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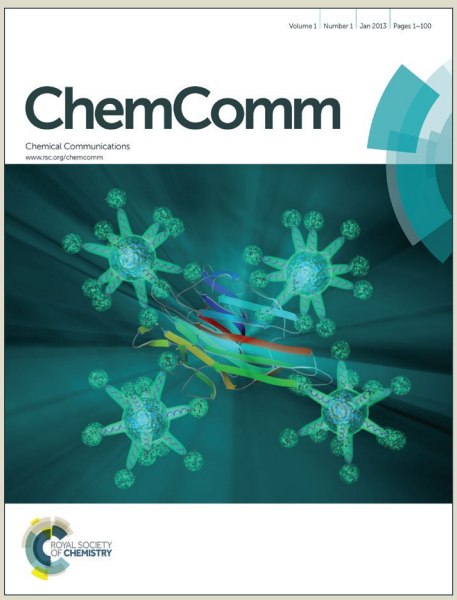

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Target-protected dumbbell molecular probe mediated cascade rolling circle amplification strategy for the sensitive assay of DNA methyltransferase activity

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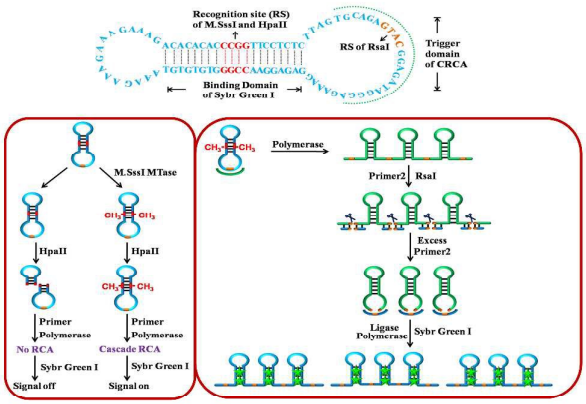
A novel fluorescence detection system is developed for DNA methyltransferase (MTase) activity assay based on target-protected dumbbell molecular probe mediated cascade rolling circle amplification, showing excellent specificity and sensitivity with a detection limit of 0.0024 U/mL, and potential application in the quantitatively monitoring MTase activity and screening of anticancer drugs.

DNA methyltransferase (MTase) is responsible for maintaining methylation pattern in the genome, which plays crucial roles in the regulation of gene expression and the maintainence of genomic stability.[1] Abnormalities in MTase activity may lead to aberrant DNA methylation parttern, which can silence the tumor suppressor genes and promote cancerous transformations.[1a,2] Thus, DNA MTase has become predictive biomarker and therapeutic target for various types of cancers.[2d,3] Moreover, aberrant activity of MTase usually occurs before other signs of malignancy, and thus exhibits potential use for early cancer diagnosis.[1a,2b] Therefore, accurate and sensitive monitoring of MTase activity and inhibitor screening for MTase activity inhibition represent a valuable strategy to both clinical diagnostics and therapy.

Traditionally, radioactive labeling strategy,[4] high performance liquid chromatography,[5] gel electrophoresis,[6] and immune-based assays[7] have been well established for MTase activity analysis. Except these methods, various new strategies with improved detection sensitivity, specificity and simplicity have been developed, including fluorescencet assays,[8] electrochemical assays,[9] colorimetric assays,[10] bioluminescence assays,[11] chemiluminescence assays[12] and electrogenerated chemiluminescence assays.[13] In most of these novel assays, DNA molecular probes play crucial roles for specific target recognition and signal amplification due to their unique molecule recognition capabilities and design flexibility.



Scheme 1 Schematic illustration of M.SssI MTase activity assay based on target-protected dumbbell molecular probe mediated cascade rolling circle amplification strategy

Firstly, DNA molecular probes are endowed with double recognition functions for DNA MTase and methylation-sensitive restriction enzyme to discriminate methylated and unmethylated DNA molecular probes. Furthermore, trigger DNA fragment sequence that could mediate a amplification strategy is incorporated into DNA molecular probes to amplifiy the discrimination event. Currently, most reported DNA molecular probes are double-stranded DNA (dsDNA) or hairpin DNA,[8-13] and after the action of MTase and enzymatic digestion, trigger DNA fragment is released or intact probe is retained to amplify the methylation event. Although these strategies have achieved sensitive DNA MTase activity assay, there are some limitations that affect the detection accuracy and sensitivity. The trigger DNA fragment in dsDNA or hairpin DNA molecular probes is partially sealed, thus hybridization between DNA molecular probes and subsequent signal amplification probes or nonspecific interaction of probes and nuclease in amplification system may lead to nonspecific amplification.[8c,d, 12] Moreover, other DNA probes (such as padlock probe,[12b,] hairpin probe[8c,d,12a] or labeled signal probe[8,9b]) are usally required to trasduce the recognition event to amplified signal output, and the indirect detection mode may lead to reduced sensitivity and complicated probe design.

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In view of the above limitations, an accurate and sensitive DNA MTase activity assay was developed based on target-protected sealed dumbbell molecular probe (D-probe)-mediated cascade rolling circle amplification (CRCA) strategy (Scheme 1). Firstly, D-probe serves as enzyme-linkage recognition probe of MTase and endonuclease. Next, methylated D-probe maintains its integrity and acts as the circular template for CRCA. Finally, mutiple D-probe copies of CRCA products bind with multimolecules labeling of Sybr Green I (SG), leading a cooperative amplified fluorecence signal for detection. Compared to the reported methods based on dsDNA or hairpin DNA molecular probes, the strategy is unique in characteristics: (i) D-probe here serves together as DNA MTase and endonuclease recognition probe, CRCA template, and signal probe, thus avoids the demand of multiple probes design. (ii) Once unmethylated D-probe is cleaved into two parts by endonuclease, the CRCA could be effectively blocked, thus lowers the nonspecific amplification. (iii) Cooperative amplification coupling with the CRCA and multimolecules labeling of SG can guarantee an improvement of sensitivity for MTase activity assay. (iiii) After the enzyme-linkage recognition of DNA MTase and endonuclease, the D-probe could be directly amplified by CRCA process. The more straight forward detection mode may induce more simple and sensitive MTase activity assay.

As shown in Scheme 1, the design of D-probe and the overall working principle (using M.SssI MTase as a model enzyme) are illustrated in detail. The D-probe adopts a stable dumbbell structure by intramolecular DNA hybridization and possesses mutiple functions. Firstly, the stem of the D-probe is designed with symmetric sequence 5′-CCGG-3′ (in red) to recognize both the M.SssI MTase and HpaII endonuclease. The M.SssI MTase can transfer a methyl group from S-adenosyl-L-methionine (SAM) to cytiosines in the symmetrical sequence 5′-CCGG-3′, forming 5′-CCmGG-3′. The HpaII endonuclease can cleave the duplex symmetrical sequence 5′-CCGG-3′ between cytiosines, but the cleavage can be blocked by methylated CpG. Additionally, one loop acts as the trigger domain of CRCA and tetranucleotide sequence of 5′-GTAC-3′ (in orange) is embedded for RasI to mediate the CRCA. Furthetmore, the stem of D-probe could bind with SG for signal output. The proposed strategy for M.SssI MTase involves two pricipal processes, one is enzyme-linkage recognition of M.SssI MTase and HpaII, the other is CRCA process and signal generation. In the first step, when M.SssI MTase is absent, the D-probe with symmetrical sequence 5′-CCGG-3′ in the stem can be recognized and cleaved into two parts between the cytosines by HpaII. Thus the following CRCA process is blocked and lead to a negligible fluorescence signal. However, in the presence of M.SssI MTase, the cytosines in the stem of D-probe (5′-CCGG-3′) is methylated, forming 5′-CCmGG-3′, and the cleavage by HpaII would be blocked. Thus, the methylated-protected CRCA process can proceed as following. Firstly, the methylation protected intact D-probe hybridizes with primer1 (P1) and starts the linear RCA by phi29 DNA polymerase, forming a long concatenated sequence copy of D-probe. Next, excess primer2 (P2) is introduced to form multiple double-strand segments along the RCA products, and RsaI (who can recognize and nick the double-strand symmetric 5′-GT↓AC-3′ sequence. The symbol '↓' represents the nicking site) recognizes its sites and cleaves sequences of double strand formation, converting

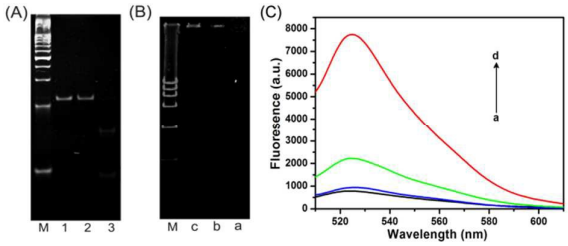

Fig. 1 (A) Nondenaturating PAGE analysis of the methylation of D-probe by M.SssI and the subsequent cleavage by HpaII. Lane M, DNA ladder marker; lane 1, D- probe (0.5 μM); lane 2, D-probe (0.5 μM) + M.SssI (100 U/mL) + HpaII (25 U/mL); lane 3, D-probe (0.5 μM) + HpaII (25 U/mL). (B) Agarose gel electrophoresis analysis of amplification products. Lane a, control system without M.SssI ; Lane b, LRCA system with M.SssI but without RsaI and P2; Lane c, CRCA system with M.SssI. $C_{M.SssI}$ = 16 U/mL (C) Fluorescence emission spectra for M.SssI activity assay under different conditions. curve a, D-probe+P1+P2; curve b, control system, curve c, LRCA system, curve d, CRCA system. $C_{M.SssI}$ = 16 U/mL.

the long RCA products to multiple new D-probe monomers. Subsequently, RsaI is heat-inactivited, which also plays the role of annealing, causing the fragmented P2 to dissociate from the new D-probe monomers and intact P2 anneals to new D-probe monomers. It is noted that P2 preferentially hybridizes to both ends of the same monomer, forming circular monomer products rather than linear concatemers through ligation of T4 DNA ligase.[14] Next, the newly multiple circular D-probe monomers and P2 would trigger a new round of RCA and hence realizes the CRCA, leading to much more copies of D-probe. Finally, mutimolecules of SG bind with D-probe copies and generate distinct enhanced fluorescence signal. Therefore, it is conceivable that the sensitive and accurate analysis of M.SssI MTase activity could be achieved by measuring the fluorecnece signal.

To investigate the feasibility of the proposed strategy for M.SssI MTase activity assay, the nondenaturating PAGE experiment for methylation process was carried out, firstly. As shown in Fig. 1A, in the absence of M.SssI MTase, two bands with faster migration rate were observed (lane 3), indicating the fact that unmethylated D-probes were cleaved into two parts by HpaII. While in the presence of both M.SssI MTase and HpaII, only one band (lane 2) with similar migration rate to original D-probe (lane 1) was observed, suggesting that the methylation reaction occured, and the methylated D-probes could not be cleaved by HpaII.

To further confirm whether the methylation-protected D-probes could be amplified by CRCA process, agarose gel electrophoresis experiment was used to monitor the amplification products. As shown in Fig. 1B, no distinct band appeared in lane a, indicating no significant RCA process proceed because the fact that D-probes were cleaved into two parts by HpaII and could not trigger the CRCA process. As a comparison, there was a bright band with high molecular weight in lane b and c, respectively, suggesting the occurrence of RCA process. Moreover, it was also observed that lane c showed a larger and brighter band than lane b, proving that much more RCA products were produced by CRCA than linear

RCA (LRCA) with one round rolling circle amplification.

Finally, fluorescence measurements were also performed to monitor the progress of the reaction and further confirm the feasibility of the M.SssI MTase activity assay. As shown in Fig. 1C, in the presence of only D-probe, P1 and P2, a negligible fluorescence signal was observed (curve a), which may produce by the D-probe or the hybrid of P1/P2. In the control system without M.SssI MTase, the fluorescence signal barely changed (curve b), suggesting the fact that in the absence of M.SssI MTase, the unmethylated D-probes were cleaved by HpaII and thus blocked the CRCA process. Importantly, the barely enhanced signal indicated that the CRCA was blocked effectively and ensured a negligible nonspecific amplification. With the further addition of M.SssI MTase into the reaction system, fluorescence enhancements were observed in both the system of LRCA (curve c) and CRCA (curve d), indicating the occurrence of methylation of D-probe and the subsequent methylation-protected D-probe mediated LRCA or CRCA process. Moreover, more significant signal enhancement of CRCA system than LRCA system further verified the more robust amplification ability of CRCA. These results were consistent with those of gel electrophoresis experiment, commonly demonstrating that the proposed target-protected D-probe mediated CRCA strategy for MTase activity assay is feasible.

In order to achieve the best sensing performance, corresponding experimental conditions including the stem length of D-probe, methylation time, HpaII cleavage time, RsaI cleavage time, the concentrations of P1, HpaII endonuclease, dNTPs, P2 and RsaI endonuclease were optimized, respectively (Fig. S2–S3 in detail, ESI†). Subsequently, the sensitivity and dynamic range of the developed strategy for M.SssI MTase activity were evaluated under the optimized conditions. Fig. 2A showed the fluorescence responses for different M.SssI MTase concentrations. It was observed that the fluorescence intensity gradually increased with the increase of M.SssI MTase concentration from 0 to 100 U/mL. In Fig. 2B (insert), it can be seen that the fluorescence intensity had a good linear fit to the logarithm of M.SssI MTase concentration ranging from 0.01 to 50 U/mL. The linear relationship could be described as $\Delta F = 4389.9 + 1674.7 \lg C$ with a correlation coefficient of $R = 0.995$ ($\Delta F = F - F_0$, where F and F_0 were fluorescence intensities of the system with and without M.SssI MTase, respectively). According to the principle of three times standard deviation over the blank signal, a detection limit of 0.0024 U/mL was evaluated, which was lower than most of the reported dsDNA or hairpin DNA molecular recognition probe mediated RCA methods (Table S2). Meanwhile, a control experiment for M.SssI MTase activity detection was also conducted in the case of LRCA with one round RCA (Fig. S4, ESI†). The detection limit was calculated to be 0.08 U/mL, which was much higher than that of CRCA system. These results indicated again the robust amplification ability of CRCA, which ensured a more sensitive strategy for the MTase activity assay. In addition, the intra-assay and inter-assay precision of the proposed method were examined in the presence of 0.025, 1.0, 25 U/mL for three times, respectively. The relative standard deviation (RSD)

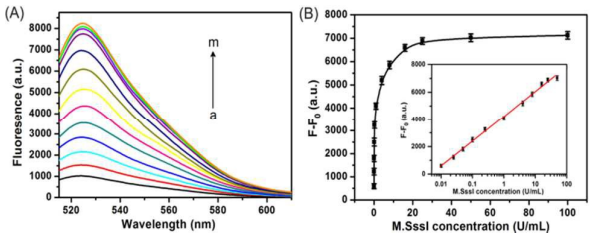

Fig. 2 (A) Fluorescence emission spectra towards different concentrations of M.SssI MTase. From a to m were 0, 0.010, 0.025, 0.050, 0.10, 0.25, 1, 4, 8, 16, 25, 50, 100 U/mL, respectively. (B) Changes of ΔF versus M.SssI MTase concentration. Inset shows the linear relationship of fluorescence intensity with the logarithm of M.SssI concentration.

obtained from intra-assay were 4.6%, 4.0%, and 4.6% at 0.025, 1.0, 25 U/mL M.SssI, respectively. And RSD from inter-asssy were 6.3%, 6.5% and 6.2%. These results demonstrated that the proposed method may be used for the sensitive detection of M.SssI MTase activity with good reproducibility.

To investigate the selectivity of the proposed method, other cyosine MTase including AluI, HaeIII, and HhaI were used as the interference methyltransferases. AluI, HaeIII, and HhaI may methylate the cytosine residues in the double-stranded symmetric sequence of 5′-AG**C**T-3′, 5′-GG**C**C-3′ and 5′-G**C**GC-3′, respectively.[15] As shown in Fig. S5 (ESI†), remarkable relative fluorescence intensity was only observed in the presence of M.SssI, suggesting the excellent selectivity of the proposed strategy towards M.SssI activity that evidently contribute to the fact that cyosine in HpaII recognition sequence of 5′-CCGG-3′ cannot be methylated by AluI, HaeIII, or HhaI and unmethylated D-probe would be cleaved off by HpaII.

To further examine the possibility of the proposed sensing platform for cellular MTase activity profiling and practical sample analysis, HeLa cell extracts were added in the buffer to simulate the intracellular complex system during the test procedure. The fluorescence intensity and the logarithm of MTase concentration also exhibited a linear relationship similar to that in solution (Fig. S6, ESI†). Moreover, the recoveries of three different concentrations of M.SssI MTase (0.025, 1.0 and 25 U/mL) were calculated to be 92%, 91%, 98%, respectively (Table S3), which are excellent (close to 100%) and comparable to traditional methods[5,6]. These results demonstrated that the proposed sensing system works well in the intracellular-mimicking system and could be further used for real sample analysis.

Since the regulation of the DNA MTase activity may block the aberrant DNA methylation or kill the cancer cells, the screening of DNA MTase inhibitors for activity inhibition has attracted increasing attention in disease treatment. In this research, the capacity of our method in evaluating and screening the inhibitors of M.SssI was inveatigated, and two anticancer drugs (5-Aza (5-azacytidine) and 5-Aza-dC (5-aza-2′-deoxycytidine)) were chosen as the model inhibitors. Given HpaII, RsaI, T4 DNA ligase and Phi29 DNA polymerase involving in the whole process, it is necessary to investigate the effects of the two drugs (both 5 μM) on them. And results showed

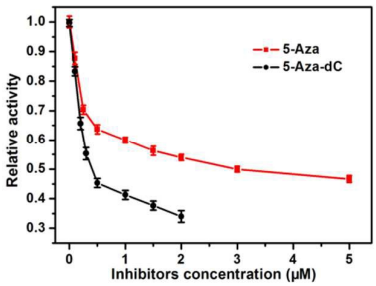

Fig. 3 Inhibitory effects of anticancer drugs with different concentrations on the M.SssI MTase activity.

that the effects were negligible (Fig. S7, ESI†). Thus, we further evaluated the inhibit effects of the two drugs on M.SssI activity by comparing the fluorescence signal under various doses of the drugs (within 5 μM). The relative activity (RA) of M.sssI MTase was estimated by the following equation:

$$RA = \frac{F_i - F_0}{F_t - F_0}$$

where F_0, F_t and F_i are the fluorescence intensities in the absence of M.SssI MTase, in the presence of M.SssI MTase, in the presence of both M.SssI MTase and inhibitors, respectively. Fig. 3 showed the activity of M.SssI decreased with the increasing concentration of 5-Aza and 5-Aza-dC, indicating the weakening of DNA methylation. The IC_{50} values, which represent the inhibitor concentration required to cause a 50% decrease of M.SssI activity, were found to be 0.46 μM and 3.0 μM for 5-Aza-dC and 5-Aza, respectively. It was observed that the inhibition of the two drugs were in significant dose-dependent manner and 5-Aza-dC exhibited higher inhibition efficiency than 5-Aza, which were consistent with the prior report.[7,8b] These results indicate that the developed method has potential application in screening MTase inhibitors and quantitatively monitoring the activity of MTase and then may serve to antibiotics and anticancer therapeutics.

In conclusion, we have developed a target-protected dumbbell molecular probe (D-probe) mediated cascade rolling circle amplification (CRCA) strategy for accurate and sensitive DNA methltransferase (MTase) activity assay. The sufficient cleavage of HpaII and the dependence on circular template of CRCA improves the specificity of the amplification. The introduction of cooperative amplification of CRCA and multimolecules labeling of Sybr Green I (SG) improves the sensitivity of detection. The multifunctional D-probe design avoids complex probe design. A wide linear range from 0.01 to 50 U/mL and a low detection limit of 0.0024 U/mL for M.SssI activity analysis are obtained with the proposed strategy. We have also demonstrated that the strategy is an effective tool for recognizing target MTase in the cell extracts–containing samples. More importantly, the inhibition effects of 5-Aza-dC and 5-Aza can be evaluated conveniently showing potential application in quantitatively monitoring the activity of MTase and screening of anticancer drugs. These results indicate that the sensing strategy represent a valuable strategy to both clinical diagnostics and therapy.

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Notes and references

1 (*a*) S. B. Baylin and J. G. Herman, *Trends Genet.*, 2000, **16**, 168; (*b*) X. Cheng and R. J. Roberts, *Nucleic Acids Res.*, 2001, **29**, 3784; (*c*) P. A. Jones and D. Takai, *Science,* 2001, **293**, 1068; (*d*) W. Reik, W. Dean and J. Walter, *Science*, 2001, **293**, 1089.

2 (*a*) P. A. Jones and P. W., Laird, *Nat. Genet.*, 1999, **21**, 163; (*b*) P. M. Das and R. Singal, *J. Clin. Oncol.*, 2004, **22**, 4632; (*c*) V. Shukla, X. Coumoul, T. Lahusen, R. H. Wang, X. L. Xu, A. Vassilopoulos, C. Y. Xiao, M. H. Lee, Y. G. Man, M. Ouchi, T. Ouchi and C. X. Deng, *Cell Res.*, 2010, **20**, 1201; *(d)* H. Heyn and M. Esteller, *Nat. Rev. Genet.*, 2012, **13**, 679.

3 (a) K. Mutze, R. Langer, F. Schumacher, K. Becker, K. Ott, A. Novotny, A. Hapfelmeier, H. Hofler and G. Keller, *Eur. J. Cancer*, 2011, **47**, 1817; (*b*) V. Singh, P. Sharma and N. Capalash, *Curr. Cancer Drug Targets*, 2013, **13**, 379.

4 S. Pradhan, A. Bacolla, R. D. Wells and R. J. Roberts, *J. Biol. Chem.*, 1999, **274**, 33002.

5 I. Reenila, P. Tuomainen and P. T. Mannisto, *J. Chromatogr. B: Biomed. Sci. Appl.*, 1995, **663**, 137.

6 M. F. Fraga, R. Rodriguez and M. J. Canal, *Electrophoresis*, 2000, **21**, 2990.

7 M. Wang, Z. N. Xu, L. J. Chen, H. S. Yin, S. Y. Ai, *Anal. Chem.*, 2012, **84**, 9072.

8 (*a*) J. Li, H. Yan, K. Wang, W.Tan and X. Zhou, *Anal. Chem.*, 2007,**79**, 1050; (*b*) L. J. Ji, Z. W. Cai, Y. D. Qian, P. Wu, H. Zhang, C. X. Cai, *Chem. Commun.*, 2014, **50**, 10691; (*c*) X. W. Xing, F. Tang, J. Wu, J. M. Chu, Y. Q. Feng, X. Zhou and B. F. Yuan, *Anal. Chem.*, 2014, **86**, 11269; (*d*) Y. Zhang, W. J. Xu, Y. P. Zeng, C. Y. Zhang, *Chem. Commun.*, 2015, **51**, 13968.

9 (*a*) S. Baek, B. Y. Won, K. S. Park and H. G. Park, *Biosens. Bioelectron.*, 2013, **49**, 542; (*b*) N. B. Muren and J. K. Barton, *J. Am. Chem. Soc.*, 2013, **135**, 16632.

10 (*a*) G. Song, C. Chen, J. Ren and X. Qu, *ACS Nano,* 2009, **3**, 1183; (*b*) T. Liu, J. Zhao, D. M. Zhang and G. X. Li, *Anal. Chem.*, 2010, **82**, 229; (*c*) Y. X. Zhao, F. Chen, M. L. Lin, C. H. Fan, *Biosens. Bioelectron.*, 2014, **54**, 565.

11 C. Jiang, C. Y. Yan, C. Huang, J. H. Jiang and R. Q. Yu, *Anal. Biochem.*, 2012, **423**, 224.

12 (*a*) S. Bi, T. T. Zhao, B. Y. Luo and J. J. Zhu, *Chem. Commun.*, 2013, **49**, 6906; (*b*) Y. P. Zeng, J. Hu, Y. Long and C. Y. Zhang, *Anal. Chem.*, 2013, **85**, 6143.

13 Y. Li, X. Luo, Z. Yan, J. B. Zheng and H. L. Qi, *Chem. Commun.*, 2013, **49**, 3869.

14 F. Dahl, J. Baner, M. Gullberg, M. Mendel-Hartvig, U. Landegren and M. Nilsson, *PNAS*, 2003, **13**, 4548.

15 H. Deng, X. Yang, S. P. X. Yeo and Z. Gao, *Anal. Chem.*, 2014, **86**, 2117.