G-quadruplex-based colorimetric assay of S1 nuclease activity and inhibition

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A label-free colorimetric assay based on the catalysis of G-quadruplexes/hemin DNAzyme was developed for highly sensitive and specific detection of S1 nuclease activity and inhibition. In this system, G-riched DNA is used as the substrate for G-quadruplex. G-riched DNA binds hemin to form the G-quadruplexes/hemin DNAzyme with peroxidase-like activity, which catalyzes the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonate disodium salt (ABTS) by H_2O_2 to generate colored $ABTS^{\bullet+}$. In the presence of S1 nuclease, G-riched DNA was cleaved into small fragments, resulting in fewer formation of G-quadruplexes and low absorbance. The difference of absorbance before or after enzymatic digestion can be used to monitor S1 nuclease activity and inhibition. This sensing platform can detect S1 nuclease activity in a linear range of 0.03~5 U/mL with a detection limit of 0.014 U/mL. The present strategy enables also "naked-eye" detection of S1 nuclease activity with an actual detection limit of 0.03 U/mL, which makes it more convenient than other methods that rely on instrumentation. More importantly, the adenosine triphosphate (ATP) is found to well inhibit the activity of S1 nuclease when using certain G-quadruplex DNA as substrate, which provides a promising application in drug screening based on the inhibition of S1 nuclease.

1. Introduction

Endonucleases can hydrolyze the phosphodiester bonds in DNA or RNA.[1,2] They are essential in a variety of fields such as biotechnology and pharmacology, as well as in many important biological processes involving DNA replication, DNA recombination, DNA repair, molecular cloning, and polymerase chain reaction assay.[3-5] Moreover, inhibitors that can inhibit endonucleases activities are widely recognized as candidates for series of drugs development.[6] Consequently, the assays of endonuclease activity and inhibition are of great importance in the fields of biosensing, clinical diagnostics and drug discovery. Traditional analytical methods for the detection of nuclease activity are high performance liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE), radioactive labeling and enzyme-linked immunosorbent assay (ELISA).[7-9] However, these conventional protocols are time-intensive, DNA-consuming, laborious, discontinuous, and usually require radioactive labeling of substrates which have limited shelf life.[1]

To address these challenges, several alternative techniques for the assay of nuclease activity including fluorescence-based methods (fluorescence quenching, fluorescence resonance energy transfer (FRET), and nanoparticle-enhanced fluorescence polarization),[10-12] electrochemical approaches,[13] and gold nanoparticles (AuNPs)-based colorimetric assay[6] have been conducted. Although these new approaches have been identified to be successful and shown great advantages, there still exist undesirable features. As for fluorescence-based methods, a majority of approaches either require labeled DNA substrates or modified nanoparticles, which make the assay complex, expensive, and time-consuming. Therefore, the fabrication of label-free, low cost, and simple bioprobes has become highly focused. Many of AuNP-based methods have been developed for signal read-out and the development of detector-free diagnostic kits. However, AuNPs are sensitive to the salt concentration in buffer solutions used for enzymatic reaction, which would limit the application of AuNPs in nuclease assay. Thus it still remains a challenge to develop efficient, facile, label-free and amenable to high-throughput screening strategies by "bare-eye" to assay nuclease

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activity.

Recently, the use of G-quadruplexes as biological sensing elements has attracted significant interest due to its rapidness, simplicity, and easily detected by the "bare-eye", especially no need to use expensive analytical instruments.[14-16] The G-quadruplex-based DNAzyme displays peroxidase-like activity, which endow G-quadruplexes to be utilized for the colorimetric, chemiluminescence, or fluorescent detection of a series of analytes including metal ions, small molecules, DNA and proteins.[17-23] S1 nuclease, which is a widespread multifunctional endonuclease, can selectively catalyze and cleave single strand DNA (ssDNA) to small mono- or oligonucleotide fragments.[24-26] Thus it has been used as a tool in molecular biology and biotechnology.[27-29] Several intriguing strategies based on fluorescence or nanoparticles colorimetric probes have been developed for the detection of S1 nuclease activity.[1,26-32] To our best knowledge, no report is available for the label-free colorimetric assay of S1 nuclease activity based on G-quadruplexes.

By taking advantage of label-free colorimetric assay and G-quadruplexes, we designed a G-quadruplex based colorimetric method for the detection of S1 nuclease activity and inhibition. In the present work, G-riched DNA does not need any chemical modification or labeling. The S1 nuclease activity can be easily and simply detected by the "bare-eye" or with ultraviolet–visible (UV−vis) spectroscopy. This sensing platform exhibited high sensitivity and specificity toward S1 nuclease, which is not only sensitive and reliable, but also simple and economical in operation.

2. Experimental section

2.1 Materials and reagents

G-riched DNA with specific sequences (5'-GGGTAGGGCGG GTTGGG-3') was synthesized and purified by Sangon Biotech-nology Co. Ltd. (Shanghai, China). The G-riched DNA was used as provided and diluted in Tris-HCl buffer solution (pH 7.4, 10 mM) to give stock solutions of 50 μM. The G-riched DNA was heated to 90 °C for 10 min, and slowly cooled down to room temperature before use. S1 nuclease, 2,2'-azino-bis(3-ethylbenzo-thiazoline)-6-sulfonate disodium salt (ABTS), H_2O_2, sodium 4-(2-hydroxyethyl) piperazine-1-ethanesulfonate (HEPES), Adeno-sine triphosphate (ATP), tris(hydroxymethy-l) aminomethane (Tris), KCl, and hemin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Water was purified with a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA) and used throughout the work.

2.2 Procedure for the nuclease activity assay

A 12.5 μL volume of reaction solution containing 2 μM G-riched DNA, S1 nuclease buffer (0.2 M CH_3COONa, 1.5 M NaCl, 10 mM $ZnSO_4$, pH 4.5) and S1 nuclease with various concentrations was incubated at 37 °C for 60 min. Then, the solution was diluted with 175 μL of HEPES buffer solution (pH 7.4, 25 mM HEPES, 200 mM NaCl, 20 mM KCl). Subsequently, 10 μL of hemin solution was added to obtain a mixture and give final concentration of 0.14 μM hemin, and incubated for 60 min at room temperature to form the hemin/G-quadruplex structures. Finally, the ABTS and H_2O_2 solutions were added to obtain a total volume of 250 μL, and final concentrations of ABTS and H_2O_2 were 2 mM and 2 mM, respectively.

2.3 Procedure for the nuclease inhibition assay

A 12.5 μL volume of reaction solution containing 2 μM G-riched DNA, S1 nuclease buffer (0.2 M CH3COONa, 1.5 M NaCl, 10 mM ZnSO4, pH 4.5), S1 nuclease with various concentrations and different concentration of ATP was incubated at 37° C for 60 min. The followed procedure was same with the experiment mentioned above for S1 nuclease activity assay.

2.4 Absorption spectra and absorbance measurements

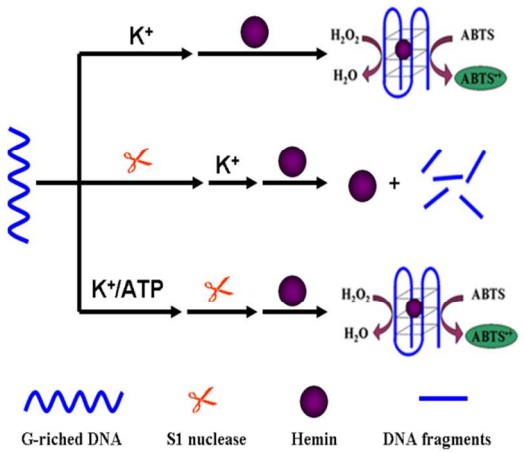
UV−vis absorption spectra and absorbance measurements were performed using a Cray 60 UV−visible spectrophotometer (Agilent Technologies). The absorption spectra of the solution were measured in the wavelength range from 380 nm to 500 nm at a fixed time interval of 5 min. The absorbance measurements were performed at 420 nm.

2.5 Circular dichroism experiments

Three samples were used for circular dichroism (CD) assays. Sample one was prepared by incubating the G-riched DNA (5 μM) solution at 37 °C for 60 min; sample two was prepared by incubating the S1 nuclease solution (80 U/mL) at 37 °C for 60 min; sample three was prepared by incubating the mixture solution of G-riched DNA (5 μM) and S1 nuclease (80 U/mL) at 37 °C for 60 min. Sample (130 μL each) assays were performed using a J-810 CD spectrophotometer equipped with a programmable, thermoelectrically controlled cell holder (JASCO, Japan). CD spectra were collected from 200 nm to 320 nm. The measurements were made at room temperature using a square quartz cell with a 1 cm path length. CD spectra were recorded with a response of 2 s, at 2.0 nm bandwidth. All experiments were repeated in triplicate.

3. Results and discussion

3.1 Probe design



Scheme 1 Schematic representation of the sensing system for the analysis of S1 nuclease activity and inhibition.

The working principle of G-quadruplex-based colorimetric assay of

S1 nuclease activity and inhibition is illustrated in Scheme 1. In this system, G-riched DNA is used as the digestion substrate to construct G-quadruplex for label-free assay of S1 nuclease. K^+ was used to promote G-quadruplex formation. In the absence of S1 nuclease, G-riched DNA binds K^+ and hemin to form the G-quadruplexes/hemin DNAzyme with peroxidase-like activity, which catalyzes the oxidation of ABTS by H_2O_2 to generate colored $ABTS^{\bullet+}$. However, G-riched DNA, in the presence of S1 nuclease, was cleaved into small fragments, resulting in fewer formation of G-quadruplexes and low absorbance/color response of $ABTS^{\bullet+}$. Therefore, the cleavage of G-riched DNA by S1 nuclease can be monitored by observing the absorbance/color changes of $ABTS^{\bullet+}$ before or after enzymatic digestion. Consequently, we can easily detect the S1 nuclease activity by the "bare-eye" or with UV−vis spectroscopy. Furthermore, it is known that K^+ can stabilize the G-quadruplex structure[33] and ATP can inhibit the activity of S1 nuclease,[34] which cannot be cleaved by S1 nuclease, thus causing the increase of colorimetric signal. As illustrated in Scheme 1, if the cleavage process is conducted in the presence of K^+ or ATP before S1 nuclease, G-riched DNA would remain intact due to the K^+ protecting the DNA from nuclease digestion. Whereas, small fragments are generated and the absorbance/color response of $ABTS^{\bullet+}$ is low after enzymatic digestion.

3.2 Feasibility study

To demonstrate the feasibility of the present strategy, the UV−vis absorption spectra under different conditions were investigated. As shown in Fig. 1, in the absence of the G-riched DNA, the UV−vis absorption spectra of the mixture solution of $ABTS/H_2O_2$ and hemin (black curve) was similar to that of the addition of S1 nuclease to mixture solution of $ABTS/H_2O_2$ and hemin (red curve). In contrast, when the G-riched DNA was added into the $ABTS/H_2O_2$ and hemin mixture solution, the absorbance intensity at 420 nm was increased about 740 % and with a significant color change from colorless to green (blue curve), which belong to the characteristic absorption spectrum of colored $ABTS^{\bullet+}$ oxidized by the hemin/G-quadruplexes.[35] However, in the presence of S1 nuclease, the absorbance of colored $ABTS^{\bullet+}$ at 420 nm was significantly decreased (green curve), causing a remarkable color change (insert in Fig.1). These results demonstrated that the absorbance reduction was attributed to the S1 nuclease activity. Thus, the present strategy can be used for assay of S1 nuclease activity.

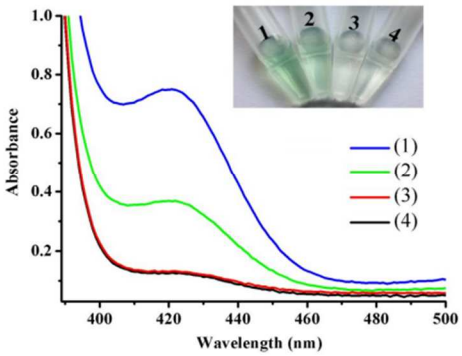


Fig. 1 UV−vis absorption spectra of sample solutions under different conditions: (1) G-riched DNA+hemin+ABTS+H_2O_2; (2) G-riched DNA+S1 nuclease+hemin+ABTS+H_2O_2; (3) S1 nuclease+hemin+ABTS+H_2O_2; (4) hemin+ABTS+H_2O_2. The concentrations of G-riched DNA, S1 nuclease, hemin, H_2O_2 and ABTS were 0.1 μM, 5.0 U/mL, 0.14 μM, 2.0 mM and 2.0 mM, respectively.

3.3 CD characterization

In order to illustrate the mechanism of the strategy, the evidence for forming G-quadruplexes and occurrence of enzymatic digestion reaction with S1 nuclease was provided by CD analysis. As can be seen from Fig. S1 (ESI), the CD spectrum of mixture of G-riched DNA and hemin had a negative peak at approximately 240 nm, a small positive peak around 260 nm (green curve). It is well-documented that the spectra of parallel quadruplexes have a dominant positive peak at around 265 nm and a negative peak at around 240 nm.[36,37] This result demonstrated that a parallel quadruplex was formed after incubating the mixture of G-riched DNA and hemin at 37 °C for 60 min. After the G-riched DNA interacting with S1 nuclease, the intensity of the peaks around 240 and 260 nm significantly decreased (red curve). This indicated that the G-riched DNA cleavage reaction really occurs, as a result, small G-quadruplexs were formed.

3.4 Optimization of assay conditions

In order to achieve the performance of the proposed G-quadruplex-based colorimetric assay for S1 nuclease activity and inhibition, the assay conditions such as the cleavage reaction time and concentration of hemin were optimized. The effect of the cleaving reaction time from 10 min to 100 min on the S1 nuclease activity assay was tested. The S1 nuclease cleaves G-riched DNA into small segments, which result in absorbance decrease of system. In general, extending the reaction time can improve the cleavage efficiency. Therefore, it is expected that higher sensitivity for S1 nuclease activity assay could be achieved by prolonging the cleaving time. Fig. S2a (ESI) indicates that the relative absorbance intensity ΔA ($\Delta A=A_0-A$, where A_0 is the absorbance in the absence of S1 nuclease, and A is the absorbance in the presence of S1 nuclease) increased with the increase of cleaving reaction time. Additionally, it can also be observed that ΔA was intensified with increasing the reaction time from 0 to 60 min, and kepting constant after 60 min. By weighing both the sensitivity and the assay time, the cleaving reaction time of 60 min was selected for subsequent studies. Subsequently, the effect of the concentration of hemin on S1 nuclease activity detection was also tested. As shown in Fig. S2b (ESI), ΔA value increased with the increase of the concentration of hemin, and maximal ΔA value was consistent with the concentration of hemin at 0.14 μM. However, with further increasing the concentration of hemin, the ΔA value decreased. As a result, 0.14 μM was chosen as the optimized the concentration of hemin.

3.5 Assay of S1 nuclease activity

Fig. 2 shows the change of absorption spectra upon adding different concentrations of S1 nuclease into the G-riched DNA system. As expected, the absorbance values are gradually decreased as the concentration of S1 nuclease increases from 0.03 U/mL to 10 U/mL. The inset (b) in Fig. 2 shows a good linearity between the ΔA and S1

nuclease concentration when the S1 nuclease concentrations were from 0.03 U/mL to 5 U/mL. The regression equation is $\Delta A=0.07323C+0.03019$ ($R^2=0.9931$), where ΔA and C represent the absorbance difference and the concentration of S1 nuclease, respectively. According to 3σ rule, a detection limit (LOD) of 0.014 U/mL was estimated, which is lower than that of many methods previous reported (Table 1). Thus, this facile, sensitive and cost efficient colorimetric method is promising for fast detection of the activity of S1 nuclease without the aid of any sophisticate instruments.

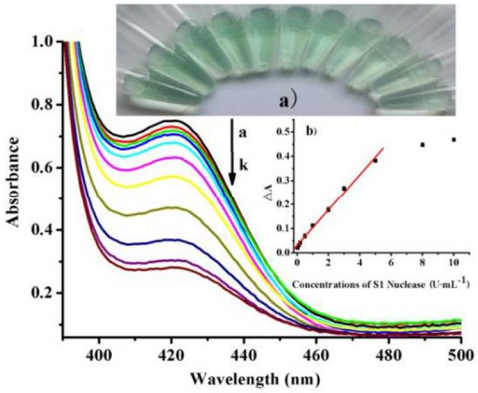


Fig. 2 UV−vis absorption spectra of the sensing system for the analysis of different concentrations of S1 nuclease. From a to k, the concentrations of S1 nuclease were 0, 0.03, 0.1, 0.2, 0.5, 1, 2, 3, 5, 8 and 10 U/mL, respectively. Inset (a) is photographs of color change. Inset (b) is the linear range for the detection of S1 nuclease activity.

Table 1 Comparison of analytical methods for the detection of S1 nuclease activity

Type	Dynamic ranges (U/mL)	Sensitivity (U/mL)	Ref.
Label-free fluorescence	0～80.0	0.092	28
Fluorescence quenching based on carbon nanotube	0.6～8.0	0.08	30
G-quadruplex based fluorescent assay	0.04～0.4	−	1
Colorimetric method based on gold nanoparticles	0～30.0	≈ 4.3	29
Peptide-mediated fluorescence energy transfer	0～12.0	−	31
Graphene/Au-NP hybrid based colorimetric biosensors	1～12.0	≈ 0.5	32
G-quadruplex-based colorimetric assay	0.03～5.0	0.014	This work

3.6 The inhibition of S1 nuclease activity

Taking ATP as a model which is known as the inhibitor of S1 nuclease,[34] we employed the assay to detect the inhibition effect of ATP on S1 nuclease activity. First, we studied that whether ATP exhibited inhibition effect on the hemin/G-quadruplexes catalyzed ABTS-H_2O_2 reaction. The results indicate that the effect of ATP (up to a concentration of 6.0 mM) on the hemin/G-quadruplexes catalyzed ABTS-H_2O_2 system was negligible (Fig.3a). Subsequently, inhibition effects of different concentrations of ATP on S1 nuclease activity were evaluated. As shown in Fig. 3b, the absorbance of the system increased gradually with the increase of ATP concentration. This phenomenon implied that the cleavage reaction of ssDNA by S1 nuclease was prohibited. And it can been seen that the activity of S1 nuclease sharply decreased with ATP concentration increasing (Fig.3b inset). In addition, it was also observed that the necessary ATP concentration to reach maximal absorbance was about 2.0 mM. This indicates that 2.0 mM ATP can prohibit completely the cleavage reaction of ssDNA by S1 nuclease.

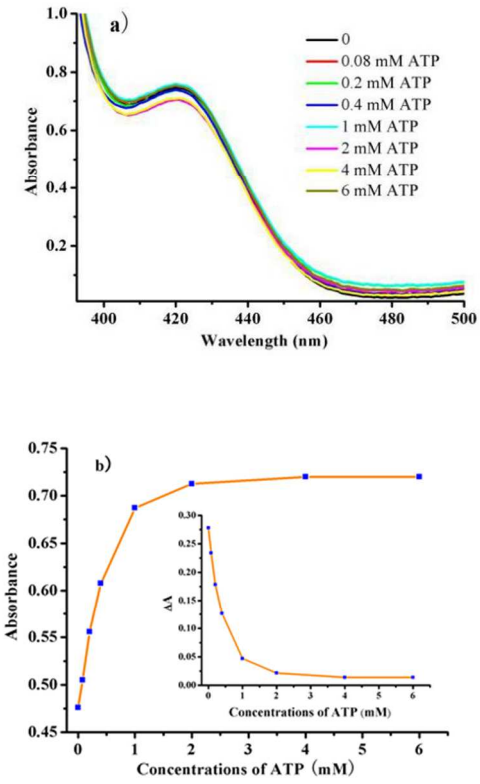


Fig. 3 a) The effect of ATP on the hemin/G-quadruplexes catalyzed ABTS/H_2O_2 reaction; b) inhibition effects of different concentrations of ATP on ssDNA cleavage by S1 nuclease. Concentration of ssDNA was 0.1 μM; concentration of S1 nuclease was 3.0 U/mL.

Thus, the proposed method could be employed to investigate the S1 nuclease inhibition, which provides a promising application in drug screening based on the inhibition of S1 nuclease, and may further be applied to screen simply other S1 nuclease inhibitors.

3.7 Specificity of the assay for S1 nuclease activity

To evaluate the specificity of the colorimetric assay for S1 nuclease activity, we challenged the method with target S1 nuclease and several kinds of other common proteins such as GOX, Exo III, Dpan I and Nb.BbvcI. As shown in Fig. 4, only the target S1 nuclease can cause a dramatic absorbance decrease, while other enzymes or proteins did not lead to obvious variation of absorbance even under the same condition. These results imply that the G-riched DNA is selectively cleaved by S1 nuclease and is resistant to other enzymes or proteins mentioned above. It also demonstrates clearly that the designed strategy exhibits high specificity for S1 nuclease.

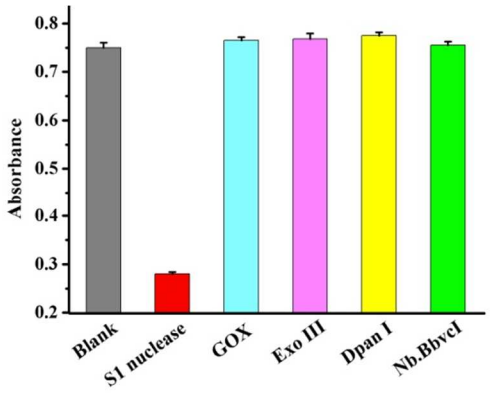


Fig. 4 Absorbance of the system at 420 nm toward S1 nuclease, GOX, Exo III, Dpan I, Nb.BbvcI and Blank. The concentration of S1 nuclease was 10 U/mL, and others were 40 U/mL each.

3.8 Detection of S1 nuclease activity in human serum

To evaluate the feasibility of the present method in complex biological matrixes, real sample analysis was challenged by human serum obtained from the hospital of Guilin. Human serum samples were diluted in a 1:100 ratio with S1 nuclease buffer (pH 7.5, 20 mM Tris-HCl, 50 mM NaCl, 0.1 mM $ZnCl_2$ and 50% glycerol), and then was spiked with standard solutions containing different concentrations of S1 nuclease. The analytical results are shown in Fig. 5. As can been seen, the absorbance at 420 nm decreases with the increase of concentration of S1 nuclease (Fig. 4, inset), and exhibits a linear relationship toward the concentrations of S1 nuclease ranged from 0.06 U/mL to 1.0 U/mL (the regression equation is $\Delta A=0.2072C+2.5\times10^{-5}$, $R^2=0.9870$). The assay results of S1 nuclease activity in human serum samples indicate that the method possess excellent applicability for real sample analysis.

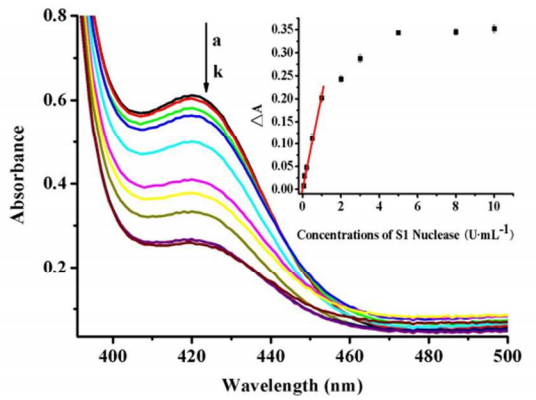


Fig. 5 UV−vis absorption spectra of the sensing system for the analysis of different concentrations of S1 nuclease in human serum. From a to k, the concentrations of S1 nuclease were 0, 0.06, 0.1, 0.2, 0.5, 1, 2, 3, 5, 8 and 10 U/mL, respectively. Inset is the linear range for the detection of S1 nuclease activity.

4. Conclutions

In conclusion, we have developed a label-free colorimetric assay based on the catalysis of G-quadruplexes/hemin DNAzyme for highly sensitive and specific detection of S1 nuclease activity and inhibition. Compared with traditional analytical methods for the detection of S1 nuclease activity, the assay presented here has several excellent features as following: first, the assay avoids complicated modifications or chemical labeling, and only involves G-quadruplex DNA as substrate and signal responser, which is simple and cost efficiency. Second, the use of the present strategy enables the “bare-eye” detection of S1 nuclease activity. Third, the assay can be conducted in aqueous solution without troublesome separation procedures. Furthermore, this assay was applied for the determination of S1 nuclease activity in human serum, which demonstrated that the G-quadruplex-based technologies can be applied to a wide range of fields such as biology, biomedicine, and bioanalysis.

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