**Introduction**

Ribonucleic acid (RNA) plays many important roles in biological processes, and the analysis of sequence specific RNA has a major impact in diverse areas, such as clinical diagnosis and biological research.1–4 Although the structure and recognition chemistry of RNA is similar to DNA, the hydroxyl group at position 2 of RNA makes it much less stable than DNA, so that many DNA detection methods are too harsh to practically apply to RNA. Traditional methods for RNA analysis are carried out by Northern blotting,5 in situ hybridization,6 and microarray hybridization.7 However, all these approaches have several drawbacks that limit their implementation for wider and more versatile applications. For example, low sensitivity and selectivity; inconvenience; large amounts of sample required; complicated experimental processes; or the using of hazard and radioactive reagents.

The invention of the polymerase chain reaction (PCR) technique has made a tremendous impact on the RNA assay. With reverse-transcription polymerase chain reaction (RT-PCR) magnification, a trace amount of RNA could be amplified and large amounts of copies of a target RNA sequence were produced across several orders of magnitude. However, the PCR technique for RNA detection encountered problems of complicated procedures, high cost and easy contamination, which have been hampering its application in many laboratories.8–14 Therefore, continuing efforts have been made to seek ideal tools for fast, sensitive, cost-effective detection of sequence specific RNA.

Several isothermal amplification strategies have been developed to overcome the limitations of PCR-based methods, such as loop-mediated isothermal amplification (LAMP),15 rolling circle amplification (RCA),16 and exponential amplification reaction (EXPAR).13,17 Nicking enzyme signal amplification (NESA) is based on nicking endonuclease (NEases); the method can be used to recognize specific nucleotide sequences in double-stranded DNA (dsDNA) and catalyze the cleavage of only one strand of a dsDNA at a fixed position relative to the recognition sequence. The NEase cleavage of the probe induces the signaling element to generate detection signals, and the target DNA or RNA is concurrently recycled for the next round of events. NESA has shown great potential in biosensor amplification.18,19

**Keywords** NESA, G-quadruplex DNAzyme, BMP6 mRNA

(Received July 30, 2014; Accepted September 22, 2014; Published November 10, 2014)
Table 1  The oligonucleotide sequences

| Oligonucleotide | Sequence |
|-----------------|----------|
| Target RNA      | 5'-AGG AAG GCCG UCG UGC GC-3' |
| Template T*-X-T* | 5'-GCA GGA CAG CGC CCT CTT AAT AAG AGA GCA GCC CCT TTC CT-3' |
| Haipin probe    | 5'- TTC CAG AAA GCC CCA CTG CCC ACT CTG CAG ACT TCG GGT TGG AAT TTT GCG AGG ACA GCG CCT TCC TTC T-3' |
| M1-5'           | 5'-AGU AGC GCCG UCG UGC GC-3' |
| M1-3'           | 5'-AGA AAG GCCG UCG UGC GC-3' |
| M1-M            | 5'-AGA AAG UCC GCC UGC GC-3' |
| M2              | 5'-AGA AAG UGG CUG UAC UGA GC-3' |
| M3              | 5'-AGA UGG CUG UAC UGA GC-3' |
| M4              | 5'-AGC AAG UCG CUG UAC UGA GC-3' |

Experimental

Reagents and chemicals

The Nicking endonuclease (Nt.BstNBI), Vent (exo-) DNA polymerase, and deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs (Beverly, MA). The RNase inhibitor and diethyl pyrocarbonate (DEPC)-treated water were obtained from Takara Bio Inc. (Dalian, China). A supply of 3,5,5,5'-tetramethylbenzidine (TMB) was obtained from Guangzhou Mbchem Technology Co., Ltd. (Guangzhou, China). Other chemicals were purchased from standard commercial sources and were of analytical grade. In order to create and maintain an RNase-free environment, DEPC-treated water was used in all experiments. A Synergy HT Multi-Mode Microplate Reader (Winooski, VT) was used to record the absorbance spectrum and absorbance of samples. All buffer solutions used in this work were prepared in our lab using distilled water purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA).

The RNA sequence and the oligonucleotide sequences were synthesized and purified through HPLC by Takara Bio Inc. (Dalian, China). The oligonucleotide sequences are listed in Table 1.

Two-stage NESA reaction

The two-stage NESA reaction mixtures for RNA assay were prepared separately on ice as Part A, Part B, and Part C. Part A consisted of a 200 nM T*-X-T* template, 300 μM dNTPs, 0.5 × Nt.BstNBI buffer (25 mM Tris–HCl, pH 7.9, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM dithiothreitol) and different concentrations of target RNA. Part B consisted of 0.8 U/μL RNase inhibitor, 0.8 U/μL NL.BstNBI nicking endonuclease, 0.1 U/μL Vent (exo-) polymerase, 1 × ThermoPol buffer (20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100) and DEPC-treated water. Part C consisted of a 300 nM hairpin probe, 0.8 U/μL NL.BstNBI nicking endonuclease, 0.1 U/μL Vent (exo-) polymerase, 1 × ThermoPol buffer and DEPC-treated water.

Part A solution was first heated at 95°C for 3 min to denature the T*-X-T* template and the RNA target. After part A was cooled down to 55°C, part B solution was added. The reaction of Part A with Part B was performed at 55°C for 30 min for the exponential amplification to generate the single strand fragment T₀.

After the reaction finished, part C was added immediately to the reaction mixtures; the reaction was performed at 52°C in a total volume of 20 μL for 30 min.

The reaction mixture and 10 μL of hemin stock solution (1 μM) were added to 50 μL of reaction solution containing 10 mM HEPES (pH 7.4), 8 mM KCl, 80 mM NaCl, 0.02% Triton X-100 (w/v), and 0.04% DMSO (v/v), then vortexed to mix well. The mixture was incubated at room temperature for 1 h, the hemin then intercalated into the G-quadruplex to form HRP-mimicking G-quadruplex DNAzyme. After that 10 μL TMB-H₂O₂ solution was added to the mixture and incubated at room temperature for 5 min. The reaction was terminated with 50 μL of H₂SO₄ (2 M). The reaction solutions were transferred to the 96-well microplate to monitor UV-vis absorption spectra.

To optimize the molar ratio of the two templates, various ratios of T*-X-T* template to hairpin probe (the total concentration of two templates was 500 nM) was added to the part A and part C solutions, respectively. The concentration of target RNA was 1 nM. The amplification products were analyzed with the same method described above.

To optimize the concentration of hemin, different concentrations of hemin were added to the reaction solution and the optical responses were analyzed with the same method described above.

Results and Discussion

Principle of RNA detection

The principle of two-stage NESA signal amplification for RNA assay is illustrated in Fig. 1. The two-stage NESA involves two templates and two-stage amplification reactions under isothermal conditions. The T*-X-T* template, used for exponential amplification, consists of three domains: two repeat RNA binding domain T*, and an endonuclease recognition domain X. Two repeat domain T* is separated by domain X. The hairpin probe has a stem-loop structure and also has three domains: domain T* at 3' end, domain X and domain G* (at 5' end) which includes the sequence that is complementary to the G-quadruplex DNAzyme. As a proof of concept, a short RNA sequence related to bone morphogenetic protein 6 (BMP6) was chosen as the detection target.

In the presence of target RNA, it recognizes and hybridizes with the domain T* of the T*-X-T* template, causing the target RNA to become extended along the template to form a complete duplex in the presence of Vent (exo-) DNA polymerase and dNTPs. Following the polymerization, a stable double-stranded DNA duplex is produced and the replicated strand contains a recognition site for the nicking enzyme Nt.BstNBI. The cleavage of the upper DNA strand by nicking enzyme Nt.BstNBI generated a single-stranded DNA T₀. DNA T₀ has the same sequence as the target RNA except for the change of U to T, and the change of ribonucleotides to deoxyribonucleotides. Through the exponential NESA amplification comprising extension, cleavage, and the release of DNA T₀, a large number of DNA T₀ is released (first-stage NESA).

The releasing DNA T₀ might function as the primer to anneal with the domain T* of the hairpin probe, subsequently a new linear NESA reaction is initiated. The polymerization reaction
is initiated upon hybridization between DNA T₀ and the domain T* of the hairpin probe at 3’ terminus in the presence of polymerase and dNTPs. During the cyclic reaction containing hybridization of DNA T₀, polymerization, cleavage, and the release of G-quadruplex sequence, a large amount of fragments of HRP-mimicking G-quadruplex DNAzyme was produced (second-stage NESA). Once hemin is added into the reaction mixture after amplification, The G-quadruplex interacts with hemin and generates the activated DNAzyme that can catalyze the oxidation reaction of 3,3’5,5’-tetramethylbenzidine (TMB) by H₂O₂, thus the target RNA can be sensitively detected using a spectrophotometer.

In the absence of target RNA, no G-quadruplex is produced, leading to a negative signal.

**Signal amplification by first-stage NESA**

The first-stage NESA (exponential amplification, EA) was used to produce a large amount of DNA T₀ and transfer the target RNA sequence to a more stable DNA sequence. In order to confirm that the first-stage NESA does take place as expected for amplification of RNA detection signal, we investigated the absorption spectrum response to 1 nM and 1 fM target RNA with and without EA. In the case without EA, a mixed reaction buffer solution was added into the tube without the T*-X-T* template. Figure 2 shows the typical absorption spectrum response of 1 nM and 1 fM target RNA with EA and without EA. In the presence of 1 n M target RNA, the peak absorbance of the sample with EA was about 3 times greater than that without EA. When the target RNA decreased to as low as 1 fM, the peak absorbance of the sample with EA still had an obvious signal response, while the peak absorbance of the sample
The first-stage NESA is of central importance to improve the detection sensitivity. Optimization of the experimental conditions was observed. However, when the ratio of the T*-X-T* template was 0.2, 0.3, 0.5, 0.6, 0.7, and 0.8, the absorbance decreased even in the presence of 1 nM target RNA. The lower optical response at lower molar ratio (less than 0.4) of the T*-X-T* template can be explain by the following two reasons: (1) The low concentration of the T*-X-T* template led to low DNA T0 and G-quadruplex production. (2) The high concentration of the hairpin probe increases the background signal due to the non-specific amplification by DNA polymerase. The lower optical response at higher molar ratio (higher than 0.4) of the T*-X-T* template can contribute to two factors. (1) The high concentration of the T*-X-T* template produced a large amount of DNA T0, but the low concentration of hairpin can not efficiently convert the DNA T0 to G-quadruplex. (2) The high concentration of the T*-X-T* template might lead to the high background signal due to the non-specific amplification by DNA polymerase. Therefore, the optimal molar ratio of the T*-X-T* template was chosen to be 0.4 in the following experiments.

Another factor that affects the sensitivity of the assay is the concentration of hemin. As shown in Fig. 3B, the absorbance increased gradually with the increasing of hemin concentration from 0.6 to 1.0 μM in the presence of 1 nM target RNA, indicating more hemin intercalated in the G-quadruplex DNAzyme. Correspondingly, the catalytic activity of the detection system was greatly enhanced and the optical intensity produced by TMB increased greatly. Further increasing the concentration of hemin resulted in a decrease of absorbance. Higher concentration of hemin led to the high background signal from hemin itself. Therefore, 1.0 μM of hemin was selected as the working solution throughout the experiments to obtain the highest signal.

Quantitative analysis of target RNA

Under optimum conditions, we examined the sensitivity and dynamic range of the assay with eight concentrations (0, 0.01 fM, 1 fM, 100 fM, 1 nM, 100 nM, and 10 μM) of target RNA by a spectrophotometer. As shown in Fig. 4A, the absorbance increased as the concentration of the target RNA increased in the range from 0.01 fM to 10 μM. In logarithmic scales, the absorbance change ΔA (ΔA = A – A0, A: sample absorbance; A0: background absorbance) exhibits a linear correlation with RNA concentration over a range of 5 orders of magnitude from 1 fM to 100 nM (Fig. 4B). The regression equation is ΔA = 2.0197 + 0.1242 Ln [mRNA], with a regression coefficient (R²) of 0.9896. An obvious absorbance change (more than three times the standard deviation of the blank) was observed when 0.01 fM target RNA was present. Therefore, 0.01 fM of target RNA is considered as the detection limit (LOD), which is 2 orders of magnitude than the existing assay based on NESA and DNAzyme amplification.17

Specificity of the RNA assay

The specificity of the RNA assay was investigated by testing a number of synthetic target sequences with one, two, three, and four mutations, such as: perfectly matched target RNA (PM), a single-base mismatched target RNA at 5’ end. (M1-5’), a single-base mismatched target RNA at 3’ end. (M1-3’), a single-base mismatched target RNA in the middle of the sequences (M1-M), two-base mismatched target RNA (M2), three-base mismatched target RNA (M3), four-base mismatched target RNA (M4), and blank. As shown in Fig. 5, the absorbance response from perfectly matched RNA was about 5 times greater than that of a

---

Without EA was almost the same as the negative control. Linear NESA can amplify the detection signal if the target RNA is high enough (such as 1 nM). But the sensitivity of the linear NESA is considerably low. These experimental results showed that the first-stage NESA is of central importance to improve the detection sensitivity.

Optimization of the experimental conditions

In this assay, the first T*-X-T* template was used to exponentially amplify the target RNA and produce a large amount of DNA T0 fragment. The hairpin probe was used to convert the RNA and DNA T0 fragment to the HRP-mimicking G-quadruplex DNAzyme. Since the total concentration of the two templates was kept constant in the assay, the ratio of the two templates is one of the most important factors to affect the sensitivity and selectivity of the assay. In addition, the two templates contained the same domain of T*, which can competitively hybridize with RNA and T0 fragment, consequently influencing the production of the G-quadruplex DNAzyme. Therefore, it was necessary to optimize the molar ratio of the two templates.

We have investigated different molar ratios (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8) of the T*-X-T* template with 1 nM target RNA. The results are presented in Fig. 3A, at a molar ratio of the T*-X-T* template of 0.4, the maximum optical response was observed. However, when the ratio of the T*-X-T* template was 0.2, 0.3, 0.5, 0.6, 0.7, and 0.8, the absorbance decreased even in the presence of 1 nM target RNA. The lower optical response at lower molar ratio (less than 0.4) of the T*-X-T* template can be explain by the following two reasons: (1) The low concentration of the T*-X-T* template led to low DNA T0 and G-quadruplex production. (2) The high concentration of the hairpin probe increases the background signal due to the non-specific amplification by DNA polymerase. The lower optical response at higher molar ratio (higher than 0.4) of the T*-X-T* template can contribute to two factors. (1) The high concentration of the T*-X-T* template produced a large amount of DNA T0, but the low concentration of hairpin can not efficiently convert the DNA T0 to G-quadruplex. (2) The high concentration of the T*-X-T* template might lead to the high background signal due to the non-specific amplification by DNA polymerase. Therefore, the optimal molar ratio of the T*-X-T* template was chosen to be 0.4 in the following experiments.

Another factor that affects the sensitivity of the assay is the concentration of hemin. As shown in Fig. 3B, the absorbance increased gradually with the increasing of hemin concentration from 0.6 to 1.0 μM in the presence of 1 nM target RNA, indicating more hemin intercalated in the G-quadruplex DNAzyme. Correspondingly, the catalytic activity of the detection system was greatly enhanced and the optical intensity produced by TMB increased greatly. Further increasing the concentration of hemin resulted in a decrease of absorbance. Higher concentration of hemin led to the high background signal from hemin itself. Therefore, 1.0 μM of hemin was selected as the working solution throughout the experiments to obtain the highest signal.

Quantitative analysis of target RNA

Under optimum conditions, we examined the sensitivity and dynamic range of the assay with eight concentrations (0, 0.01 fM, 1 fM, 100 fM, 1 nM, 100 nM, and 10 μM) of target RNA by a spectrophotometer. As shown in Fig. 4A, the absorbance increased as the concentration of the target RNA increased in the range from 0.01 fM to 10 μM. In logarithmic scales, the absorbance change ΔA (ΔA = A – A0, A: sample absorbance; A0: background absorbance) exhibits a linear correlation with RNA concentration over a range of 5 orders of magnitude from 1 fM to 100 nM (Fig. 4B). The regression equation is ΔA = 2.0197 + 0.1242 Ln [mRNA], with a regression coefficient (R²) of 0.9896. An obvious absorbance change (more than three times the standard deviation of the blank) was observed when 0.01 fM target RNA was present. Therefore, 0.01 fM of target RNA is considered as the detection limit (LOD), which is 2 orders of magnitude than the existing assay based on NESA and DNAzyme amplification.17

Specificity of the RNA assay

The specificity of the RNA assay was investigated by testing a number of synthetic target sequences with one, two, three, and four mutations, such as: perfectly matched target RNA (PM), a single-base mismatched target RNA at 5’ end. (M1-5’), a single-base mismatched target RNA at 3’ end. (M1-3’), a single-base mismatched target RNA in the middle of the sequences (M1-M), two-base mismatched target RNA (M2), three-base mismatched target RNA (M3), four-base mismatched target RNA (M4), and blank. As shown in Fig. 5, the absorbance response from perfectly matched RNA was about 5 times greater than that of a
single-base mismatched RNA (M1-5′, M1-3′ or M1-M) in the presence of 1 nM RNA. The dramatic absorbance difference between perfectly matched RNA and mismatched RNA indicated that this RNA assay has good specificity to distinguish a single-base mismatched RNA target.

Conclusions

In conclusion, we have developed a convenient and feasible RNA sensing system for quantitative detection of RNA based on two-stage NESA and DNAzyme amplification. Compared to other reported methods based on these detection strategies, the sensitivity of the RNA detection assay developed here is higher than the existing assay based on NESA and DNAzyme amplification. By using this assay, as low as 0.01 fM target RNA could be detected and the linear range was from 1 fM to 100 nM. Besides, this proposed approach is label-free and the optical signal could be easily recorded by a spectrophotometer. Based on these advantages, this new RNA detection approach is expected to serve as a platform for searching for target sequences in samples of genomic RNA. We believe that this RNA assay approach will find wide application in molecular biology and clinical diagnosis.

Acknowledgements

Financial support for this study was provided by the National Natural Science Foundation of China (project: 81160219).

References

1. B. R. Cullen, Mol. Cell, 2004, 16, 861.
2. J. A. Lednicky, Arch. Pathol. Lab. Med., 2003, 127, 30.
3. W. W. Fischer, Nature, 2008, 455, 1051.
4. K. Hirota, T. Miyoshi, K. Kugou, C. S. Hoffman, T. Shibata, and K. Ohta, Nature, 2008, 456, 130.
5. A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen, and Z. Havelka, Nucleic Acids Res., 2004, 32, e175.
6. J. T. Pena, C. Sohn-Lee, S. H. Rouhanifard, J. Ludwig, M. Hafner, A. Mihailovic, C. Lim, D. Holoch, P. Berninger, M. Zavolan, and T. Tuschl, Nat. Methods, 2009, 6, 139.
7. L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, and J. M. Johnson, Nature, 2005, 433, 769.
8. J. Baner, M. Nilsson, M. Mendel-Hartvig, and U. Landegren, Nucleic Acids Res., 1998, 26, 5073.
9. R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich, Sciences (N.Y.), 1988, 239, 487.
10. R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim, Sciences (N.Y.), 1985, 230, 1350.
11. B. C. Yin, Y. Q. Liu, and B. C. Ye, Anal. Chem., 2013, 85, 11487.
12. J. Li, B. Yao, H. Huang, Z. Wang, C. Sun, Y. Fan, Q. Chang, S. Li, X. Wang, and J. Xi, Anal. Chem., 2009, 81, 5446.
13. Y. Zhang and C. Y. Zhang, Anal. Chem., 2012, 84, 224.
14. G. L. Wang and C. Y. Zhang, Anal. Chem., 2012, 84, 7037.
15. C. Li, Z. Li, H. Jia, and J. Yan, Chem. Commun. (Cambridge, U. K.), 2011, 47, 2595.
16. Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang, and Y. Zhang, Angew. Chem., Int. Ed. Engl., 2009, 48, 3268.
17. X. P. Wang, B. C. Yin, P. Wang, and B. C. Ye, Biosens. Bioelectron., 2013, 42, 131.
18. P. J. Asiello and A. J. Baeumner, Lab Chip, 2011, 11, 1420.
19. H. Zhang, F. Li, B. Dever, X.-F. Li, and X. C. Le, Chem. Rev., 2012, 113, 2812.
20. L. Qiu, C. Wu, M. You, D. Han, T. Chen, G. Zhu, J. Jiang, R. Yu, and W. Tan, J. Am. Chem. Soc., 2013, 135, 12952.
21. Y. Li, X. Li, X. Ji, and X. Li, Biosens. Bioelectron., 2011, 26, 4095.
22. J. Liu, C. Y. Lu, H. Zhou, J. J. Xu, Z. H. Wang, and H. Y. Chen, Chem. Commun. (Cambridge, U. K.), 2013, 49, 6602.
23. J. Li, Q. H. Yao, H. E. Fu, X. L. Zhang, and H. H. Yang, Talanta, 2011, 85, 91.