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One-Step, Ultrasensitive, and Electrochemical Assay of microRNAs Based on T7 Exonuclease Assisted Cyclic Enzymatic Amplification

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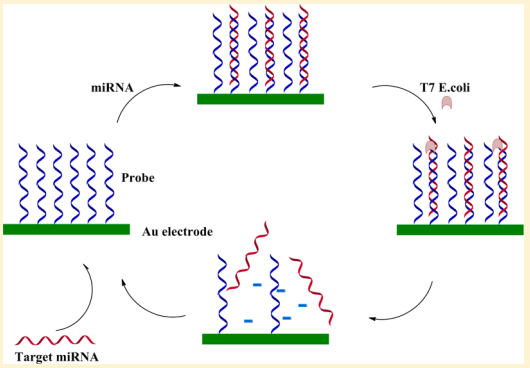
Supporting Information

ABSTRACT: Taking advantage of the special exodeoxyribonuclease activity of T7 exonuclease, a simple, sensitive, selective, and label-free microRNA biosensor based on the cyclic enzymatic amplification method (CEAM) has been proposed. First, thiol functionalized DNA probes were assembled onto a gold nanoparticles modified gold electrode surface through a Au−S bond, followed by hybridizing with target miRNA. Subsequently, DNA in RNA/DNA duplexes was digested by T7 exonuclease, which can release the microRNA molecules from the electrode surface and return into the buffer solution. Meanwhile, the released microRNA can further hybridize with the unhybridized DNA probes on the modified electrode surface. On the basis of it, an isothermal amplification cycle is realized. The T7 exonuclease-assisted CEAM achieved a low detection limit of 0.17 fM. Moreover, this assay presents excellent specificity with discriminating only a single-base mismatched microRNA sequence. Furthermore, this work can also be applied to detect avian leukemia based on the decreased expression level of microRNA-21.



MicroRNAs (MiRNAs) are a kind of short endogenous nonprotein coding RNA, which play a critical role in gene expression of most eukaryotic organisms from plants to animals.[1] More and more evidence has demonstarted that abnormal repression of miRNA is associated with many diseases, including diabetes,[2] cancer,[3,4] and stroke induced tissue injury.[5] However, miRNA detection is also challenged from their short lengths, low abundance, susceptibility to degradation, and sequence similarity among family members. The current widely used standard methods for miRNA analysis are Northern blotting,[6,7] real-time PCR,[8] and microarrays.[9] Northern blotting is viewed as a standard assay method for miRNAs detection, but it is a time- and sample-consuming and semiquantitative detection method with low sensitivity and throughput. Without an elaborate separation and enrichment process, it is difficult to achieve sufficient sensitivity in biological sample analysis. The real-time PCR (RT-PCR) approach shows high detection sensitivity (about 100 fM) and good specificity, but it generally requires complex and tedious steps for miRNAs isolation and purification. It also needs to reverse transcription to cDNA prior to the amplification step. Furthermore, the apparatus for RT-PCR is very expensive. Analysis techniques based on microarray make multiple miRNA analysis feasible, but it frequently suffers from poor reproducibility and inaccuracy, currently resulting from cross hybridization and nonspecific adsorption. Thus, the development of fast, specific, sensitive, and convenient methods for miRNA analysis is urgently needed.

Recently, a great deal of effort has been devoted for miRNAs analysis. The methods developed for the detection of miRNAs mainly include mass spectrometry,[10] capillary electrophoresis,[11] electrochemiluminescence,[12] Raman spectroscopy,[13,14] and colorimetry.[15] Though sensitive, these methods tend to be complicated and require advanced instrumentation or expensive reagents for signal amplification. Then, some amplification mechanism has to occur. Rolling circle amplification (RCA) is a simple, reliable, and isothermal enzymatic process. This technique can produce long single-strand DNA with tandem repeats, but it suffers from its tedious reaction time and low sensitivity which cannot satisfy the requirement of miRNA detection.[16] Wen et al. applied the RCA technique for the miRNA assay with a low detection limit of 2 aM; however, the entire experiment was required over 6 h of reaction time.[17] The isothermal exponential amplification reaction (EXPAR) is another signal amplification technique with high sensitivity,[18−20] but it is expensive and imprecise.

The cyclic enzymatic amplification method (CEAM) based on nucleases, in which one target leads to many cycles of target dependent nuclease cleavage of reporter probes for output signal amplification, is a recently developed method for simple, rapid, sensitive, and inexpensive nucleic acid detection.[21−23] Cui et al. developed a novel DNase I-based CEAM for miRNA analysis.[24] This assay suffers from high fluorescence background, high detection limit (5 nM), and expensive cost due to the combination with fluorescence. Herein, we fabricated a

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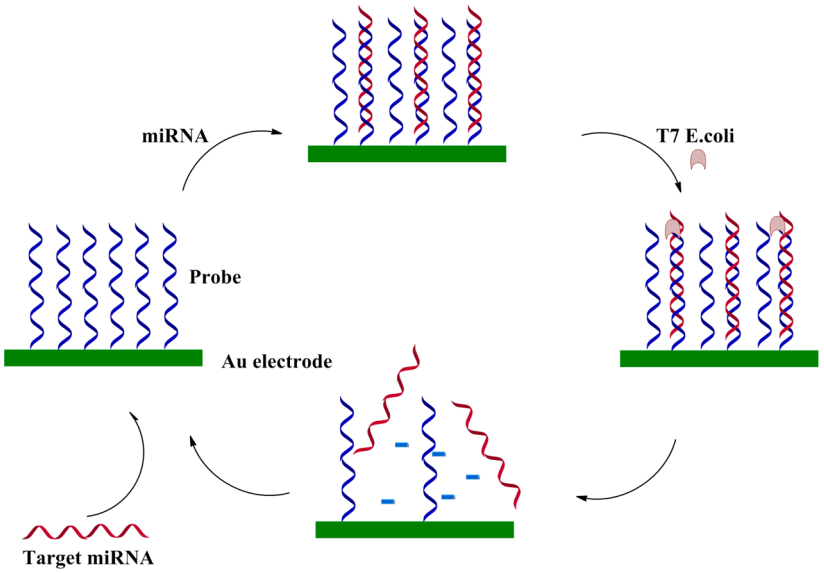
Scheme 1. Biosensor Fabrication and miRNA Detection Principle Based on T7 Exonuclease Assisted Signal Amplification



simple and yet ultrasensitive electrochemical biosensor for miRNA detection with signal amplification by T7 exonuclease assisted target recycling. T7 exonuclease, a sequence-independent nuclease, catalyzes the removal of 5′ mononucleotides from the 5′ termini of double stranded DNA, while its activity on single-stranded DNA is limited.[25] Furthermore, it acts on DNA or RNA in an RNA/DNA complex from 5′ to 3′, while it is not active on double stranded RNA or single stranded RNA.[26] Recently, T7 exonuclease-based CEAM has been widely used for nucleic acid detection based on fluorescence.[27,28]

As illustrated in Scheme 1, thiol functionalized DNA probes are first assembled onto a gold nanoparticles modified gold electrode (AuNPs/Au) through the Au−S bond (thiol is marked in the 3′ terminal); then, the electrode is further hybridized with target miRNA. Subsequently, the probe DNA in RNA/DNA duplexes is specifically digested by T7 exonuclease. Meanwile, the microRNA molecules are released from the electrode surface and return into the buffer solution. Then, the released miRNA molecules can also hybridize again with the unhybridized probes on the modified electrode. On the basis of it, an isothermal amplification cycle is achieved. This recycling mechanism leads to the decrease of the impedance of electrode surface and significantly amplifies the electrochemical signal.

EXPERIMENTAL SECTION

Reagents and Materials. T7 exonuclease and 10× NEBuffer 4 (500 mM KAc, 200 mM Tris acetate buffer, 100 mM magnesium acetate, 10 mM dithiothreitol, pH 7.9 at 25 °C) were obtained from New England Biolabs (USA) and used without further purification. Mercaptoropronic acid (MPA), sodium citrate, disodium ethylenediaminetetraacetic acid (EDTA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$) were from Aladdin (China). Diethypyrocarbonate (DEPC) was provided by Amresco (USA). The DNA sequence was obtained from Sangon Biotech (China) and used as received. All miRNA sequences were from TaKaRa (China) with HPLC purification. The base sequences used in the study are as follows: thiol-capped DNA, 5′-TCAACATCAGTCTG-ATAAGCTA-SH-$(CH_2)_6$-3′; target miRNA-21, 5′-UAGC-UUAUCAGACUGAUGUUGA-3′; single-base mismatched miRNA, 5′-UAGCUUAUCACACUGAUGUUGA-3′; three-base mismatched miRNA, 5′-UCGCUUAUCACACUGAU-GUCGA-3′; noncomplementary miRNA, 5′-ACCGCACA-GGUGGAAUCUAACG-3′. The oligonucleotides were diluted in 10 mM TE buffer (pH 7.4), to give stock solutions of 100 μM. The sequences DNA and miRNA were stored at −20 and −80 °C, respectively. All other reagents were analytically pure grade.

Ten mM Tris−HCl (pH 7.4) containing 1.0 mM EDTA, 1.0 M NaCl, and 1.0 mM TCEP was used as probe immobilization buffer; 1× SSC (pH 7.4) was employed as miRNA hybridization buffer. 10 mM Tris-HCl (pH 7.4) was used as washing buffer, and 10 mM PBS (pH 7.4) containing 1 mM $[Fe(CN)_6]^{3-/4-}$ was selected as detection solution. To avoid the effect of RNase on the stability of miRNAs, all centrifuge tubes (Axygen, USA) and tips (Axygen, USA) were autoclaved, and the redistilled deionized water treated with DEPC was used throughout this work.

Biosensor Fabrication. The Au electrode was thoroughly burnished with 30 nm Al_2O_3 powder and successively ultrasonicated in double distilled deionized water and anhydrous ethanol for 5 min, respectively. After drying under N_2, the pretreated bare Au electrode was further immersed into 3 mM $HAuCl_4$ solution containing 0.1 M KNO_3 for electrodeposition of AuNPs using the amperometry technique at −0.2 V for 250 s; then, the obtained AuNPs/Au electrode was rinsed with double distilled deionized water and dried at room temperature. Afterward, 5 μL of probe immobilization buffer containing 0.5 μM thiol modified DNA probe was dropped on the AuNPs/Au surface and incubated for 12 h at room temperature. The electrode (DNA/AuNPs/Au) was then washed thoroughly with washing buffer to remove the unconjugated DNA on the substrate. After that, 5 μL of probe immobilization buffer containing 1.65 μM MPA was dropped on the DNA/AuNPs/Au surface and incubated for 1 h to seal the electrode. Then, the hybridization of the complementary miRNA-21 was performed by incubating the electrode with the hybridization buffer containing different concentrations of miRNA-21 for 2 h at 37 °C. The electrode

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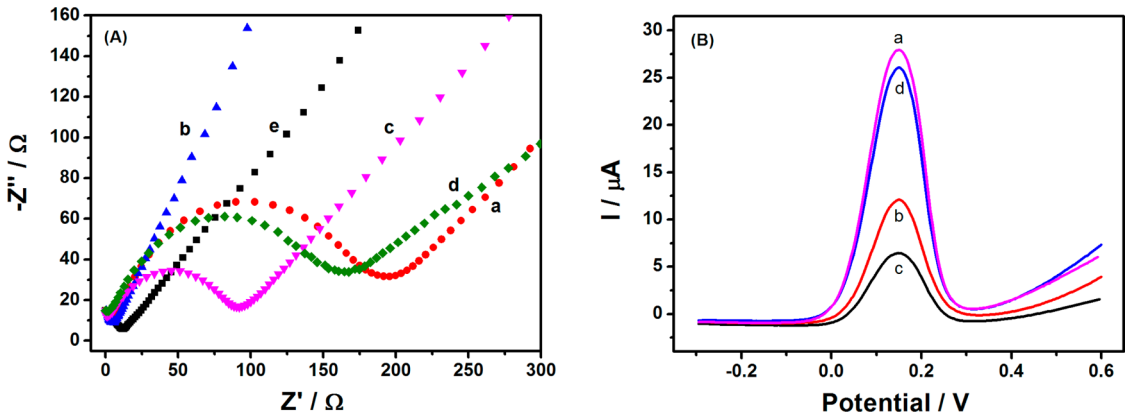

Figure 1. (A) Nyquist plot for different electrodes in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl solution with the frequency range from 10^{-1} to 10^5 Hz: (a) the bare Au electrode, (b) AuNPs/Au, (c) DNA/AuNPs/Au, (d) miRNA-DNA/AuNPs/Au, and (e) T7/miRNA-DNA/AuNPs/Au. 60 min T7 exonuclease incubation in buffer solution containing 0.05 U of the T7 exonuclease, 1 pM target miRNA. (B) The DPV responses of AuNPs/Au (a), DNA/AuNPs/Au (b), miRNA-DNA/AuNPs/Au (c), and T7/miRNA-DNA/AuNPs/Au (d).

(noted as miRNA-DNA/AuNPs/Au) then was rinsed thoroughly with 0.1× SSC to wash off the unhybridized complementary miRNA-21. Finally, miRNA-DNA/AuNPs/Au was incubated with 5 μL of 1× NEBuffer 4 containing T7 exonuclease for 60 min, followed by rinsing with washing buffer. The electrode was denoted as T7/miRNA-DNA/AuNPs/Au.

Electrochemical Determination. Electrochemical determination was carried out using a CHI660C electrochemical workstation (USA). A bare or modified Au electrode was used as working electrode. A saturated calomel electrode (SCE) was used as the reference electrode, and a platinum wire was used as the auxiliary electrode. Electrochemical impedance spectroscopy (EIS) was performed in 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) containing 0.1 M KCl with the frequency from 10^{-1} to 10^5 Hz. Differential pulse voltammetry (DPV) was recorded in 10 mL of 10 mM PBS (pH 7.4) containing 1 mM $Fe(CN)_6^{3-/4-}$ (1:1). The parameters were as follows: increment potential, 0.004 V; pulse amplitude, 0.05 V; pulse width, 0.05 s; sample width, 0.0167 s; pulse period, 0.2 s; quiet time, 2 s.

Sample Analysis. First, DF-1 chicken fibroblast cells were cultured in DMEM medium containing 1% FBS. Subsequently, DF-1 cells were treated with ALV-J China strain (NX0101) for 2 h, and then, those cells were cultivated in fresh medium with 1% FBS for 7 days. Finally, cells were harvested for RNA extraction. This extraction procedure was according to the RNA extraction kit (TaKaRa, Dalian, China). As control, RNA was also extracted from normal DF-1 cells.

RESULTS AND DISCUSSION

In this work, we presented a cyclic enzymatic amplification method for miRNA-21 detection based on T7 exonuclease triggered signal amplification. The EIS technique is used to characterize the modification process of the biosensor, where the $[Fe(CN)_6]^{3-/4-}$ was used as redox probe. As seen in Figure 1A, the electron transfer resistance (R_{et}) of the bare Au electrode was about 185 Ω (curve a). The R_{et} value decreased greatly when AuNPs were modified on the bare Au electrode surface, and only a straight line was obtained in the whole frequency region (curve b), indicating that AuNPs have good conductivity and facilitate the electron-transfer process of redox probe. Moreover, the active surface area of the electrode was also increased through the immobilization of AuNPs, which could improve the capture efficiency of probe DNA. When probe DNA was assembled on the AuNPs/Au surface, a small semicircle was observed and the R_{et} value was about 95 Ω (curve c). After hybridizing with miRNA-21, the R_{et} value was further increased to 160 Ω (curve d). These increases could be explained as the electrostatic repulsion effect between the negatively charged phosphoric acid backbone of oligonucleotides and redox probe. Subsequently, the R_{et} value decreased, and an almost straight line was obtained when miRNA-DNA/AuNPs/Au was incubated with T7 exonuclease (curve e), indicating the efficiency digestion of T7 exonuclease toward the hybridized DNA probe. Under the incubation with T7 exonuclease, most DNA probes in DNA-miRNA duplexes were cleaved off, and the complementary miRNA-21 were released to further hybridize with the unhybridized probe DNA. Through these processes, the isothermal amplification cycle was achieved, from which one complementary miRNA-21 molecule could cleave thousands of probe DNA during T7 exonuclease digestion. Thus, the amount of oligonucleotides decreased greatly on the substrate electrode. However, the resistance for curve e is higher than that at AuNPs/Au (curve b) due to the residues of the thiol linker in probe DNA and the sealing agent of MPA. These results also demonstrated that a sensing interface was successfully fabricated.

To demonstrate the working principle of the method, DPV was performed in 10 mL of detection solution. As shown in Figure 1B, AuNPs/Au presented a strong reduction peak with the current of 28.11 μA (curve a). Then, the reduction peak current (I_{pc}) decreased to 12.58 and 7.41 μA for DNA/AuNPs/Au (curve b) and miRNA-DAN/AuNPs/Au (curve c), respectively. The reason could be ascribed to the electrostatic repulsion and steric hindrance of oligonucleotides toward $[Fe(CN)_6]^{3-/4-}$. However, the I_{pc} increased significantly (curve c) after miRNA-DNA/AuNPs/Au was incubated with T7 exonuclease, signifying a quick cleavage of the probe and recycling of miRNA. These EIS and DPV results confirmed the CEAM indeed took place as expected, and this method can be used to detect miRNA with high sensitivity.

Under optimal conditions (Figures S1 and S2 in the Supporting Information), we tested the current signal with different miRNA-21 concentrations using this method. For interpreting the results of the proposed method, the current change ($\Delta I = I_2 - I_1$) was compared, where I_1 was the current

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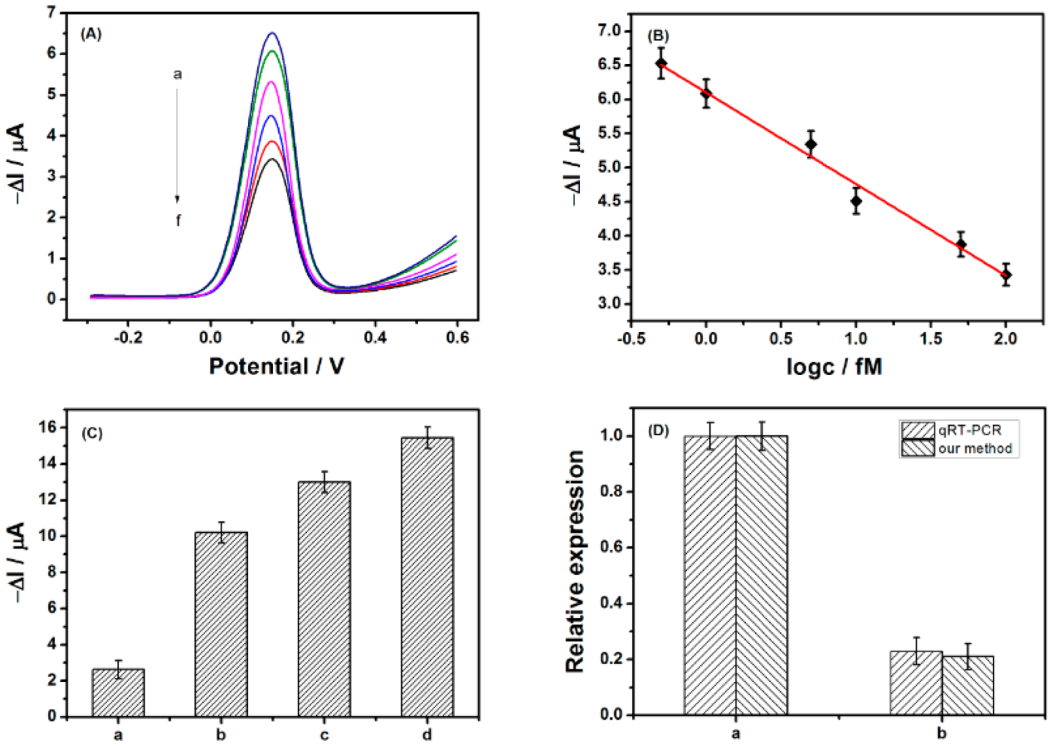

Figure 2. (A) Current change response of T7/miRNA-DNA/AuNPs/Au with different hybridization of miRNA in 10 mL of detection solution. MiRNA concentration (from a to f): 0.5, 1, 5, 10, 50, and 100 fM. (B) The calibration curve of the current change versus logarithm value of miRNA concentration. (C) Comparison of current change after the probe was hybridized with complementary (a), single-base mismatched (b), three-base mismatched (c), and noncomplementary (d) miRNA (c = 1 pM). (D) The relative expression level of miRNA-21 in DF-1 chicken fibroblast cells infected with (b) and without (a) infected subgroup J ALVs.

of AuNPs/Au and I_2 was the current of T7/miRNA-DNA/AuNPs/Au in 10 mL of detection solution. Figure 2A illustrated the current observed upon different concentrations of miRNA-21. As shown in Figure 2B, the reduction peak current exhibited a good linear relationship with the logarithm value of the target miRNA-21 concentration within the range from 0.5 to 100 fM. The linear regression equations were obtained as $I_{pa} = -1.34 \log c + 6.10$ ($R = 0.993$), and the limit of detection was calculated to be 0.17 fM (S/N = 3), which is higher that that obtained at DNA/AuNPs/Au microRNA biosensor (0.035 nM, Figure S3 in the Supporting Information). The detection performance of our biosensor was also compared with some previous reports, and the results were shown in Table S1 (Supporting Information), indicating an acceptable and competitive detection sensitivity.

For confirming the good selectivity of the biosensor, we designed four types of miRNA sequences, including complementary (a), single-base mismatched (b), three-base mismatched (c), and noncomplementary miRNA (d) at the same concentration (1 pM). As shown in Figure 2C, the ΔI was 2.63 μA for probe DNA hybridizing with complementary miRNA, which was much lower than 10.21 μA (b), 13.04 μA (c), and 15.46 μA (d) for the other three miRNA sequences. These results demonstrated the CEAM had good detection specificity.

The applicability for real sample detection is also an important parameter for the assay method, so the expression levels of miRNA-21 in the total RNA extracted from DF-1 chicken fibroblast cells infected with and without subgroup J avian leukemia virus were analyzed using the proposed method. As shown in Figure 2D, the expression level of miRNA-21 decreased after the cells were infected with subgroup J avian leukemia virus. In order to test the veracity of this method, the expression level of miRNA-21 was also evaluated by qRT-PCR. The results were also shown in Figure 2D. It was clear that the results were consistent for the electrochemical biosensor and qRT-PCR, which demonstrated the detection reliability of our method. On the basis of these results, we think that miRNA may be a kind of new biomarker for avian leukosis.

CONCLUSIONS

In conclusion, we took advantage of the T7 exonuclease to create a new cyclic enzymatic amplification method and developed its application for rapid ultrasensitive miRNA detection. T7 exonuclease is a sequence-independent nuclease, which acts on the blunt or recessed DNA/RNA complex and has high catalytic activity and excellent substrate specificity. The recycling probe digestion mechanism of the assay leads to signal amplification and therefore enhances detection sensitivity. As a result, a competitive detection limit of 0.17 fM and an excellent selectivity to distinguish even single-base mismatched miRNA as well as the feasibility in real sample determination have been demonstrated with the presented assay. Moreover, this one-step, label-free method can screen not only the biomarker of avian leukemia virus but also other diseases.

ASSOCIATED CONTENT

Supporting Information

Optimum detection conditions, comparison of detection performances, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Wienholds, E.; Kloosterman, W. P.; Miska, E.; Alvarez-Saavedra, E.; Berezikov, E.; de Bruijn, E.; Horvitz, H. R.; Kauppinen, S.; Plasterk, R. H. A. *Science* **2005**, *309*, 310−311.
(2) Zhao, C.; Dong, J.; Jiang, T.; Shi, Z.; Yu, B.; Zhu, Y.; Chen, D.; Xu, J.; Huo, R.; Dai, J.; Xia, Y.; Pan, S.; Hu, Z.; Sha, J. *PLoS One* **2011**, *6*, No. e23925.
(3) Kosaka, N.; Iguchi, H.; Ochiya, T. *Cancer Sci.* **2010**, *101*, 2087−2092.
(4) Volinia, S.; Calin, G. A.; Liu, C.-G.; Ambs, S.; Cimmino, A.; Petrocca, F.; Visone, R.; Iorio, M.; Roldo, C.; Ferracin, M. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 2257−2261.
(5) Liu, D.-Z.; Tian, Y.; Ander, B. P.; Xu, H.; Stamova, B. S.; Zhan, X.; Turner, R. J.; Jickling, G.; Sharp, F. R. *J. Cereb. Blood Flow Metab.* **2009**, *30*, 92−101.
(6) Varallyay, E.; Burgyan, J.; Havelda, Z. *Nat. Protoc.* **2008**, *3*, 190−196.
(7) Válóczi, A.; Hornyik, C.; Varga, N.; Burgyán, J.; Kauppinen, S.; Havelda, Z. *Nucleic Acids Res.* **2004**, *32*, No. e175.
(8) Chen, C.; Ridzon, D. A.; Broomer, A. J.; Zhou, Z.; Lee, D. H.; Nguyen, J. T.; Barbisin, M.; Xu, N. L.; Mahuvakar, V. R.; Andersen, M. R. *Nucleic Acids Res.* **2005**, *33*, No. e179.
(9) Lim, L. P.; Lau, N. C.; Garrett-Engele, P.; Grimson, A.; Schelter, J. M.; Castle, J.; Bartel, D. P.; Linsley, P. S.; Johnson, J. M. *Nature* **2005**, *433*, 769−773.
(10) Kullolli, M.; Knouf, E.; Arampatzidou, M.; Tewari, M.; Pitteri, S. J. *J. Am. Soc. Mass. Spectrom.* **2014**, *25*, 80−87.
(11) Khan, N.; Cheng, J.; Pezacki, J. P.; Berezovski, M. V. *Anal. Chem.* **2011**, *83*, 6196−6201.
(12) Cheng, Y.; Lei, J.; Chen, Y.; Ju, H. *Biosens. Bioelectron.* **2014**, *51*, 431−436.
(13) Lopez-Tocon, I.; Otero, J.; Arenas, J.; García-Ramos, J. V.; Sanchez-Cortes, S. *Anal. Chem.* **2011**, *83*, 2518−2525.
(14) Driskell, J.; Seto, A.; Jones, L.; Jokela, S.; Dluhy, R.; Zhao, Y.-P.; Tripp, R. *Biosens. Bioelectron.* **2008**, *24*, 917−922.
(15) Zhang, Y.; Li, Z.; Cheng, Y.; Lv, X. *Chem. Commun.* **2009**, 3172−3174.
(16) Zhou, Y.; Huang, Q.; Gao, J.; Lu, J.; Shen, X.; Fan, C. *Nucleic Acids Res.* **2010**, *38*, No. e156.
(17) Wen, Y.; Xu, Y.; Mao, X.; Wei, Y.; Song, H.; Chen, N.; Huang, Q.; Fan, C.; Li, D. *Anal. Chem.* **2012**, *84*, 7664−7669.
(18) Zhang, Y.; Zhang, C.-y. *Anal. Chem.* **2011**, *84*, 224−231.
(19) Jia, H.; Li, Z.; Liu, C.; Cheng, Y. *Angew. Chem., Int. Ed.* **2010**, *49*, 5498−5501.
(20) Wang, X.-P.; Yin, B.-C.; Wang, P.; Ye, B.-C. *Biosens. Bioelectron.* **2013**, *42*, 131−135.
(21) Cui, L.; Ke, G.; Wang, C.; Yang, C. J. *Analyst* **2010**, *135*, 2069−2073.
(22) Xuan, F.; Luo, X.; Hsing, I.-M. *Anal. Chem.* **2012**, *84*, 5216−5220.
(23) Cui, L.; Zhu, Z.; Lin, N.; Zhang, H.; Guan, Z.; Yang, C. J. *Chem. Commun.* **2014**, *50*, 1576−1578.
(24) Cui, L.; Lin, X.; Lin, N.; Song, Y.; Zhu, Z.; Chen, X.; Yang, C. J. *Chem. Commun.* **2012**, *48*, 194−196.
(25) Kerr, C.; Sadowski, P. D. *J. Biol. Chem.* **1972**, *247*, 311−318.
(26) Shinozaki, K.; Tuneko, O. *Nucleic Acids Res.* **1978**, *5*, 4245−4262.
(27) Wang, H.-B.; Ou, L.-J.; Huang, K.-J.; Wen, X.-G.; Wang, L.-L.; Liu, Y.-M. *Can. J. Chem.* **2013**, *91*, 1266−1271.
(28) Wang, H.-b.; Wu, S.; Chu, X.; Yu, R.-Q. *Chem. Commun.* **2012**, *48*, 5916−5918.