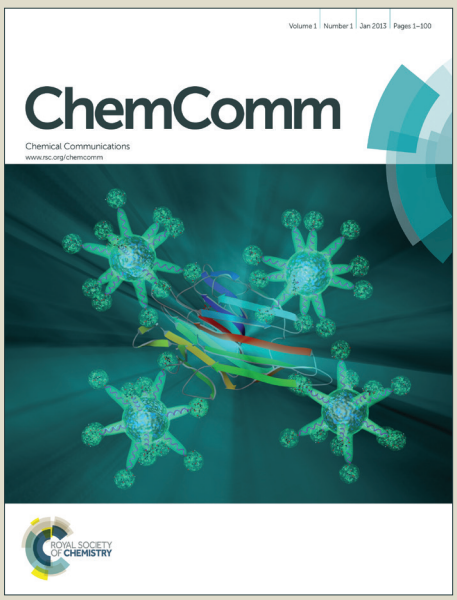
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Sensitive SERS detection of miRNA using a label-free multifunctional probe

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

DOI: 10.1039/x0xx00000x

www.rsc.org/

Hao Zhang,[a] Yu Liu,[a] Jian Gao,*[a] and Junhui Zhen*[b]

A novel surface enhanced Raman scattering (SERS) detection method is fabricated for miRNA based on a smart multifunctional probe for dual cyclical nucleic acid strand-displacement polymerization (CNDP), achieving high sensitivity, universality, rapid analysis, and good performance in real cell samples.

Micro-ribonucleic acid (miRNAs) are a class of small (18–24 nucleotides), endogenous, non-protein-coding RNA molecules.[1] Expression of miRNA has been associated with the regulation and progression of numerous cancers such as breast,[2] thyroid,[3] colorectal,[4] prostate,[5] lung,[6] and ovarian.[7] While miRNAs are critical in the regulation of cancers, many other diseases such as cardiovascular diseases,[8] neurological diseases,[9] and immunological diseases[10] also exhibit miRNA-based regulation. Monitoring changes in miRNA biomarkers can be used to signify the early onset of disease.

The studying of miRNA has generated an demand for its accurate and sensitive detection. However, miRNAs have the extremely limited size, significant sequence homology among family members and the low expression levels, thus weakening the routine techniques for quantitative analysis of miRNA.[11] Currently, several methods have been developed for miRNA detection, ranging from simple to complex and multi-step procedures, such as quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR),[12] microarrays,[13] and Northern blotting. [14]Among them, qRT–PCR[15] is the most sensitive and practical method to detect miRNA targets. However, qRT–PCR method relies on the reverse transcription of miRNA to cDNA. Likewise, miRNA is too short to be amplified by PCR directly, which makes the PCR design very sophisticated[16]. Thermal cycling of the PCR technique are time-consuming and limited to a thermostable enzyme, a laboratory setting, and dual-labelled fluorescent probes, such as Taqman probes,[17]are usually needed to determine the specificity of amplification.

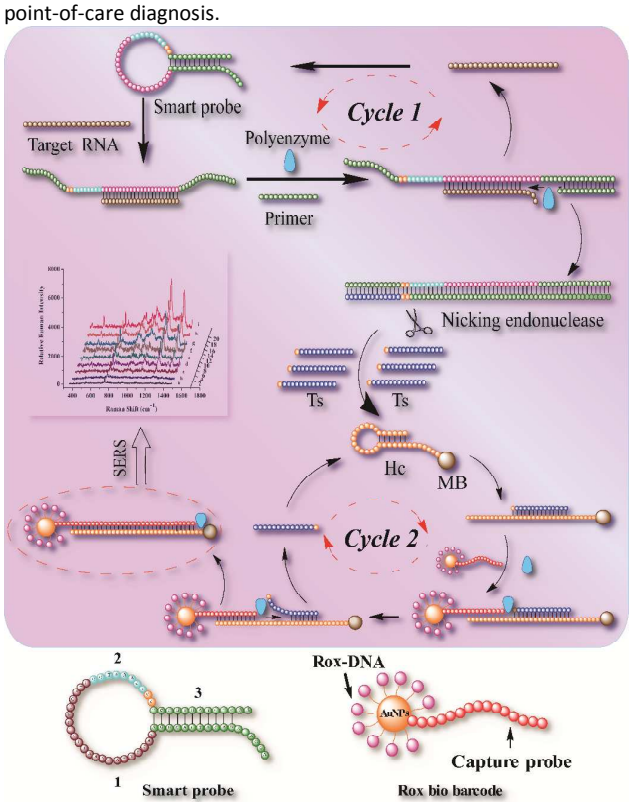
Therefore, isothermal nucleic acid amplification of miRNA, rolling-cycle amplification (RCA)[18] loop-mediated isothermal amplification (LAMP)[19], and exponential amplification strand-displacement amplification reaction (SDA)[20] have emerged an alternative amplification techniques. As the above mentioned reactions can be preceded at a constant temperature, there is no need of specialized instruments for miRNA detection. In addition, they have potential for 'on-site' testing. However, there are some limitations that affect the sensitivity and specificity toward target miRNA detection, such as the requirement of fluorescein-labeled recognition probe, circular template, relatively complex procedure design. In this context, the development of a simple, rapid and isothermal nucleic acid signal amplification system is highly desirable for the applications in biomolecule diagnostics.

Currently, SERS has emerged as a specific, rapid and sensitive tool for biological analysis.[21] It can facilitate molecular-level identification of samples, easy operation without complicated sample preparation, and nondestructive detection in a wide variety of matrices.[22] Therefore, SERS has been employed as an efficient biosensing method for the detection of small bioactive molecules,[23] nucleic acids,[24] proteins[25]and even mammalian cells and tissues.[26] Even though miRNAs can be directly identified by SERS.[27]However, only few SERS strategies based nucleic acid signal amplification have been reported for miRNA. Zhang et al. reported circular exponential amplification reaction (EXPAR) based SERS for simultaneous detection of multiple microRNAs.[28]

In this work, we design a new signal amplification strategy for the detection of miRNA. A label-free multifunctional probe is designed for the recognition of target miRNA molecule to initiate the following exponential amplification. MiR-203, an important biomarker for tumor growth and progression,[29] which expression is generally lower in cervical cancer cells than normal cervical tissues,[30] is selected as the model analyte. Coupling of SERS technology with multiple amplification modes enabled the rapid amplification in 100 min and sensitive detection of miR-203 in a complex biological matrix, with a detection limit of 6.3 fM. This novel sensing system is simple in design and can easily be carried

[a]Department of Chemistry, Qilu University of Technology, Jinan 250353, China. E-mail: gaojian@qlu.edu.cn
[b]School of Medicine, Shandong University, Jinan 250012, China. E-mail: zhen6576@126.com
† Footnotes relating to the title and/or authors should appear here.
Electronic Supplementary Information (ESI) available: Experimental details, UV-vis, SEM. The optimization and selectivity and additional figures. See DOI: 10.1039/x0xx00000x

out by simple mixing and incubation, offering a high potential for point-of-care diagnosis.



Scheme 1 The structure and sequences of the smart probe and sensitive SERS detection of miRNA by using label-free multifunctional probe.

A novel dual circular strand-displacement polymerization (CNDP) SERS method by using a smart probe is fabricated for miRNA. With the cooperation of the nicking endonuclease and polymerases, the amplified analysis of target through a two-cycle working mode based on bio-barcode is carried out. The design of the smart probe and principle of multiple amplification detection are elucidated in Scheme 1. The smart label-free probe facilitates the integration of multiple functional elements into a cooperative biosensing system. The "1" segment in the loop has target recognition sequences for suitable hybridization without hindering the affinity of the probe toward its target. The "2" segment is the recognition site of nicking endonucleade. The "3" segment is the stem which has self-block segment (antiprimer) and template for CNDP reactions. An antiprimer segment is added to the outermost part at the 3´-end. The antiprimer is designed to be complementary to the primer. In this case, the primer is incapable of hybridizing to antiprimer, inhibiting (namely, self-locking) the subsequent isothermal polymerization process.

In the presence of miRNA-203, the hairpin structure of the probe is unfolded via a target molecule binding event. The antiprimer at the 3´-end is released. In the presence of dNTPs, the primer can hybridize to the antiprimer and is subsequently extended by the polymerase from the 3´-end of the probe to the 5´-terminal stem segment as the template. Target miRNA is replaced with the extension of the primer on the probe and binds to another probe, triggering the target CNDP (cycle 1, Scheme 1). Furthermore, a nicking endonuclease (Nt.AlwI), which could recognize specific nucleotide sequences in double-strand DNA and cleave only one strand, was added into the reaction to obtain nicked DNA. Subsequently, the forward-cleaved DNA strand was extended by using DNA polymerase and the short DNA sequence (trigger strand, Ts) behind the nicking site was released, owing to the strand-displacement property of DNA polymerase, which triggered the downstream signal amplification (the second CNDP, cycle 2 Scheme 1B). In our strategy of signal amplification, the released DNA strand Ts was hybridized to a loop of hairpin capture DNA probe (Hc) on magnetic bead (MB), which served as not only the template of CNDP reaction, but also the signal carrier through capturing the SERS signal on MBs. Via the Ts-Hc duplex hybrid event, the hairpin structure is opened. The SERS probe is attached to Hc through the DNA 1, as the primer for DNA strand polymerization. In the process of primer extension, the SERS probes can be anchored on the surface of MBs by forming dsDNA. Ts is displaced to renew the cycle. Thus a large amount of SERS probe labeled dsDNA could be immobilized on the surface of MBs and used for SERS measurement after magnetic separation. It is conceivable that the detection sensitivity could be significantly improved by accomplishing two cycle amplification.

To test the feasibility of the SERS detection system, a series of control experiments were performed and the results are show in Fig.1. The laser excitation of the samples provided discrete vibrational peaks at 1344, 1499 and 1645 cm^{-1}, from Rox dyes molecules. The strongest Raman band at 1499 cm^{-1} was used for the quantitative evaluation of miR-203. In the absence of miR-203, the double-helix DNA immobilized on MB can't be opened, the bio-barcodes couldn't be anchored on the MBs, the subsequent cycles did not happen. So the hairpin probe kept a closed state, the SERS bio-barcode could not be captured, only small signals were observed, which should be due to the nonspecific adsorption of the bio-barcodes on the surface of MBs. In the presence of miR-203 but without Klenow polymerase or nicking endonuclease (curve b), the recognition probe can be unfolded. However, the Ts was not formed and released. Therefore, the Raman intensity still very low similar with curve a. In the presence of miR-203, polymerase, nicking endonuclease but no primer (curve c), miRNA hybridized to the probe and was subsequently extended by the polymerase. The double strand was formed and the Ts behind the nicking site was released in the joint action of nicking enzyme and DNA polymerase, which triggered the downstream signal amplification (cycle 2). Compared with the curve b a higher Raman signal was obtained. When the miR-203, Klenow polymerase, nicking endonuclease and primer were coexisted in the system, the Raman intensity was greatly raised via dual CNDP, suggesting when all reagents coexisted in the system, the Raman intensity was greatly raised, which confirmed the feasibility of this system.

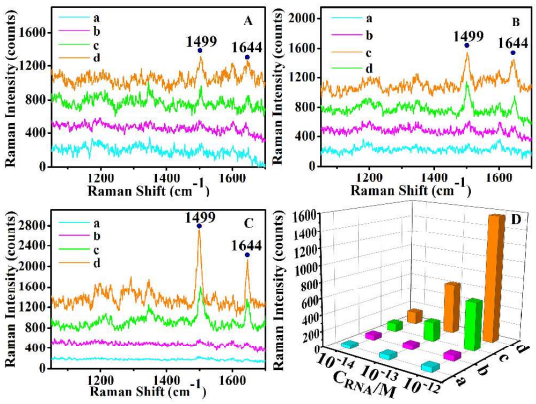


Figure 1. SERS spectra obtained from the detection of miR-203, the concentration of miR-203 was A) 10^{-14}, B) 10^{-13} and C) 10^{-12} M. (a) In the absence of miR-203; (b) in the presence of miR-203, but in the absence of Klenow polymerase or dNTPs or nicking endonuclease; (c) in the presence of miR-203, Klenow polymerase, dNTPs and nicking endonuclease, but in the absence of primer; (d) miR-203, Klenow polymerase, dNTPs, nicking endonuclease and primer were coexistent in the reaction system. (D), the comparision of all the controlled experiments, the concentration of miR-203 were 10^{-14}, 10^{-13} and 10^{-12} M, respectively.

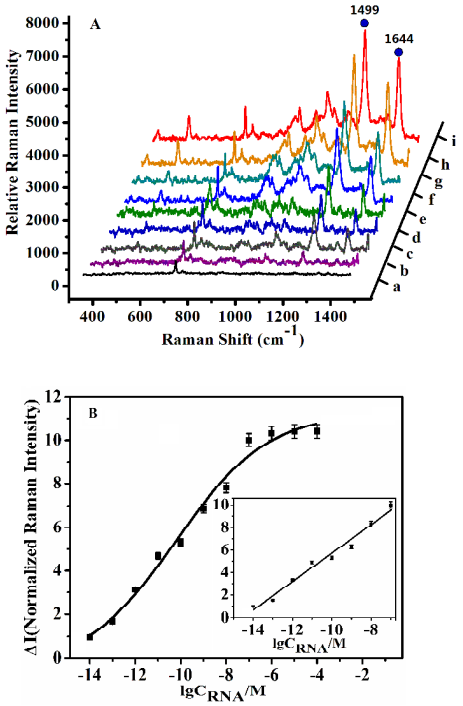


Figure 2. A) SERS spectra for increasing concentrations of miR-203 (a-i: 0, 1.0×10^{-14}, 1.0×10^{-13}, 1.0×10^{-12}, 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9} , 1.0×10^{-8}, 1.0×10^{-7} M, respectively). B) Variance of the normalized Raman intensity with the concentration of miR-203.

Under the optimized experimental condition (the details of the optimum experiments are provided in the ESI†), the detection performance of the SERS assay was investigated by using miRNA 203 with different concentrations. According to Figure 2A, the intensity of Raman scattering increased with the increase in the concentration of miRNA 203. In Figure 2B, the Raman intensity had a good linear fit to the logarithm of miRNA 203 in the range from 1.0×10^{-14} M to 1.0×10^{-7} M. The correlation equation was $\Delta I = 1.268 \lg C + 18.388$ (ΔI is the normalized Raman intensity, C is the concentration of miR-203), and the corresponding correlation coefficient (R) of calibration curve is 0.990. A relative standard deviation (RSD) of 10.1% was obtained by 11 replicate measurements of 50 nM miRNA 203, indicating a good reproducibility of the assay. The obtained detection limit of 6.3 fM (S/N=3) demonstrated high sensitivity. Compared with EXPAR-based SERS method[28], this method possesses two remarkable features as follows: (i) The template for CNDP is blocked in the stem of the hairpin probe, avoiding the cross reaction of the hybridization among the trigger strands (Ts) and the template. (ii) The problem of high background induced by excess SERS probes (bio-barcodes) is circumvented using MBs. The comparison of different methods for miRNA detection is shown in Table S2 (ESI).

To determine whether this method could be applied to target miRNA detection in real samples, miR-203 extracted from the human bronchial epithelial cancer cell lines (ACC-M, H1299 and a bronchial epithelial cell line HBE) were measured by the proposed method. Moreover, to assess the accuracy of this new method, we further compared asymmetric amplification SERS assay with qRT-PCR for miRNA. The detailed result is shown in Figure 3. The results obtained by the SERS assay agree well with the results obtained RT-PCR. The detailed description and data about RT-PCR were added in the Supporting information (see ESI, Figure. S9).

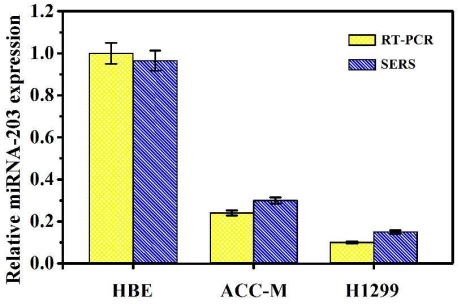


Figure 3. Concentrations of miRNA-203 in sample cells measured by SERS (yellow column) and RT-PCR (blue column).

A simple, rapid, isothermal and sensitive SERS biosensing platform for miRNA detection was developed on the basis of a smart label-free probe. The detection limit toward miRNA was achieved to be as low as 6.3 fM. The entire detection time was less than 2 h. Moreover, it exhibited the distinct advantages of simplicity in probe design and biosensor fabrication, rapidness in a recognition and detection process. Furthermore, since various recognition units might be fused in the label-free hairpin probe structure, the new platform should be fairly easy to extend for the detection of a wide spectrum of analytes including nucleic acid, protein, nuclease activity, and biological small molecules, and be associated with some other analytical techniques. Thus, it opens a promising avenue to develop the biosensing of analytes for basic research and clinical applications.

This work was supported by National Natural Science Foundation of China (No. 21375068 and 81400729), Scientific Research

Foundation for Outstanding Young Scientists of Shandong Provence (No. BS2012SW017), Science and Technology Development Plan Project of Shandong Province, China (No. 2014GSF118122) and the Basic Research Foundation of Shandong University (No. 2014JC033).

Notes and references

1 A. Ventura and T. Jacks, *Cell*, 2009, **136**, 586.
2 (a) S. Volinia, G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris and C. M. Croce, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**,2257; (b) S. Zhu, H. Wu, F. Wu, D. Nie, S. Sheng and Y. Y. Mo, *Cell Res*, 2008, **18**, 350; (c) M. V. Iorio, M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri and M. Campiglio, *Cancer Res*, 2005, **65**, 7065.
3 (a) S. Mitomo, C. Maesawa, S. Ogasawara, T. Iwaya, M. Shibazaki, A. Yashima-Abo, K. Kotani, H. Oikawa, E. Sakurai, N. Izutsu, K. Kato, H. Komatsu, K. Ikeda, G. Wakabayashi and T. Masuda, *Cancer Sci*, 2008, **99**, 280; (b) R. Visone, L. Russo, P. Pallante, I. D. Martino, A. Ferraro, V. Leone, E. Borbone, F. Petrocca, H. Alder, C. M. Croce and A. Fusco, *Endocr. Relat. Cancer,* 2007, **14**, 791.
4 (a) Y. Akao, Y. Nakagawa and T. Naoe, *Oncol. Rep*, 2006, **16**, 845; (b) B. Shi, L. Sepp-Lorenzino, M. Prisco,P. Linsley, T. deAngelis and R. J.Baserga, *Biol. Chem*, 2007, **282**, 32582.
5 (a) Y. Sylvestre, V. D. Guire, E. Querido, U. K. Mukhopadhyay, V. Bourdeau, F. Major, G. Ferbeyre and P. J. Chartrand, *J. Biol. Chem*, 2007, **282**, 2135; (b) X. B. Shi, L. Xue, J. Yang, A. H. Ma, J. Zhao, M. Xu, C. G. Tepper,C. P. Evans, H. J. Kung, R. W. White and deVere, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 19983.
6 (a) S. M. Johnson, H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K. L. Reinert, D. Brown and F. Slack, F. *J. Cell*, 2005, **120**, 635; (b)M. Fabbri, R. Garzon, A. Cimmino, Z. Liu, N. Zanesi, E. Callegari, S. Liu, H. Alder, S. Costinean, C. Fernandez-Cymering, S. Volinia, G. Guler, C. D. Morrison, K. K. Chan, G. Marcucci, G. A. Calin, K. Huebner and C. M. Croce, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 15805.
7 (a) S. Shell, S. M. Park, A. R. Radjabi, R. Schickel, E. Kistner, D. A. Jewell, C. Feig, E. Lengyel and M. E. Peter, *Proc. Natl. Acad. Sci.U. S. A.,* 2007, **104**, 11400; (b) A. Giannakakis, R. Sandaltzopoulos, J. Greshock, S. Liang, J. Huang, K. Hasegawa, C. Li, A. O'Brien-Jenkins, D. Katsaros, B. L. Weber, C. Simon, G. Coukos and L. Zhang, *Cancer Biol. Ther.,*2008, **7**, 255.
8 S. Ikeda, S. W. Kong, J. Lu, E. Bisping, H. Zhang, P. D. Allen, T. R. Golub, B. Pieske and W. T. Pu, *Physiol. Genomics*, 2007, **31**,367.
9 R. Fiore, G. Siegel and G. Schratt, *Biochim. Biophys. Acta, Gene Regul. Mech,* 2008, **1779**, 471.
10 K. D. Taganov, M. P. Boldin, K. J. Chang and D. Baltimore, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 12481.
11 M. Martinez-Pacheco, A. Hidalgo-Miranda, S. Romero-Cordoba, M. Valverde and E. Rojas, *Gene*, 2014, **533**, 508.
12 (a) N. Redshaw, T. Wilkes, A. Whale, S. Cowen, J. Huggett and C. A. Foy, *Biotechniques*, 2013, **54**, 155; (b) E. Sonkoly, T. Wei, P. C. J. Janson, A. Saaf, L. Lundeberg, M. Tengvall-Linder, G. Norstedt, H. Alenius, B. Homey, A. Scheynius, M. Stahle and A. Pivarcsi, *PLoS One*, 2007, **2** , e610; (c) A. M. Krichevsky and G. J. Gabriely, *Cell, Mol. Med,* 2009, **13**, 39.
13 (a) A. Git, H. Dvinge, M. Salmon-Divon, M. Osborne, C. Kutter, J. Hadfield, P, Bertone and C. Caldas, *RNA*, 2010, **16**, 991; (b) S. G. Jensen, P. Lamy, M. H. Rasmussen, M. S. Ostenfeld, L. Dyrskjøt, T. F. Ørntoft and C. L. Andersen, *BMC Genomics*, 2011, **12**, 435; (c) E. Varallyay, J. Burgyan and Z. Havelda, *Nat. Protoc*, 2008, **3**, 190.
14 (a) A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen and Z. Havelda, *Nucleic Acids Res*, 2004, **32**, e175; (b) G. A. Calin, C. Sevignani, C. D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini and C. M. Croce, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 2999; (c) J. R. Vieregg, H. M. Nelson, B. M. Stoltz, N. A. Pierce, *Am. Chem. Soc*, 2013, **135**, 9691.
15 R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim, *Science*, 1985, **230**, 1350.
16 (a) H. Jia, Z. Li, C. Liu, and Y. Cheng, *Angew. Chem. Int. Ed. Engl*, 2010, **49**, 5498; (b) C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen and K. Guegler, *Nucleic Acids Res*, 2005, **33**, e179.
17 L. G. Lee, C. R. Connell and W. Bloch, *Nucleic Acids Res*, 1993, **21**, 3761.
18 (a) A. Lagunavicius, E. Merkiene, Z. Kiveryte, A. Savaneviciute, V. Zimbaite-Ruskuliene, T. Radzvilavicius and A. Janulaitis, *RNA*, 2009, **15**, 765; (b) E. Merkiene, E. Gaidamaviciute, L. Riauba, A. Janulaitis and A. Lagunavicius, *RNA*, 2010, **16**, 1508; (c) H. Y. Liu, L. Li, L. L. Duan, X. Wang, Y. X. Xie, L. L. Tong, Q. Wang and B. Tang, *Anal. Chem*, 2013, **85**, 7941.
19 (a) T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe and N. Amino, *Nucleic Acids Res*, 2000, **28**, E63; (b) T. C. Cardoso, H. F. Ferrari, L. C. Bregano, C. SilvaFrade, A. C. Rosa and A. L. Andrade, *Mol. Cell Probe*, 2010, **24**, 415.
20 (a) Y. Y. Zhao, L. Zhou and Z. Tang, *Nat. Commun*, 2013, **4**, 1493;(b) C. Shi, Q. Liu, C. P. Ma, and W. W. Zhong, *Anal. Chem*, 2014, **86**, 336.
21 S. J. Ye, Y. N. Mao, Y. Y. Guo and S. S. Zhang, *Chem. Commun*, 2014, **55**, 43.
22 (a) S. M. Nie and S. R. Emory, *Science*, 1997, **275**, 1102; (b) J. F. Li, Y. F. Huang, Y. Ding, Z. L. Yang, S. B. Li, X. S. Zhou, F. R. Fan, W. Zhang, Z. Y. Zhou, D. Y. Wu, B. Ren, Z. L. Wang and Z. Q. Tian, *Nature*, 2010, **392**, 7287; (c) S. J. Ye, Y. N. Mao, Y. Y. Guo and S. S. Zhang, *Trends Anal. Chem.*, 2014, **55**, 43.
23 K. E. Shafer-Peltier, C. L. Haynes, M. R. Glucksberg and R. P. Van Duyne, *J. Am. Chem. Soc.*, 2003, **125**, 588.
24 (a) X. Qian, X. Zhou and S. M. Nie, *J. Am. Chem. Soc.*, 2008, **130**, 14934; (b) T. Kang, S. M. Yoo, I. Yoon, S. Y. Lee and B. Kim, *Nano Lett.*, 2010, **10**, 1189.
25 S. J. Ye, Y. Y. Wu, W. Zhang, N. Li and Bo Tang, *Chem. Commun*, 2014, **50**, 9409.
26 (a) K. V. Kong, Z. Lam, W. D. Goh, W. K. Leong and M. Olivo, *Angew. Chem., Int. Ed.*, 2012, **51**, 9796; (b) M. Salehi, L. Schneider, P. Strobel, A. Marx, J. Packeisen and S. Schlucker, *Nanoscale*, 2014, **6**, 2361.
27 (a) J. D. Driskell, A. G. Seto, L. P. Jones, S. Jokela, R. A. Dluhy, Y. P. Zhao and R. A. Tripp, *Biosens. Bioelectron*, 2008, **24**, 917;(b) J. D. Driskell and R. A. Tripp, *Chem. Commun.*, 2010, **46**, 3298.
28 L. P. Ye, J. Hu, L. Liang and C. Y. Zhang, *Chem. Commun.*, 2014, **50**, 11883
29 (a) F. Degliangeli, P. Kshirsagar, P. P. Brunetti and R. Fiammengo, *J. Am. Chem. Soc.* 2014, **136***,* 2264;(b) Q. Zhang, D. W. Piston and R. H. Goodman, *Science,* 2002, **295***,* 1895; (c) S. J. Lin, P. A. Defossez and L. Guarente, *Science*, 2000, **289**, 2126; (d) S. Bruzzone, A. De Flora, C. Usai, R. Grae and H. C. Lee, *Biochem. J.* 2003, **375***,* 395; (e) A. Wilkinson, J. Day and R. Bowater, *Mol. Microbiol.* 2001, **40***,* 1241.
30 X. Y. Zhua, K. J. Erb, C. Y. Mao, Q. Yan, H. J. Xu, Y. B. Zhang, J. H. Zhu, F. Cui, W. X. Zhao and H. Shi, *Cell Physiol Biochem*, 2013, **32,**64.