A Novel DNAzyme Signal Amplification-Based Colorimetric Method for RNase H Assays

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Abstract

A simple visual strategy was developed for the RNase H colorimetric measurement using DNAzyme-mediated signal amplification. When RNase H was presented, the RNA strand of the duplex formed by the G-rich DNA sequence (G-Rich) and its complementary RNA sequence (cp-RNA) was digested, releasing G-Rich to form HRP-mimicking DNAzymes of the G-quadruplex/hemin complexes in the presence of hemin. These DNAzymes catalyze the oxidation reaction of the substrate of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to produce green color products of ABTS•+, allowing for the detection of RNase H. A horseradish peroxidase (HRP)-mimicking DNAzyme of the G-quadruplex/hemin complex was used to mediate the signal amplification in the sensing strategy, resulting in high selectivity and sensitivity. This proposed colorimetric method shows a low detection limit of 0.04 U/mL, with a detection range of 0.1 to 3 U/mL. Moreover, this colorimetric method has been successfully used for RNase H assays in complicated biosamples, such as cell lysates. These results indicate that our colorimetric method not only detects RNase H in an ideal system but also in real samples.

Keywords RNase H, DNAzyme, G-quadruplex/hemin, colorimetric, signal amplification.
Introduction

RNase H (Ribonuclease H), a highly conservative endogenous ribonuclease that attracts significant attention and is studied worldwide due to its biological functions, has been implicated in diseases\(^1\)\(^-\)\(^3\), primarily because RNase H participates in biological processes involving replication\(^4\),\(^5\), repair\(^6\), and reverse virus transcription\(^7\),\(^8\). RNase H can only specifically hydrolyze the phosphodiester bonds in the RNA strand of a heterogenous DNA/RNA duplex\(^9\),\(^10\). In past decades, detection and quantification methods of RNase H, like gel\(^11\) or capillary\(^12\) electrophoresis, HPLC\(^13\), and fluorometry\(^14\) have been mainly constructed of cumbersome and infrastructure-heavy analyses, thus limiting the application of these methods in resource-poor environments. A simple robust method for RNase H measurement is still urgently needed in both biomedical research and clinical diagnostics.

Since RNA was found to have catalytic functions, the theory that all enzymes are proteins has been discarded\(^15\). Subsequently, many nucleic acids with catalytic functions were not only found in nature but also by systematic evolution of ligands by exponential enrichment (SELEX)\(^16\),\(^17\). As one kind of nucleic acid enzyme, DNAzymes are capable of catalyzing biochemistry reactions\(^18\)-\(^20\). DNAzymes are more popular with researchers than protein enzymes, due to their cost-effectiveness, activity, and stability\(^21\),\(^22\). The DNA sequence containing rich guanine can fold to form a G-quadruplex (G-qua)\(^23\). Introducing hemin forms a G-quadruplex/hemin conjugate, a new kind of DNAzyme that mimics horseradish peroxidase (HRP) to catalyze the oxidation reactions of the substrates\(^24\).

We have developed a simple visual method to detect RNase H via DNAzyme-mediated signal amplification. As depicted in Scheme 1, in the absence of RNase H, there is no obvious phenomenon for a system of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide (H\(_2\)O\(_2\)) (ABTS-H\(_2\)O\(_2\)) containing duplexes resulting from the G-rich DNA sequence and complementary RNA sequence (G-Rich/cp-RNA). However, when RNase H was presented, the cp-RNA strand of G-Rich/cp-RNA
duplex was hydrolyzed by RNase H. With continuous RNase H hydrolysis, increasing G-Rich sequences left in the solution can fold into G-quadruplexes. HRP-mimicking G-quadruplex/hemin complexes catalyze ABTS to form green ABTS·⁺, subsequently realizing colorimetric measurements of RNase H via DNAzyme-mediated signal amplification visually or with UV-vis spectrophotometry.

Materials and Methods

Materials and Instrumentation
The G-rich DNA sequence (G-rich: 5’-GGG TAG GGC GGG TTG GGT-3’), the complementary RNA sequence (cp-RNA: 5’-ACC CAA CCC GCC CUA CCC-3’), and RNase H were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Hemin was purchased from Yuanye Bio-Technology Co., Ltd (Shanghai, China). ABTS was purchased from Sangon Biotech Co., Ltd (Shanghai, China). G-Rich and RNase H were prepared in sterile water as stock solutions, and cp-RNA was prepared in DEPC-treated water as a stock solution. All other chemicals were prepared in high-purity water as stock solutions or commercially available and used without further purification.

UV-vis absorption was recorded at 418 nm on a Hitachi U-4100 UV-vis spectrophotometer (Kyoto, Japan).

Preparation of cell lysates
Cell lysates were prepared as previously described 25. Briefly, a normal cell line (L02) and tumor cell lines (MCF-7, Hela, and HepG2) were first incubated in DMEM medium. Then, 1×10⁶ cells were harvested to 0.5 mL ice-cold cell lysis buffer. The samples were then centrifuged at 12,000 rpm for 10 min at 4 °C. Finally, the supernatants were collected as cell-free lysates and stored at -20 °C before use.

Preparation of the probe system
A solution of G-Rich (20 μM) and cp-RNA (24 μM) in 20 mM Tris-HCl buffer (pH 7.8,
40 mM KCl, 8 mM MgCl$_2$, and 0.05% Triton X-100) was first placed in 90 °C for 5 min, and then incubated at 37 °C for 2 h to guarantee complete hybridization of G-Rich with the cp-RNA. Before use, the prepared G-Rich/cp-RNA duplex probe system was stored at 4 °C.

**RNase H activity assay with the probe system**

Four μL of the prepared G-Rich/cp-RNA duplex probe system were diluted into 395 μL with 20 mM of Tris-HCl buffer (pH 7.8, 40 mM KCl, 8 mM MgCl$_2$, and 0.05% Triton X-100). After that, 5 μL of sample solutions with and without RNase H were added. The mixture solution was then incubated at 37 °C for 1 h, and the freshly prepared hemin (final concentration of 400 nM) was added to the reaction solution and further incubated for 0.5 h at 37 °C. Finally, both H$_2$O$_2$ (final concentration of 1 mM) and ABTS (final concentration of 1 mM) were quickly introduced into each of the above reaction solutions, and the absorbance levels were continuously monitored at 418 nm.

**Gel electrophoresis**

A mixture solution (100 μL) of G-Rich (300 nM) and cp-RNA (300 nM) in 20 mM of Tris-HCl buffer (pH 7.8, 40 mM KCl, 8 mM MgCl$_2$, 0.05% Triton X-100) was first placed at 90 °C for 5 min and then at 37 °C for 2 h. RNase H (final concentration of 100 U/mL) was added to 50 μL of the above G-Rich/cp-RNA duplex solution and further incubated at 37 °C for 1 h. 20% polyacrylamide gel was submerged in 1x TBE buffer, and four samples (G-Rich, cp-RNA, G-Rich/cp-RNA duplex treated with/without RNase H) were loaded at various sample wells of the gel. The voltage was set at 110 V for 90 min. SYBY Green II (Invitrogen, San Diego, CA, USA) dyes were utilized to stain the gel.

**Results and Discussion**
Feasibility study of the colorimetric sensing method

In this colorimetric sensing method for RNase H assay, cp-RNA strands of G-Rich/cp-RNA duplexes were efficiently hydrolyzed by RNase H. The released G-Rich sequences fold into G-quadruplexes, later combining with hemin to form G-quadruplex/hemin conjugates mimicking HRP to catalyze the redox reaction of ABTS and producing green products of ABTS·-. To investigate the strategic feasibility, the ABTS-H_2O_2 system was first used to study the prepared probe system. As shown in Figure 1A, when only hemin was presented in the ABTS-H_2O_2 system solution, its absorbance at 418 nm was at a lower level (curve 1) and the solution was colorless (picture 1), due to hemin having little activity to catalyze the ABTS-H_2O_2 system. When both G-quadruplex and hemin were presented in the ABTS-H_2O_2 system solution, a significant increase in absorbance at 418 nm was observed (curve 2) and the solution's color was dark green (picture 2), because the hemin combined with a G-quadruplex to form the HRP-mimicking DNAzyme of the G-quadruplex/hemin conjugate. However, when cp-RNA was presented and the G-Rich/cp-RNA duplex was formed, the ABTS-H_2O_2 system solution containing "hemin + G-Rich/cp-RNA duplexes" was colorless (picture 3) and its absorbance at 418 nm was still at a lower level (curve 3) because there were no free G-quadruplexes to combine with hemin to form the conjugate. When the G-Rich/cp-RNA duplexes were first treated with RNase H, the ABTS-H_2O_2 system solution containing "hemin + G-Rich/cp-RNA duplexes treated with RNase H" was also dark green (picture 4), and its absorbance at 418 nm also showed a significant increase (curve 4), which was mainly due to the fact that RNase H can hydrolyze the cp-RNA strand of the G-Rich/cp-RNA duplexes. Then the released G-Rich sequence was combined with hemin to form conjugates, catalyzing the redox reaction of ABTS to produce green ABTS·-. In conclusion, only when the free G-Rich sequence is available can the HRP-mimicking DNAzyme of the G-quadruplex/hemin conjugate be formed, rapidly initiating the reaction between ABTS and H_2O_2.
Polyacrylamide gel analysis was performed to verify method feasibility. Figure 1B reveals that the band patterns of G-Rich (lane 1), cp-RNA (lane 2), and G-Rich/cp-RNA (lane 3) were all in different horizontal locations, and the luminescence of G-Rich/cp-RNA was brighter than that of G-Rich and cp-RNA, indicating that the designed G-Rich and cp-RNA can hybridize with each other to form the G-Rich/cp-RNA duplex. The band pattern and horizontal location of the "G-Rich/cp-RNA duplex treated with RNase H" sample (lane 4) were almost the same as that of the "G-Rich" sample (lane 1), indicating that RNase H can hydrolyze the cp-RNA strand of the formed G-Rich/cp-RNA duplex into RNA residues, resulting in only the G-Rich sequence in solution. These consistent results demonstrate that the detection of RNase H via DNAzyme-mediated signal amplification is viable.

Optimization of the colorimetric sensing method

One can see from the designed sensing strategy for RNase H assay (Scheme 1) that the sensing performance of the colorimetric method is affected by background signals, which are dependent on free G-DNA sequences, as well as hemin. To complete the hybridization of G-Rich with cp-RNA, the amount of cp-RNA should be larger than that of G-Rich. However, too much cp-RNA can decrease the sensitivity of the colorimetric method. To guarantee high sensing performance, an appropriate molar ratio of G-rich/cp-RNA should be utilized. The molar ratio of G-rich/cp-RNA was optimized by using an ABTS-H$_2$O$_2$ system. As shown in Figure 2A, the change in absorbance ($A_1-A_0$, where $A_1$ and $A_0$ are the absorbances at 418 nm of the sample solutions after/before adding RNase H, respectively) of the ABTS-H$_2$O$_2$ system solution initially increased with decreasing G-rich/cp-RNA molar ratios and reached a maximum value of ca. 1.26 at a G-rich/cp-RNA molar ratio of 1:1.2. The "$A_1-A_0$" value of the ABTS-H$_2$O$_2$ system solution decreased when the G-rich/cp-RNA molar ratio was lower than 1:1.2. However, the background signal depends on the free G-rich sequence, which can be reduced by increasing the amount of cp-RNA. These results indicate that the "$A_1-A_0$" value of the ABTS-H$_2$O$_2$
system solution decreases if the cp-RNA amount is excessive. Therefore, an appropriate molar ratio of G-rich/cp-RNA was determined to be 1:1.2 in this colorimetric sensing system and was adopted in the assay.

Hemin, alone, has some enzymatic character, which can slightly catalyze the oxidation reaction of the substrates. Hence, a significant concentration of hemin will lead to a high background signal, while a low concentration of hemin is not enough to make the G-Rich sequences generate complete G-quadruplex/hemin complexes, thus decreasing the method sensitivity. Accordingly, hemin concentration was also optimized to improve the performance of the colorimetric sensing method for RNase H assay. As shown in Figure S1 and Figure 2B, the absorbances at 418 nm of both the "G-Rich/cp-RNA duplexes" sample solution and the "G-Rich/cp-RNA duplexes treated with RNase H" sample solution were increased with increasing hemin concentration (Figure S1). The absorbance change (A₁-A₀ at 418 nm) reached a maximum at 400 nM hemin (Figure 2B), corroborating that an appropriate concentration of hemin aids the colorimetric sensing method. 400 nM of hemin was employed in the subsequent sensing assay.

**Sensitivity and specificity of the colorimetric sensing method**

This method was first used under optimal conditions for the determination of RNase H at different concentrations. One can see from Figure 3 that the absorbance at 418 nm (Figure 3A) and the intensity of the green color (Figure 3B) of the sample solutions gradually increased with increasing concentrations of RNase H (0 ~ 15 U/mL). The absorbance change value of "A₁-A₀" was linearly dependent on the concentration of RNase H over the range of 0.1 ~ 3 U/mL (y = 0.2576x−0.02282, R² = 0.991) (Figure 3C). According to the formula LOD = 3SD/slope (SD: standard deviation of the blank samples; slope: the slope of the calibration curve), it was found that the detection limit was 0.04 U/mL. The performance of the developed colorimetric sensing method was also compared with that of other current methods for RNase H (27-29) (Table 1), which showed the better detection sensitivity of the sensing method developed here. Moreover, as the
concentration of RNase H is in the range of 57–96 U/mL in normal human serum or the patient's serum\(^3^0\), several microlitres (μL) of serum sample will be enough to realize the RNase H measurement in real applications. These results demonstrate that there is sufficient sensitivity in this method.

Five different kinds of enzymes, including EXO I, EXO III, DNase I, S1, and RNase A were tested to further investigate method selectivity. One can see from Figure 4 that the absorbance of the sample solution significantly increased when 5 U/mL of RNase H was used, while only slight increases of the absorbance intensity were observed for the other enzymes (25 U/mL), whose concentrations were five times higher than that of the RNase H. Results indicate that the proposed colorimetric sensing method demonstrates high specificity for RNase H detection over other interference enzymes.

**RNase H assay in cell lysates with the colorimetric sensing method**

To investigate the feasibility of the proposed method for RNase H measurement in real samples, RNase H in cell lysates obtained from four different cell lines (L02, HeLa, HepG2, and MCF-7) were detected using this method. One can see that compared with the blank sample, significant increases in absorbance at 418 nm were observed for all four cell lysates (Figure 5A), and their RNase H concentrations, calculated using the previously established linear equation, were 3.284 ± 0.185 U/mL for L02, 8.496 ± 2.409 U/mL for Hela, 9.992 ± 0.261 U/mL for HepG2, and 11.85 ± 0.854 U/mL for MCF-7, respectively (Table 2, Figure 5B). The measurement variation is similar to the ELISA method (37.75 IU/L for L02, 50.64 IU/L for Hela, 55.04 IU/L for HepG2, and 60.03 U/L for MCF-7) (Table 3, Figure 5B). Meanwhile, RNase H concentration levels in the MCF-7 cell line were highest, followed in succession by the HepG2 cell line, Hela cell line, and L02 cell line. This result is consistent with those reported in a previous work\(^3^1\). However, we also found that the concentration values of RNase H in cell lysates detected by the proposed colorimetric sensing method were different from those obtained by the ELISA method, likely in that the ELISA measures protein content of RNase H, but the
colorimetric sensing method measures RNase H activity.

Conclusions

Herein, we have developed a sensitive and selective colorimetric sensing strategy for the measurement of RNase H based on the HRP-mimicking DNAzyme of G-quadruplex/hemin conjugates. The successful quantification of RNase H in cell lysates verified the capability of this strategy to detect RNase H in complex and real biological samples. This colorimetric sensing method was not dependent on large-scale and expensive instrumentation, and, thus, can be applied in real biological systems in resource-limited environments.

Conflicts of interest

There are no conflicts to declare.

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Captions

Scheme 1 Schematic illustration of the detection of RNase H via DNAzyme-mediated signal amplification.

Figure 1 (A) Absorbance changes at 418 nm and pictures (insert) of the ABTS-H$_2$O$_2$ system solutions with different elements: curve 1. 400 nM of hemin; curve 2. 200 nM of G-rich sequences + 400 nM of hemin; curve 3. 200 nM of G-rich/cp-RNA duplexes + 400 nM of hemin; curve 4. 200 nM of G-rich/cp-RNA duplexes treated with 1 U/mL of RNase H + 400 nM of hemin. (B) Polyacrylamide gel electrophoresis: lane 1, 200 nM of G-rich; lane 2, 200 nM of cp-RNA; lane 3, 200 nM of G-rich/cp-RNA duplex; lane 4, 200 nM of G-rich/cp-RNA duplex treated with 1 U/mL of RNase H.

Figure 2 (A) Absorbance changes (A$_1$-A$_0$) at 418 nm of the ABTS-H$_2$O$_2$ system solutions in the presence of the G-Rich/cp-RNA duplexes with different molar ratios of G-Rich/cp-RNA (The concentration of hemin is 400 nM). (B) Absorbance changes (A$_1$-A$_0$) at 418 nm of the ABTS-H$_2$O$_2$ system solutions in the presence of G-Rich/cp-RNA duplexes (The molar ratio of G-Rich/cp-RNA is 1:1.2) and hemin with different concentrations. "A$_1$" and "A$_0$" represent the absorbance at 418 nm of the reaction solutions before and after treatment with 1 U/mL of RNase H, respectively.

Figure 3 (A, B) Absorbance change at 418 nm (A) and the pictures (B) of the sensing system solutions (1 mM of ABTS-H$_2$O$_2$, 200 nM of G-Rich/cp-RNA duplexes, 400 nM of hemin) upon addition of different concentrations of RNase H (0, 0.1, 0.5, 1, 2, 3, 5, 10 and 15 U/mL). (C) Calibration curve of the colorimetric sensing method for the detection of RNase H.

Figure 4 (A, B) Absorbance changes at 418 nm (A) and the corresponding bar graph
representation (B) of the sensing system solutions (1 mM of ABTS-H$_2$O$_2$, 200 nM of G-Rich/cp-RNA duplexes, 400 nM of hemin) upon addition of different enzymes (1 U/mL of RNase H; 5 U/mL of RNase A, DNase I, EXO I, EXO III, and S1).

**Figure 5** (A) Absorbance changes at 418 nm of the sensing system (1 mM of ABTS-H$_2$O$_2$, 200 nM of G-Rich/cp-RNA duplexes, and 400 nM of hemin) upon addition of different cell lysates obtained from L02, HeLa, HepG2, and MCF-7 cell lines, respectively. (B) The concentration of RNase H for different cell lines detected by different methods (ELISA: blue bar, which are results from Table 1 and Figure S2; Our sensor: yellow bar, which are results from Table 2 and Figure 5A).

**Table Captions**

**Table 1** Comparison of the detection performances among different assays for RNase H.

**Table 2** Calculation of the concentration of RNase H with ELISA.$^a$

$^a$Note: 10 μL cell lysate was added into the 50 μL detection system. $C_{\text{RNase H}}$ was obtained by the equation: $y = 0.01137x - 0.0005$ (Figure S2 in “Supporting Information”).

**Table 3** Calculation of the concentration of RNase H with our method.$^a$

$^a$Note: 100 μL cell lysate was added into the 400 μL sensing system solution (1 mM of ABTS-H$_2$O$_2$, 200 nM of G-Rich/cp-RNA duplexes, and 400 nM of hemin). $C_{\text{RNase H}}$ was obtained by the equation: $y = 0.2576x - 0.02282$ (Figure 3C).
Figure 2

Figure 3
Figure 4

Figure 5
### Table 1

| Methods                | Detection range (U/mL) | Detection limit (U/mL) | Refs. |
|------------------------|------------------------|------------------------|-------|
| Fluorescence analysis  | 1 – 80                 | 0.5                    | [27]  |
|                        | 0.2 – 4                | 0.2                    | [28]  |
|                        | 0 – 20                 | 2                      | [29]  |
| Colorimetric method    | 0.1 – 3                | 0.04                   | This study |

### Table 2

| Cell lysate | $A_{1}-A_0$ | Average | $C_{RNase H}$ (IU/L) | 5 $C_{RNase H}$ (IU/L) |
|-------------|-------------|---------|----------------------|------------------------|
| L02         | 0.087       | 0.087   | 0.085                | 7.549                  | 37.75                |
| HeLa        | 0.105       | 0.121   | 0.118                | 10.129                 | 50.64                |
| HepG2       | 0.138       | 0.127   | 0.109                | 11.009                 | 55.04                |
| MCF-7       | 0.136       | 0.131   | 0.141                | 12.005                 | 60.03                |

### Table 3

| Cell lysate | $A_{1}-A_0$ (at 418 nm) | Average | $C_{RNase H}$ (U/mL) | 4$C_{RNase H}$ (U/mL) |
|-------------|-------------------------|---------|----------------------|-----------------------|
| L02         | 0.184                   | 0.177   | 0.189                | 0.821                 | 3.284                |
| HeLa        | 0.386                   | 0.446   | 0.524                | 2.124                 | 8.496                |
| HepG2       | 0.601                   | 0.642   | 0.621                | 2.498                 | 9.992                |
| MCF-7       | 0.684                   | 0.722   | 0.740                | 2.963                 | 11.85                |