CHEMISTRY
A European Journal

A Journal of
ChemPubSoc Europe

Accepted Article

Title: A Novel One Pot Fluorescence Derivatization Strategy for Highly Sensitive MicroRNA Analysis

Authors: Shulin Zhao, Li Pan, Huaisheng Zhang, Jingjin Zhao, Ifedayo Victor Ogungbe, and Yi-Ming Liu

To be cited as: *Chem. Eur. J.* 10.1002/chem.201905639

Link to VoR: http://dx.doi.org/10.1002/chem.201905639

Supported by
ACES

WILEY-VCH

10.1002/chem.201905639

A Novel One Pot Fluorescence Derivatization Strategy for Highly Sensitive MicroRNA Analysis

Li Pan, [a] Huaisheng Zhang, [a] Jingjin Zhao, [a,b] Ifedayo Victor Ogungbe,[a] Shulin Zhao,*[b] and Yi-Ming Liu*[a]

Abstract: MicroRNAs (miRNAs) modulate the expression of over 30% of mammalian genes during development and apoptosis, abnormal expression of miRNAs may lead to a range of human pathologies. Therefore, analysis of miRNAs is significantly valuable for disease diagnostics. In this work, a novel one pot fluorescence derivatization strategy was developed for miRNA analysis. The mechanism of the derivatization reaction was explored by using instrumental methods, including liquid chromatography, fluorescence, and mass spectrometry. Highly fluorescent N^6-ethenoadenine (ε-adenine) was formed and detached from the miRNA sequence through the reaction of adenine in nucleic acids with 2-chloroacetaldehyde (CAA) at 100 ℃. This is the first experimental evidence that the cooperation of formed ε-adenine and water-mediated H-bond interaction between the proton at 2′ and the oxyanion at 3′ positions stabilized the oxocarbenium significantly, which make the depurination and derivatization of miRNA highly effective. Based on this derivatization strategy, a facile and sensitive high-performance liquid chromatography (HPLC) method was developed for quantitative assay of miRNAs. In combination with magnetic solid phase extraction (MSPE), the HPLC method was proven useful for the determination of microRNAs at sub pico-molar level in serum samples.

Introduction

MicroRNAs (miRNAs) are a class about 22 nucleotides noncoding-RNAs which play significant roles in many cellular processes such as cell proliferation, migration, and apoptosis. And miRNAs serve as RNA silencers and gene post-transcriptional regulators.[1,2] Abnormal expression or alteration of miRNAs also leads to a range of human pathologies, including cancer, which suggest that the miRNAs are reliable disease markers.[3,4] Therefore, the development of assays to quantify miRNAs for clinical diagnosis has generated a lot of interest. Current standard methods for miRNA assay are northern blotting and microarray with high-throughput and multiplex miRNA gene expression screening capability.[5,6] However, it's well documented that these methods have low assay sensitivity. In order to improve the sensitivity, various amplification strategies have been proposed, such as reverse transcription quantitative polymerase chain reaction (RT-qPCR),[7,8] rolling circle amplification,[9] exponential amplification reaction,[10] hybridization chain reaction,[11] catalytic hairpin assembly,[12] loop-mediated isothermal amplification,[13] strand-displacement amplification,[14] and duplex-specific nuclease signal amplification.[15] Among these amplification methods, labeling the probes with fluorescence dyes is one of the essential requirement to realize signal amplification. However, these labeling procedures are complicated, time-consuming and involves expensive labeling reagent.[16-19] In addition, fluorescence dye used in these labeling procedures itself is fluorescent which leads to high background. Therefore, a simple, fast, zero background, low-cost labeling method for miRNA detection is highly desired.

As a nucleophilic reagent with no fluorescence itself, 2-chloroacetaldehyde (CAA) was used to label adenine and its analog forming the corresponding fluorescence products, which can realize the quantification of these small molecules,[20,21] and the labeling reaction offers a high derivative yield. It can be used to detect DNA lesions involved adenine. However, this labeling reaction is inseparable from tedious operation and time consuming,[22] such as multiple step enzymatic hydrolysis or acid hydrolysis to obtain free ε-adenine. Moreover, CAA was only proposed for labeling nucleic acids to detect DNA, and detection sensitivity for DNA was μM level.[23]

Herein, a novel one pot fluorescence derivatization method for miRNA is established. The one-pot derivatization reaction is a green and highly efficient approach due to the reduction of work-up procedures and purification steps. With the aid of DNA, dAMP and AMP, the mechanism of one-pot fluorescence derivatization reaction was also investigated and elucidated. The formed ε-adenine and hydrogen bond significantly stabilized the oxocarbenium of miRNA, making ε-adenine leave easily. The ε-adenine as a report reflects the concentration of microRNA. Based on this founding, a facile and cost-effective method for absolute quantification of microRNAs was developed. CAA can effectively derivatize and de-purine miRNAs in one pot reaction, producing quantitatively fluorescent products, i.e. etheno-adenine (ε-adenine) and etheno-guanine (ε-guanine). After being separated from other fluorescent species in the derivatization solution by high performance liquid chromatography (HPLC), ε-adenine that had a much higher fluorescence yield comparing to ε-guanine was quantified. Experimental conditions were studied to achieve optimal analytical figures of merit. To attest the applicability of the present method in clinical analysis, it was utilized to determine miRNA-21 in serum samples. An effective magnetic solid phase extraction (MSPE) of target miRNA from sample matrix by using a complementary DNA probe immobilized on magnetic beads was developed to ensure assay specificity for the miRNA target, and further improve the sensitivity of analysis.

[a] *Dr. L. Pan, Dr. H. Zhang, Prof. J. Zhao, Prof. I. V. Ogungbe, Prof. Y. M. Liu*
Department of Chemistry and Biochemistry, Jackson State University, 1400 Lynch Street, Jackson, Mississippi 39217, United States
E-mail: yiming_liu@jsums.edu
[b] *Prof. J. Zhao, Prof. S. Zhao*
State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, Guangxi Normal University, Guilin 541004, China
E-mail: zhaoshulin001@163.com
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/chem.xxxxxxxxx.

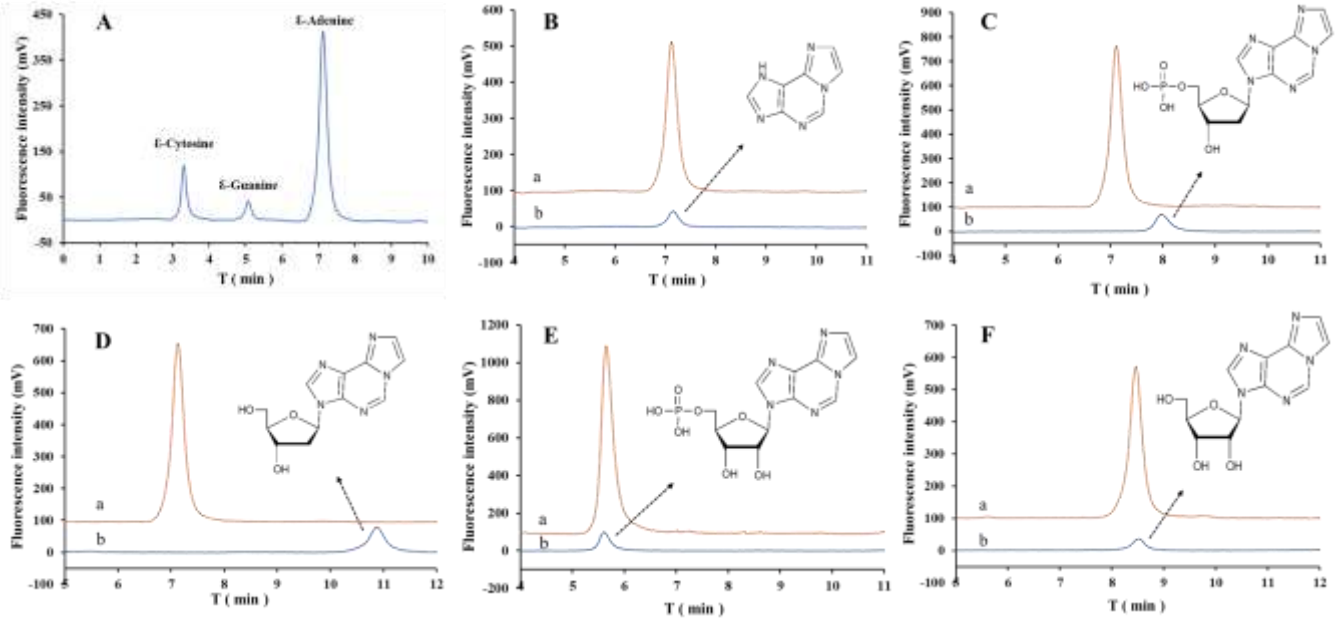


Figure 1. Chromatograms of reaction products under different conditions. The mixture of 95 μM cytosine, 3.6 μM guanine and 0.36 μM adenine was reacted with CAA at 100°C for 15 min (A). Adenine, dAMP, dAdo, AMP and Ado were reacted with CAA at 100 ℃ for 15 min (curve a) and 37 ℃ for 1 h (curve b), respectively (B-F). The structure of each fraction was detected by MS (Figure S1, Supporting Information). Reaction condition: 2 μL of adenine (1 mM), dAdo (3 mM), Ado (0.7 mM), dAMP (1.6 mM), or AMP (1.6 mM) were reacted with CAA (100 μL) in 1xPBS (898 μL) at 37 ° C for 1 h; 5 μL of Adenine (0.1mM), dA (0.3 mM), A (0.07 mM), dAMP (0.16 mM), or AMP (0.16 mM) were reacted with CAA (50 μL) in 1xPBS (495 μL) at 100 °C, for 15 min.

Results and Discussion

Study of reaction mechanism for the fluorescence derivatization

In the one pot reaction between miRNA and CAA at 100 ℃, the formed fluorescence derivatives include ε-adenine, ε-cytosine and ε-guanine. The uracil base can not be derivatized by CAA.[24] However, the fluorescence produced by ε-cytosine is too weak to be used for quantification.[20] Therefore, the excitation-emission spectra of ε-adenine and ε-guanine were investigated. The results indicate that its maximum excitation and emission wavelengths are 275 nm and 411 nm, respectively, and these derivatives can be easily separated and detected by HPLC-FD. As shown in Figure 1A, ε-cytosine, ε-guanine and ε-adenine have retention time of 3.2 min, 5.1 min, 7.1 min, respectively. The fluorescence yield ratio of ε-cytosine, ε-guanine and ε-adenine was 1:87:870, which demonstrated that the ε-adenine provides the highest fluorescence yield. Thus, ε-adenine can be used as fluorescence signal for miRNAs analysis in the one-pot reaction, and ε-adenine was further investigated as a quantification target for the depurination. DNA, AMP and dAMP were chosen as control to figure out the one pot reaction mechanism.

The retention times of the etheno-adducts formed when CAA is reacted with adenine, dAMP, dAdo, AMP and Ado at 37 ℃ for 1 h were determined using HPLC and their identities were verified by MS (Figure S1B-F, Supporting Information). The adenine, dAMP, AMP, dAdo and Ado were converted to ε-adenine, ε-dAMP, ε-AMP, ε-dAdo and ε-Ado, respectively (Figures 1B-F, curve b). However, ε-adenine was formed when adenine, dAMP and dAdo were reacted with CAA at 100 ℃ for 15 min, but the reaction with AMP and Ado led to the formation of ε-AMP and ε-Ado, respectively (Figures 1B-F, curve a). Since similar etheno-adducts were obtained at 37 ℃ and 100 ℃ for AMP and Ado, the 2′ hydroxyl group may play a role in the stabilization of ε-AMP and ε-Ado under ambient reaction conditions (37 ℃ and 100 ℃ at atmospheric pressure). Despite the fact the AMP was not depurinated when reacted with CAA at 100 ℃, we found that miRNA is quickly depurinated, just like DNA, when reacted with CAA (Figure 2A and Figure 2B). Moreover, the identity of ε-guanine was verified through detecting fraction at 5.1 min by MS. The mass spectrum was shown as Figure S2 (supporting information)

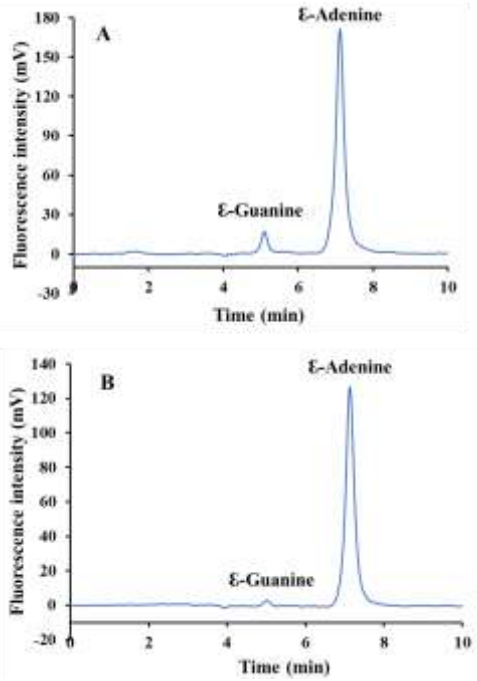


Figure 2. Chromatograms of reaction products obtained from 20 nM miRNA (A) (5'-GUA AGG CAU CUG ACC GAA GGC A-3') and 15 nM DNA (B) (5'-TCA ACA TCA GTC TGA TAA GCT A-3') reacted with CAA at 100°C for 15 min, respectively.

In order to find out the transition state of this one pot reaction, the reaction kinetic of dAMP, AMP, DNA and microRNA with CAA were subsequently investigated, respectively. The reaction kinetic curves of dAMP with CAA were shown as Figure 3A. As can be seen, the fluorescence intensity of product (ε-dAMP) arrives the maximum at 5 mins and depurated completely at 15 mins. Meanwhile, the fluorescence intensity of the product (ε-adenine) increased gradually with the reaction time, and reached the maximum at 15 min, remains almost unchanged within 25 min. From these two dynamic curves, 5 min was the time required to form ε-dAMP, and 10 min was the time for the following depuration step of ε-dAMP. Therefore, it can be concluded that it involved two step successive reactions, forming ε-dAMP and subsequent depurination. In other words, modification of adenine in dAMP occurred firstly, then, the formed ε-adenine part weakens the N-glycosidic bond to trigger the successive depurination reaction. It is worth noting that the maximum fluorescence intensity of ε-adenine was stronger than fluorescence intensity of ε-dAMP, and the reason was further researched by quantification of the products using HPLC-ESI-TQ-MS/MS in multiple-reaction monitoring (MRM) mode. In the MRM mode, the peak only appeared when the parent and daughter ions were both detected through setting up the appropriate parent and daughter ions, so the influence caused by other substances could be avoided. Quantification was performed by the MRM mode after setting up the parameters of molecule ion→fragment ion (ε-Adenine m/z 160 → m/z 106, ε-dAMP m/z 356.5 → m/z 160.23, ε-dAdo m/z 276.28 → m/z 160.09, ε-Ado m/z 292.24 → m/z 160.03, ε-AMP m/z 372.63 →m/z 160.04, Adenine m/z 136 → m/z 119, Adenine m/z 136 → m/z 119, dAMP m/z 332 →m/z 136, AMP m/z 348 → m/z 136). In order to track the intermediate products in the reaction process of dAMP with CAA, the reaction was terminated at 3 min. Then the products were identified and quantified. As shown in Figure S3a (Supporting Information), from the abundance of each product, dAMP (m/z 348→m/z 136) remains around 15 % with the order of magnitude of 10^5 which demonstrated that the reaction was stopped in the early stage of the reaction process. This is an ideal period for detection of all intermediate products. The major product is ε-dAMP (m/z 356→m/z 161) with the order of magnitude of 10^6. The abundance of adenine is 10^5 which is similar as the blank control without CAA Figure S3b (Supporting Information), thus it illustrated that the adenine originated from dAMP depurination spontaneously. Partial of ε-adenine came from the spontaneously generated adenine. This also gives a reasonable explanation for why the peak intensity of ε-adenine is stronger than the peak intensity of ε-dAMP though ε-adenine and ε-dAMP had the very similar fluorescence yield. The ε-adenine (m/z 160 → m/z 106) is the smallest abundance, and it's hard to say where it exactly originates from, which has two possibilities, one is from the adenine spontaneously degraded from dAMP, and the other is from the formed un-stabilized ε-dAMP.

The reaction kinetic curves of AMP with CAA were shown as Figure 2B. The fluorescence intensity of product ε-AMP dramatically increased to the maximum within 5 min then kept almost same. Comparing the AMP and dAMP, cleavage of the glycosyl bond after reaction of CAA with dAMP occurred and completed within 15 min at 100 ℃, whereas the loss of ribose following reaction of AMP was extremely slow, revealing the 2'-OH group in AMP imparts stability to the glycosidic bond. Exploring the reason for 2'-OH stabilizing N-glycosidic bond of AMP to impede the depurination of ε-AMP, the oxocarbonium ion intermediate would be considered. Because the electronegativity of 2'-OH, the oxocarbonium ion intermediate is even less stable for 2'-OH nucleosides, which protect them from depurination. The observations provide a strong evidence for the inductive stabilization of the ε-AMP adduct by the presence of a 2′ hydroxyl group.[25,26] Moreover, the identity of formed product by the reaction of AMP and CAA at 100℃ was evaluated by LC-MS shown as Figure S4 (Supporting Information). The majority product is ε-AMP with magnitude of 10^7, and other products including adenine (m/z 136 → m/z 119), ε-adenine (m/z 160 → m/z106), AMP (m/z 348 → m/z 136) have totally account for 1% which was negligible. It is understandable that 2'-OH significantly stabilized the N-glycosidic bond to prevent adenine or ε-adenine releasing from AMP or ε-AMP, respectively, though the formed ε-adenine from AMP decrease the stability of N-glycosidic bond. The result is well consistent with the HPLC results.

The reaction kinetic curves of DNA and miRNA with CAA were shown as Figure 3C and Figure 3D, respectively. When DNA and miRNA react with CAA at 100 ℃, both of them produce ε-adenine. and they present similar kinetic curve. Comparing the dynamic curves of miRNA and DNA, maximal fluoresce intensity of them all were achieved at 15 min. This kinetic time is in good agreement with the reaction time of dAMP with CAA previously investigated. Moreover, the kinetic curve of miRNA also was validated through monitoring the ε-adenine concentration (*m/z* 160→m/z 106) by using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). As shown in Figure S5 (Supporting Information), when miRNA react with CAA at 100 ℃, ε-adenine concentration reached the maximum at 15 min. However, no ε-adenine was detected when the reaction was carried out at 37 ℃. Therefore, two consecutive reactions involved in the one-pot reaction of DNA or miRNA were verified. And it's well known that RNA is much less vulnerable to depurination since the electronegative 2' oxygen renders the oxocarbonium ion intermediate less stable.[27,28] However, it's interesting that when miRNA react with CAA at 100℃, it did produce ε-adenine. This suggests that the stabilizing effect of the 2′ hydroxyl group is absent or minimal in the etheno-miRNA intermediate. The loss of inductive stabilization in the etheno-miRNA intermediate is most likely a water-mediated H-bond interaction between the proton at 2′ and the oxyanion at 3′ positions.[29,30] The formation of hydrogen bond abated the electronegativity of 2'-OH, contributing to the stability of oxocarbonium and thus depurination becomes relatively easier. However, for AMP, hydrogen bonding between the 2'-hydroxyl and the 3'-oxygen (or vice versa) in nucleosides has not been detected in water except in organic aprotic solvents has been proved.[31,32] It can be concluded that there is either no hydrogen bond between the 2'- and 3'-oxygen in the monoanion or that this does not contribute toward the stability of the anion in water.[28]

Mechanism pathway for one pot reaction of miRNA with CAA

Mechanism pathway of one pot reaction of microRNA with CAA was described as follows. CAA is an electrophile reagent whose carbonyl group attack exocyclic 6-amino group of adenosines of microRNA to initiate the condensation,[33-35] leading to an unstable intermediate, and the reaction is reversible. This intermediate then undergoes intramolecular cyclization to a stable intermediate, as the result of alkylation of endocyclic N_1, and this process is irreversible. This stable intermediate undergoes the dehydration to the

10.1002/chem.201905639

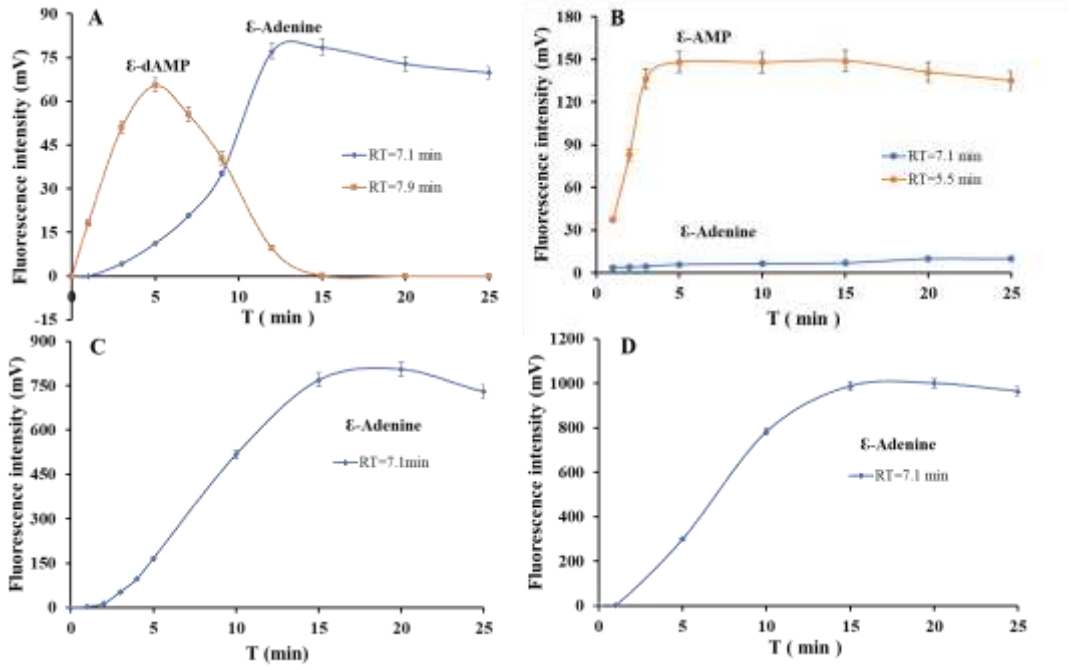


Figure 3. The reaction kinetic curves of 6.5×10^{-8} M dAMP (A), 1.2×10^{-7} M AMP (B), DNA (C) and microRNA (D) reacted with CAA at 100℃, respectively.

intermediate N^6-etheno, following protonation reaction. Evidence for adding H at N7 position is that the most stable tautomer in the gas phase is calculated to be the "N7" tautomer 7 (proton resides on the N7).[36] Protonation at N-7 can strongly catalyzes the hydrolysis of the N-glycosidic bond, making the adenine a better leaving group has been studied using density functional theory (DFT) methods.[37] Cleavage of the C-N glycosyl bond results in the formation of the oxocarbenium ion intermediate, and an electrically neutral ε-adenine electrophile adduct. Formation of the oxocarbenium ion intermediate in the process of depurination is supported by the strong resistance of RNA to depurination. The reason is the positive charge located on the C_2 carbon of ribose due to the presence of the strongly polarized C_2-O bond. Thus, formation of the oxocarbenium ion would require formation of a structure in which the two neighboring carbon atoms ($C_1$and C_2) are positively charged, which is obviously unfavorable. Cleavage of the glycosyl bond takes place most likely by participation of one pair of nonbonding electrons of the deoxyribose ring oxygen (the pair that is in the anti-orientation to the C-N glycosyl bond due to the anomeric effect). Whether ε-adenine can leave successfully, in other words, whether the glycoside bond can be cleaved depending on whether the oxocarbenium ion intermediate can be formed. Generally, the higher electronegativity of 2' position, the less stable of oxocarbonium ion intermediate.[27,28,38] For dAMP and DNA, both of them without 2-OH experienced the etheno modification and depurination. Then, the extremely reactive oxocarbenium ion adds a water molecule, giving deoxyribose as the final product (Figure S6a-b, Supporting Information). The attack of an electrophile (CAA) at the exocyclic 6-amino of AMP results in the formation of final product ε-AMP, which is not favorable to stabilize oxocarbenium ion to break the glycosyl bond (Figure S6c, Supporting Information). However, miRNA with 2'-OH, the coordination of water-mediated H-bond interaction between the proton at 2′ and the oxyanion at 3′ positions and the formed adenine adduct stabilized the oxocarbenium ion intermediate, leading to modification and depurination occur successfully (Figure 4).

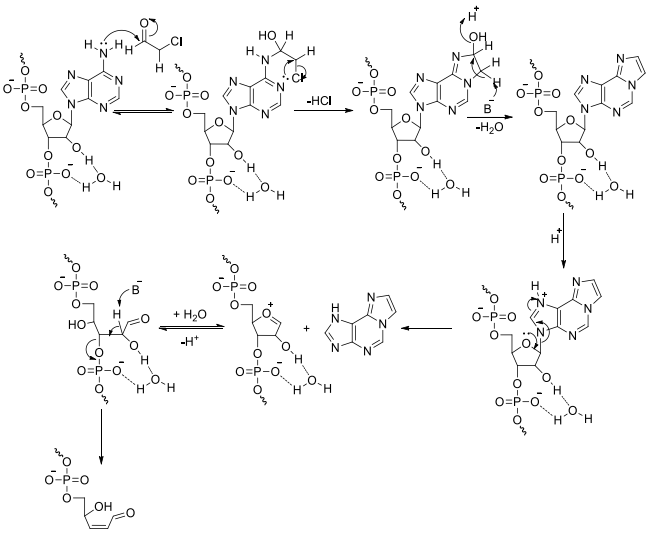


Figure 4. The mechanism pathway in the depurination of miRNA

Study on one pot reaction for miRNAs with different numbers of adenire

In order to figure out if the miRNA is able to be fully degraded into adenine in this one pot reaction, the reaction of two different kinds of miRNAs with different number of adenine bases with CAA at 100 ℃ was investigated. One is 5'-UAGCUUAUCGGACUGAUG UUGA-3' containing 5 adenine bases (5A) and the other is 5'-GUAAG GCAUCUGACCGAAGGCA-3' containing 7 adenine bases (7A). The results were shown in Figure 5, at the same concentration either 2.0 nM or 20 nM, the more adenine bases the miRNA contains, higher fluorescence peak (fluorescence intensity). Comparing miRNA with adenine, miRNA with 5 adenine bases

10.1002/chem.201905639

yields 5 times stronger fluorescence intensity as same concentration of adenine. Similarly, miRNA with 7 adenine bases yields 7 times higher fluorescence intensity as same concentration of adenine. When comparing different concentrations of miRNA, we found that 20 nM of miRNA present 10 times stronger fluorescence intensity as 2 nM does. Therefore, we draw the conclusion that the fluorescence intensity is proportional to the concentration of miRNA. It provides the basis for the quantification of miRNA.

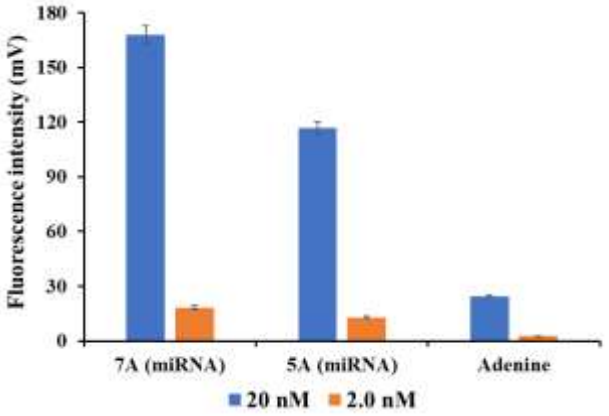
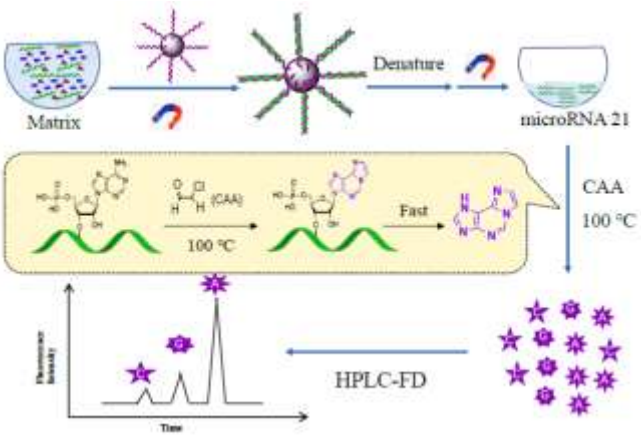


Figure 5. Study on specificity of derivatization reaction for miRNAs with different numbers of adenine bases. Standard adenine sample was carried out the same experimental procedure as miRNA.

Application of derivatization reaction for quantification of miRNA-21.

Based on the above finding, a method for detection of miRNA-21 was developed (Scheme 1). The working flow of the proposed miRNA assay is described as following: (1) DNA probe-magnetic beads were added to the sample, allowing the fully complementary target miRNA-21 be magnetically separated from the sample matrix by means of the formation of an miRNA-21-DNA probe-MB complex; (2) after the magnetic separation, miRNA-21 was released from the complex by denature; (3) miRNA-21 was then reacted with CAA, resulting in its completely derivatized and de-purined in one pot reaction of CAA, producing highly detectable fluorescent ε-adenine; and (4) ε-adenine was separated from other fluorescent derivatives and quantitatively determined by HPLC-FD.

Under the CAA derivatization and depurination conditions selected, the dynamic range and detection sensitivity for miRNA-21 assay were evaluated. Figure 6 shows HPLC-FD chromatograms obtained from CAA derivatization solutions of miRNA-21 at varying concentrations. As can be seen, ε-adenine was well separated with other fluorescent compound (ε-guanine) formed in the reaction, and the fluorescence intensity of ε-adenine was proportional to miRNA-21 concentration (inset in Figure 6). A linear calibration curve of fluorescence intensity versus miRNA-21 concentration was obtained in a concentration range from 0.28 nM to 126 nM. The linear regression equation is: FI= 6.6065 C + 1.4372, R^2=0.9999. Where FI is fluorescence intensity in mV, and C is miRNA-21 concentration in nM. The limit of detection was estimated to be 28 pM (S/N=3). To investigate the reproducibility of this HPLC-FD method, a 15 nM miRNA-21 solution was analyzed for 5 times. The fluorescence intensity and retention time of ε-adenine were recorded, respectively. The reproducibility (RSD) of fluorescence intensity was 1.2% and of retention time was 1.0% (n=5). These results indicate the proposed HPLC-FD method with CAA precolumn derivatization offers high sensitivity and good reproducibility. It can be useful for quantification of miRNAs.



Scheme1. Schematic diagram for the working flow of the proposed miRNA assay.

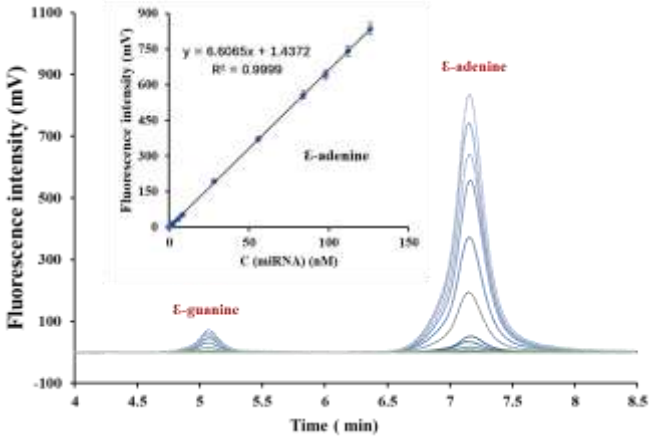


Figure 6. Chromatograms obtained from CAA derivatization solutions of miRNA-21 at concentrations from 0 to 126 nM. Inset is a plot of fluorescence intensity of ε-adenine against miRNA-21 concentration.

Selectivity of method for miRNA-21 assay

Multiple miRNAs are present in every biological specimen. Furthermore, since they chain length are short, they have highly similar sequences that differ by only a few nucleotides. Therefore, the methods that capable of performing single nucleotide polymorphism assays are high desired. In the present method, miRNA assay specificity is achieved via DNA probe/miRNA target hybridization and magnetic separation by using a DNA probe-magnetic bead conjugate. After collecting the magnetic beads easily by using an external magnet, the targeted miRNA is isolated /enriched from the sample matrix as a DNA probe/miRNA-magnetic bead complex. The complex is then denatured at 90 ℃ for 3 min, releasing miRNA into a clean chemical environment. Preparation of the conjugate from biotin-ssDNA complementary to miRNA-21 and streptavidin-magnetic beads was investigated (Figure S7 and Figure S8, Supporting Information).

The specificity of the proposed method was assessed by comparing HPLC-FD chromatograms obtained from standard solutions of miRNA-21, single-base mismatched miRNA, three-base mismatched miRNA, non-complementary miRNA, miRNA-141 and miRNA-155 (all at 28 nM). It is well known that hybridization depends on the temperature and double-stranded DNA/RNA is susceptible to denaturation at higher temperature.[38,39] Avoiding interference of base mismatch miRNAs by elevating hybridization

10.1002/chem.201905639

temperature was reported.[40] Therefore, hybridization at 50 ℃ was performed in this work. As shown in Figure 7, the fluorescence intensity from single-base mismatched microRNA and three-base mismatched microRNA were < 15% of that from miRNA-21 at the same concentration. Those from non-complementary miRNA, miRNA-141 and miRNA-155 were almost the same as that from a blank. These results indicate that the present method offers good selectivity for quantitative assay of target miRNAs.

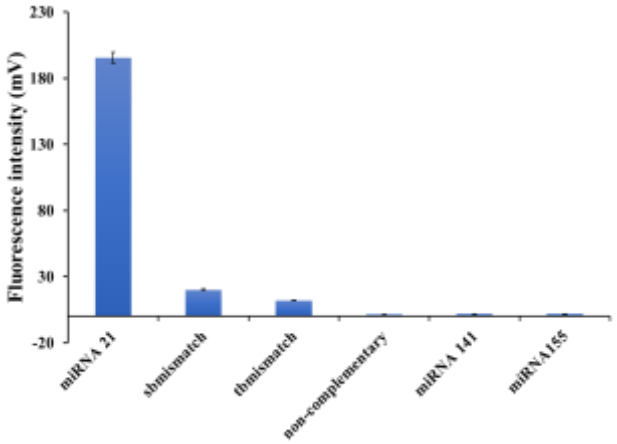


Figure 7. Specificity of the method for miRNA-21 quantification. The concentration of miRNA is 28 nM.

Quantification of miRNA-21 in serum samples

To access the applicability of the present assay in biological and clinical analysis it was applied to determine miRNA in bovine serum. In order to avoid degradation of microRNA by endogenous serum RNases, RNase inhibitor was added,[41] the RNase inhibitor was added to the 10 times diluted serum with binding buffer then the serum samples were spiked with miRNA-21 at 0.8 nM, 8.0 nM, and 80.0 nM. Since serum samples were very viscous, they had to be diluted to obtain a good efficiency of MSPE of miRNA-21 by using DNA probe-magnetic beads. Five replicate runs were conducted on 100 μL portions of each diluted serum sample to determine their miRNA-21 contents. Analytical data are summarized in Table 1. As can be seen, the recovery was found in the range of 94-106% with a good repeatability (RSD <4.0% in all three cases). These results showed that there was no interference with the quantification of miRNA-21 from the endogenous components in this sample matrix. It's worth noting that the targeted miRNAs were quantitatively isolated /enriched from this very "dirty" sample matrix by using DNA probe-magnetic beads via hybridization and magnetic separation, and that this isolation procedure was simple and quick. The biggest advantages of the present assay are its ease of operation and high repeatability, which is significantly better than PCR based quantitative assays.[7,8,42,43]

Table 1. Determination of miRNA-21 in bovine serum samples

Sample No.	Added (nM)	Found (nM)	Recovery (%)	RSD (%, n=5)
1	0	ND	-	-
2	0.80	0.85	106	3.8
3	8.0	7.5	94	2.5
4	80.0	78.0	97.5	3.3

ND: not detected.

To further improve the sensitivity of the present assay, the MSPE was utilized to enrich the miRNA-21 in serum samples. 2.0 mL bovine serum containing RNase inhibitor was spiked with miRNA at 5.0 pM, and then diluted with binding buffer (1+9). 200 μL DNA probe-magnetic beads were added in 20 mL diluted serum sample. The mixture was then incubated at 50 ℃ for 30 minutes. After the magnetic beads were collected and washed twice, miRNA-21 was released into 200 μL buffer after denatured. A portion of the solution (100 μL) was derivatized with CAA and then analyzed by HPLC-FD method. As shown in Figure S9 (Supporting Information), based on the ε-adenine fluorescence intensity of three replicate analyses, miRNA-21 concentration in serum samples was measured to be 4.9 ± 0.22 pM. Recovery was 98.0±4.4% (n=3), indicating the assay was accurate. It should be mentioned that based on the volume changes in MSPE (i.e. from 20 mL to 200 μL), miRNA-21 concentration increased 100 times. It's also worth noting that miRNA-21 concentration in these diluted serum samples was 0.5 pM, i.e. 500 fM. This detection limit is comparable or even better than some of detection method with complex enzymatic recycling amplification (Table S2).[44-49] These results indicate that the proposed assay may be useful for analysis of biological fluid samples such as urine and cell culture media to quantify miRNA targets at the fM level.

Conclusions

In summary, we report herein a one pot fluorescence derivatization strategy for microRNA with CAA, which has been proven to be a facile way to modify the adenine base and to depurinate the formed adenine adducts. This is the first experimental evidence that the cooperation of the formed ε-adenine and water-mediated H-bond interaction between the proton at 2′ and the oxyanion at 3′ positions significantly stabilized the oxocarbenium, and thus the microRNA is completely degraded into ε-adenine. Deploying this fluorescence derivatization strategy, a novel HPLC-FD method is developed for quantification of targeted miRNAs. The method involves no target conversion/cycling-based isothermal nor PCR-based signal amplification. In combination with a magnetic solid phase extraction (MSPE) of the miRNA target with its complimentary DNA probe modified magnetic beads, the proposed method is shown to be effective for quantification of trace miRNAs spiked into bovine serum samples at 500 fM without needing total RNA isolation, and with the capability of single base mismatch discrimination. Repeatability of the proposed assay (RSD) is < 4.5 %, which is better than most protocols previously reported. The proposed assay offers a facile and cost-effective means to quantify trace miRNAs in biological specimen.

Experimental Section

Chemicals and reagents

Chloroacetaldehyde dimethyl acetal, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), adenine, cytosine, guanine, hydroxymethyl aminomethane (Tris), bovine serum, triethylamine (TEA), acetic acid, ethylenediaminetetraacetic acid (EDTA), triton X-100, concentrated H_2SO_4 were obtained from Sigma-Aldrich. PBS tablets were purchased from Gibco. All microRNA or DNA were purchased from Sigma-Aldrich. And the sequences were listed in the Table S1. RNase inhibitor was purchased from New England Biolabs Ltd. Diethylpyrocarbonate (DEPC) treated water and Pierce™ Streptavidin magnetic beads (1 μm) were purchased from

Invitrogen. All miRNA stock solutions were prepared daily with DEPC-treated water in a RNase-free environment. Binding/wash buffer was composed of 10 mM Tris, 2 M NaCl, 1 mM EDTA, and 0.0005% Triton X-100 (pH 7.5). To avoid the effect of RNase on the stability of miRNA solutions, all centrifuge tubes, pipette tips, and buffers were autoclaved before use.

HPLC-FD analysis

Samples were analyzed using a Shimadzu 20A HPLC system that consists of a LC-20 AD liquid chromatograph, a DGU-20A5 degasser, a LC-20AHT auto sampler, an RF-10AXL fluorescence detector, and a CTO-20A column oven (Shimadzu, Nakagyo-Ku, Kyoto, Japan). An ODS column (C_{18}, 250 x 4.60 mm, particle size 5 μm) from Phenomenex was used. The mobile phase consisted of 0.1 M triethylammonium acetate buffer (TEAA) at pH 7.0 and acetonitrile (93:7 v/v). Separation was performed under isocratic conditions at a flow rate of 1.0 ml min^{-1}. Column temperature was maintained at 30 °C. For fluorescence detection, the excitation and emission wavelengths were set at 275 and 411 nm, respectively. Sample volume injected was 10 μL.

LC-MS analysis

C18 column (5 cm×2.1 mm, particle size 3μm) was used as separation column, 5%AcCN in water with 0.1% formic acid was used as mobile phase. Separation was performed under isocratic conditions at a flow rate of 0.2 ml min^{-1}.

Preparation of CAA [50]

Concentrated H_2SO_4 (1.0 mL) was diluted 10-times with distilled H_2O and added to 10.0 mL chloroacetaldehyde dimethyl acetal. The mixture was distilled slowly under a fume hood and the distillate fraction containing chloroacetaldehyde (at ca. 1.5 M) was collected at 80-85 °C. The reagent solution was stored in darkness at 4 °C.

Preparation of DNA probe-magnetic bead conjugate

Streptavidin-magnetic bead suspension was washed twice by binding/washing buffer, and then resuspended in binding buffer. Biotin-DNA probe solution (5×10^{-4} M, 5 μL) was added to the above solution (100 μL), and the mixture was shaken at 37 ℃ for 30 min. The magnetic beads were collected by using an external magnet. The beads were washed with 1xPBS solution three times to remove any unbound biotin-DNA probe. DNA probe-magnetic bead conjugate was resuspended in 1.0 mL binding/washing buffer.

The miRNA derivatization and depurination with CAA

A portion of sample solution (100 μL) was mixed with 100 μL 1×PBS buffer in a vial. The CAA solution prepared above (20 μL) was added. The mixture was heated at 100 °C (in a boiling water bath) for 15 min. The vial was then placed on ice to cool down. The fluorescence wavelength maxima were set at λ_{ex} = 275 nm and λ_{em} = 411 nm.

Quantitative of miRNA-21

Sample solution (100 μL) was added to DNA probe-magnetic bead solution (200 μL) in a vial. The mixture was maintained at 50 °C for 30 min to ensure a complete hybridization.[51] The mixture was magnetically separated and the decanted supernatant. The DNA/RNA-magnetic bead complex was washed twice with binding buffer and then denature at 90 ℃ for 3 min. After a magnetic separation, the supernatant containing miRNA-21 was transferred to a new vial. Then, 100 μL of 1×PBS, 20 μL of CAA solution were added. The solution was heated at 100 °C (in a boiling water bath) for 15 min. The reaction vials were removed from boiling water bath and placed on ice to cool down. Portions (10 μL) of the derivatization solution were injected into the system for HPLC-FD analysis.

Acknowledgements

Financial support from the U.S. National Institutes of Health (GM089557 to YML) and the National Natural Science Foundation of China (No. 21874030 to S. Z) are gratefully acknowledged.

Conflict of interest

The authors declare no conflict of interest.

Keywords: microRNA · etheno-adenine · one pot reaction · magnetic solid phase extraction · fluorescence detection.

[1] D. P. Bartel, *Cell* **2004**, *116*, 281-297.
[2] V. Ambros, *Nature* **2004**, *431*, 350-355.
[3] A. Lujambio, S. W. Lowe, *Nature* **2012**, *482*, 347-355.
[4] C. L. Bartels, G. J. Tsongalis, *Clin. Chem*. **2009**, *55*, 623-631.
[5] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, T. Tuschl, *Science* **2001**, *294*, 853-858.
[6] J. M. Thomson, J. Parker, C. M. Perou, S. M. Hammond, *Nat. Methods* **2004**, *1*, 47-53.
[7] H. Jia, Z. Li, C. Liu, Y. Cheng, *Angew. Chem., Int. Ed*. **2010**, *49*, 5498-5501.
[8] C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, *Nucleic Acids Res*. **2005**, *33*, e179.
[9] R. Deng, L. Tang, Q. Tian, Y. Wang, L. Lin, J. Li, *Angew. Chem., Int. Ed*. **2014**, *53*, 2389-2393.
[10] H. Jia, Z. Li, C. Liu, Y. Cheng, *Angew. Chem., Int. Ed*. **2010**, *49*, 5498-5501.
[11] H. M. Choi, J. Y. Chang, L. A. Trinh, J. E. Padilla, S. E. Fraser, N. A. Pierce, *Nat. Biotechnol*. **2010**, *28*, 1208-1212.
[12] Z. Cheglakov, T. M. Cronin, C. He, Y. Weizmann, *J. Am. Chem. Soc*. **2015**, *137*, 6116-6119.
[13] C. Li, Z. Li, H. Jia, J. Yan, *Chem. Commun*. **2011**, *47*, 2595-2597.
[14] C. Shi, Q. Liu, C. Ma, W. Zhong, *Anal. Chem*. **2014**, *86*, 336-339.
[15] B. C. Yin, Y.Q. Liu, B. C. Ye, *J. Am. Chem. Soc*. **2012**, *134*, 5064-5067.
[16] D. Proudnikov, A Mirzabekov, *Nucleic Acids Res*. **1996**, *24*, 4535-4532.
[17] R. R. Hodges, N. E Conway, L. W. McLaughlin, *Biochemistry* **1989**, *28*, 261-267.
[18] P. J. Oefner, C. G Huber, F. Umlauft, G. N. Berti, E. Stimpfl, G. K. Bonn, *Anal. Biochem*. **1994**, *223*, 39-46.
[19] L. Reyderman, S. Stavchansky, *J. chromatogr. A*. **1996**, *755*, 271-280.
[20] Z. Jahnz-Wechmann, G. R. Framski, P. A. Januszczyk, J. Boryski, *Front. Chem*. **2016**, *4*, 19.
[21] G. Avigad, S. Damle, *Anal. Chem*. **1972**, *50*, 321-323.
[22] J. Nair, A. Gal, S. Tamir, S. Tannenbaum, G. Wogan, H. Bartsch, *Carcinogenesis* **1998**, *19*, 2081-2084.
[23] N. K. Kochetkov, V. N. Shibaev, A. A. Kost, *Tetrahedron Lett*. **1971**, *22*, 1993-1996.

[24] F. Oesch, G. Doerjer, *Carcinogenesis*, **1982**, *3*,663-665.

[25] E. R. Garett, P. J. Mehta, *J. Am. Chem. Soc.* **1972**, *94*, 8532-8541.

[26] J. L. York, *J. Org. Chem.* **1981**, *46*, 2171-2173.

[27] J. L. York, *J. Org. Chem.* **1981**, *46*, 2171-2173.

[28] H. Åström, E. Limén, R. Strömberg, *J. Am. Chem. Soc.* **2004**, *126*, 14710-14711.

[29] P. H. Bolton, D.R. Kearns, *Biochim. Biophys. Acta* **1978**, *517*, 329-337.

[30] J. Pitha, *Biochemistry* **1970**, *9*, 3678-3682.

[31] P. Acharya, J. Chattopadhyaya, *J. Org. Chem.* **2002**, *67*, 1852-1865.

[32] J. Biernat, J. Ciesiolka, P. Gornicki, R. W. Adamiak, W. J. Krzyzosiak, M. Wiewiorowski, *Nucleic Acids Res.* **1978**, *5*, 789-804.

[33] W. J. Krzyzosiak, J. Biernat, J. Ciesiolka, P. Gornicki, M. Wiewiorowski, *Polish. J. Chem.* **1983**. *57*, 779.

[34] A. A. Kost, M. V. Ivanov, *Chem. Heterocyclic Comp*. **1980**, *16*, 209-221.

[35] M. Liu, M. Xu, J. K. Lee, *J. Org. Chem.* **2008**, *73*, 5907-5914.

[36] R. Rios-Font, L. Rodríguez-Santiago, J. Bertran, M. Sodupe, *J. Phys. Chem. B* **2007,** *111*, 6071-6077.

[37] V. E. Marquez, C. K. H. Tseng, H. Mitsuya, S. Aoki, J. A. Kelley, H. Ford, J. S. Roth, S. Broder, D. G. Johns and J. S. Driscoll, *J. Med. Chem.* **1990**, *33*, 978-985.

[38] J. Wang, X. Yi, H. Tang, H. Han, M. Wu, F. Zhou, *Anal. Chem*. **2012**, *84*, 6400-6406.

[39] I. Lee, S. S. Ajay, H. Chen, A. Maruyama, N. Wang, M. G. Mclnnis, B.D. Athey, *Nucleic Acids Res*. **2008**, *36*, 27.

[40] N. Xia, L. Zhang, G. Wang, Q. Feng, L. Liu, *Biosens. Bioelectron*. **2013**, *47*, 461-466.

[41] E. M. Kroh, R. K. Parkin, P. S. Mitchell, M. Tewari, *Methods*, **2010**, *50*, 298-301.

[42] C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak, K. J. Guegler, *Nucleic Acids Res*. **2005**, *33*, 179-185.

[43] N. Redshaw, T. Wilkes, A. Whale, S. Cowen, J. Huggett, C. A. Foy, *Biotech*. **2013**, *54*, 155-164.

[44] H. Xu, S. Zhang, C. Ouyang, Z. Wang, D. Wu, Y. Liu, Y. Jiang, Z. Wu, *Talanta*, **2019**, *192*, 175-181.

[45] H. Xu, Y. Zhang, S. Zhang, M. Sun, W. Li, Y. Jiang, Z. S. Wu, *Anal. Chim. Acta* **2019**, *1047*, 172-178.

[46] S. J. Zhen, X. Xiao, C. H. Li, C. Z. Huang, *Anal. Chem.* **2017**, *89*, 8766-8771.

[47] L. Qi, M. Xiao, X. Wang, C. Wang, L. Wang, S. Song, X. Qu, L. Li, J. Shi, H. Pei, *Anal. Chem.* **2017**, 89, *18*, 9850-9856.

[48] S. Xu, Y. Nie, L. Jiang, J. Wang, G. Xu, W. Wang, X. Luo, *Anal. Chem.* **2018**, *90*, 4039-4045.

[49] X. Tang, R. Deng, Y. Sun, X. Ren, M. Zhou, J. Li, *Anal. Chem.* **2018**, *90*, 10001-10008.

[50] B. Levitt, R. J. Head, D. P. Westfall, *Anal. Biochem*. **1984**, *137*, 93-100.

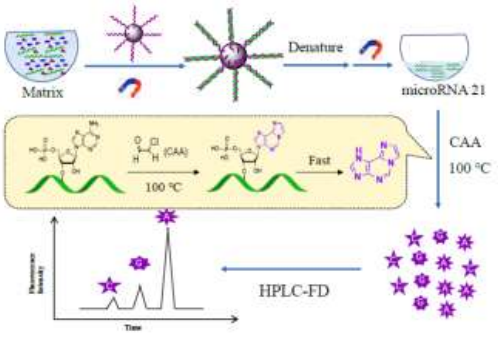
[51] X. Qiu, X. Liu, W. Zhang, H. Zhang, T. Jiang, D. Fan, Y. Luo, *Anal. Chem*. **2015**, *87*, 6303-6310.

10.1002/chem.201905639

Graphical Material

A Novel One Pot Fluorescence Derivatization Strategy for Highly Sensitive MicroRNA Analysis

Li Pan, Huaisheng Zhang, Jingjin Zhao, Ifedayo Victor Ogungbe, Shulin Zhao,* and Yi-Ming Liu*_____ Page – Page



A one pot fluorescence derivatization strategy was developed for highly sensitive miRNA analysis. Based on this derivatization strategy, a facile and sensitive high-performance liquid chromatography (HPLC) method was developed for quantitative assay of microRNAs.