.; Kotler, M.; Willner, I. *J. Am. Chem. Soc.* **2007**, *129*, 3814−3815.
(28) Lai, R. Y.; Plaxco, K. W.; Heeger, A. J. *Anal. Chem.* **2007**, *79*, 229−233.
(29) Lin, C.; Katilius, E.; Liu, Y.; Zhang, J.; Yan, H. *Angew. Chem., Int. Ed.* **2006**, *118*, 5422−5427.
(30) Chhabra, R.; Sharma, J.; Ke, Y.; Liu, Y.; Rinker, S.; Lindsay, S.; Yan, H. *J. Am. Chem. Soc.* **2007**, *129*, 10304−10305.
(31) Zhang, Y. L.; Huang, Y.; Jiang, J. H.; Shen, G. L.; Yu, R. Q. *J. Am. Chem. Soc.* **2007**, *129*, 15448−15449.
(32) Liu, J. W.; Lee, J. H.; Lu, Y. *Anal. Chem.* **2007**, *79*, 4120−4125.
(33) Sun, Z. P.; Wang, Y. L.; Wei, Y. T.; Liu, R.; Zhu, H. R.; Cui, Y. Y.; Zhao, Y. L.; Gao, X. Y. *Chem. Commun.* **2011**, *47*, 11960−11962.
(34) Yin, J. J.; He, X. X.; Wang, K. M.; Qing, Z. H.; Wu, X.; Shi, H.; Yang, X. H. *Nanoscale* **2012**, *4*, 110−112.
(35) Zhang, H. Q.; Li, X.−F.; Le, X. C. *Anal. Chem.* **2012**, *84*, 877−884.
(36) Le, X. C.; Zhang, H. Q.; Li, X.−F. U.S. Patent Application 61/238,368. PCT WO 2011/022820 A1, August 31, 2009.
(37) Holland, C. A.; Henry, A. T.; Whinna, H. C.; Church, F. C. *FEBS Lett.* **2000**, *484*, 87−91.
(38) Tasset, D. M.; Kubik, M. F.; Steiner, W. J. *Mol. Biol.* **1997**, *272*, 688−698.
(39) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564−566.
(40) Zhao, Q.; Li, X. F.; Shao, Y. H.; Le, X. C. *Anal. Chem.* **2008**, *80*, 7586−7593.
(41) Poniková, S.; Tlučková, K.; Antalík, M.; Víglaský, V.; Hianik, T. *Biophys. Chem.* **2011**, *155*, 29−35.
(42) Fredriksson, S.; Gullberg, M.; Jarvius, J.; Olsson, C.; Pietras, K.; Gústafsdóttir, S. M.; Östman, A.; Landegren, U. *Nat. Biotechnol.* **2002**, *20*, 473−477.
(43) Zuo, X. L.; Xiao, Y.; Plaxco, K. W. *J. Am. Chem. Soc.* **2009**, *131*, 59−61.
(44) Yang, X. H.; Huang, J. H.; Wang, Q.; Wang, K. M.; Yang, L. J.; Huo, X. Q. *Anal. Methods* **2011**, *3*, 59−61.
(45) Liu, Z. Y.; Zhang, W.; Hu, L. Z.; Li, H. J.; Zhu, S. Y.; Xu, G. B. *Chem.—Eur. J.* **2010**, *16*, 13356−13359.
(46) Heyduk, E.; Heyduk, T. *Anal. Chem.* **2005**, *77*, 1147−1156.
(47) Heyduk, T.; Heyduk, E. U.S. Patent Application 2009/0202990 A1, May 16, 2006.