Article

Enzyme-Activated G-quadruplex Synthesis for in situ Label-free Detection and Bio-imaging of Cell Apoptosis

Zhuoliang Liu, Xingyu Luo, Zhu Li, Yan Huang, Zhou Nie, Honghui Wang, and Shouzhuo Yao

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.6b04360 • Publication Date (Web): 11 Jan 2017

Enzyme-Activated G-quadruplex Synthesis for in situ Label-free Detection and Bio-imaging of Cell Apoptosis

Zhuoliang Liu[‡a,c], Xingyu Luo[‡a], Zhu Li[b], Yan Huang[a], Zhou Nie[a*], Hong-Hui Wang[b*], Shouzhuo Yao[a]

[a]State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P. R. China.

[b]College of Biology, Hunan University, Changsha, 410082, P. R. China.

[c]College of Science, National University of Defence Technology, Changsha, 410073, P. R. China

*Corresponding author email address: niezhou.hnu@gmail.com (Z. N.); wanghonghui@hnu.edu.cn. (H.H. W.) Tel.: +86-731-88821626; Fax: +86-731-88821848

ABSTRACT: Fluorogenic probes targeting G-quadruplex structures have emerged as the promising toolkit for functional research of G-quadruplex and biosensor development. However, their bio-sensing applications are still largely limited in in-tube detection. Herein, we proposed a fluorescent bio-imaging method based on enzyme-generated G-quadruplexes for detecting apoptotic cells at cell and tissue level, namely terminal deoxynucleotidyl transferase (TdT) activated *de novo* G-quadruplex synthesis (TAGS) assay. The detection target is genomic DNA fragmentation, a biochemical hallmark of apoptosis. The TAGS assay can efficiently "tag" DNA fragments via using their DNA double-strand breaks (DSBs) to initiate the *de novo* synthesis of G-quadruplexes by TdT with unmodified G-rich dNTP pool, followed by a rapid fluorescent readout upon the binding of thioflavin T (ThT), a fluorogenic dye highly specific for G-quadruplex. The feasibility of TAGS assay was proved by *in situ* sensitive detection of individual apoptotic cell in both cultured cells and tissue sections. TAGS assay has notable advantages, including label-free and quick detection, high sensitivity and contrast, mix-and-read operation without tedious washing, and low cost. This method not only shows the feasibility of G-quadruplex in tissue bio-analysis, but also provides a promising tool for basic research of apoptosis and drug evaluation for antitumor therapy.

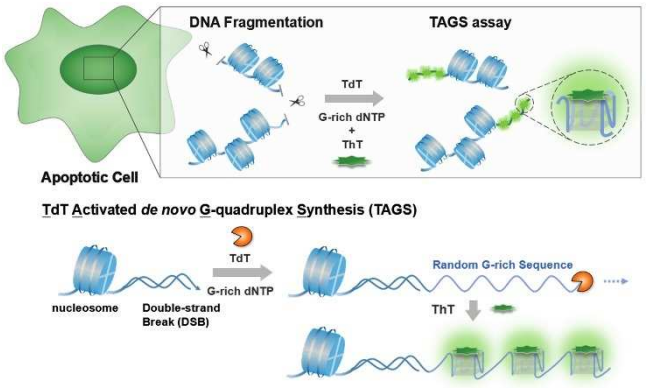
G-quadruplex is a non-canonical nucleic acid secondary structure with stacked G-tetrad assembly normally formed in guanine-rich DNA or RNA sequences[1]. Its unique four-stranded structure consisting of two or more G-tetrads is stabilized by Hoogsteen hydrogen bonding between guanines (G) in G-tetrads and π, π-stacking interaction between G-tetrad planes[2-6]. The G-quadruplex structure is highly polymorphic with various kinds of topologies and the preferential topology depends on its intrinsic sequence and the nature of bound cations. In last decade, G-quadruplexes have attracted great research interests because of their potential significance in essential biological functions, including telomere maintenance, transcriptional and translational regulation, and genome integrity. Moreover, G-quadruplex is also a latent drug targets for therapeutic applications, and its self-assembly properties have been utilized in supra-molecular chemistry and nucleic acid nanotechnology[7-12]. Besides these intriguing aspects, G-quadruplexes have also been widely exploited as responsive or signal components in nucleic acid-related biosensing applications[13]. Stimulus-trigged structural transition of G-quadruplex can directly serve for target recognition and signal transition. Furthermore, their sequence can be straightforwardly integrated with functional nucleic acid motifs and efficiently replicated by various nucleic acid amplification techniques. Therefore, G-quadruplexes are attractive building blocks for biosensor/bioassay development.

Fluorogenic probes specific for biological microenvironment are highly important for many biological applications, especially DNA/protein analysis. Comprehensive investigation on G-quadruplex/ligand interactions has discovered several potent small molecular fluorogenic ligands for "lighting-up" G-quadruplexes, such as triphenylmethane (TPM) dyes[14], triphenylamine (TPA) dyes[15], thiazole orange (TO)[16], several porphyrin derivatives[17,18], and metal complexes[19]. These molecules are capable of selectively binding with and stabilizing G-quadruplex structures, and their photophysical properties are significantly changed by the interaction with microenvironment of G-quadruplex. They provide a powerful tool for visualizing G-quadruplex, which can serve as convenient and label-free signal reporters for G-quadruplex-based biosensors. The fluorogenic dye-stained G-quadruplexes have been utilized to detect metal ion, nucleic acid, protein, and enzymatic activity[20-24]. However, most of G-quadruplex-based applications were limited in in-tube assays, while *in situ* bio-analysis attempts were seldom carried out in cells or tissue systems.

Apoptosis plays an essential role in growth regulatory network during development, and its dysfunction involves in the progression of varies of diseases such as degenerative disorders and cancers[25]. Identification and monitoring cells undergoing apoptosis are crucial for advancing apoptotic investigations and evaluation of apoptosis-targeted antitumor therapy[26,27]. One of the biochemical hallmarks of apoptosis is fragmentation of cellular genomic DNA[28]. During apoptosis, the apoptotic executor, caspase 3, cleaves and activates a specific deoxyribonuclease (DNase), leading to the cleavage of nucleosomes at internucleosomal sites and the generation of large number of DNA double-strand breaks (DSBs)[29]. The conventional methods to evaluate DNA fragmentation includes the DNA ladder assay for *ex situ* measurement and TUNEL assay for *in situ* detection[30]. Although these methods are efficient for probing cell apoptosis, their usages are still hampered by time consuming and labour-intensive procedure, multi-step of separation and washing, low sensitivity, artificial false-positive signal, and relative high cost with labeled nucleotides[31-33]. To address these issues, we envisioned that introducing functional nucleic acid with intriguing properties, for example G-quadruplex and its fluorogenic probes, into DNA fragmentation detection might provide a new opportunity for designing improved strategies for probing cell apoptosis.

Herein, we present a novel method for label-free and *in situ* bio-imaging of apoptosis at single-cell level based on enzymatic generation of G-quadruplexes, namely terminal deoxynucleotidyl transferase (TdT) activated *de novo* G-quadruplex synthesis (TAGS) assay (as shown in Scheme 1).

Scheme 1. Schematic presentation of *in situ* cell apoptosis detection utilizing TAGS assay.



TdT is a special DNA polymerase that catalyses the addition of deoxynucleotides to the 3'-OH end of DNA molecules without requirement of DNA template[9,19,34]. In this assay, the unique template-independent property TdT was utilized to synthesize randomly arrayed G-rich sequences to form G-quadruplexes starting from apoptotic DSBs. The *de novo*-generated G-quadruplexes were visualized by staining with G-quadruplex-specific probe ThT to enhance fluorescence signal, allowing sensitive "turn-on" detection of apoptosis. ThT is a water-soluble fluorogenic dye capable of selectively recognizing G-quadruplex structure from other DNA/RNA structures via its significant fluorescence lighting-up[35]. The high efficient fluorescence enhancement and ultra-low background of TdT-activated G-quadruplexes endow TAGS assay with high specificity, sensitivity, and imaging contrast, providing a feasible approach in multiple level of biological system, from culture cells to tissue sections. In addition, this method is a mix-and-read imaging assay avoiding the use of labeled nucleotides and multiple washing steps. More importantly, the rapid growth of the diversity and functionality of G-quadruplex-targeting fluorogenic probes hold great potential to endow TAGS with great versatility for various advanced bio-imaging techniques, such as near-infrared or two-photon imaging.

EXPERIMENTAL

Materials and reagents. Terminal deoxynucleotidyl transferase (TdT) was purchased from Fermentas Inc. (Vilnius, Lithuania). Deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and SYBR Green II were obtained from Sangon (Shanghai, China). Staurosporine (STS) was purchased from Selleck Chemicals. RPMI 1640 cell culture medium was purchased from Thermo Scientific Gibco (MA, USA). Human blood serum samples were obtained from Hunan Provincial People's Hospital, China. pET28 plasmid was purchased from EMD Millipore (MA, USA). TIANprep Mini Plasmid Kit, RNAsimple Total RNA Kit, and TIANamp Genomic DNA kit were purchased from TIANGEN (Beijing, China). Annexin V-FITC Apoptosis Detection Kit was purchased from KeyGEN (Nanjing, China). TUNEL Apoptosis Assay Kit was purchased from Beyotime (Jiangsu, China). Ultrapure water with an electric resistance of 18.3 MΩ was obtained from a Millipore filtration system and used throughout. All other chemicals were of analytical reagent grade and used without further purification.

DNA, RNA and cell lysates preparation. Single-strand DNA (ssDNA) oligo (5'-AAT ACA ACC TCT CA-3') was synthesized by Sangon. pET28 plasmid was transformed into Dh5α Eco.li bacterium. The bacteria containing pET18 plasmid were grown in selective LB media supplemented with kanamycin (50 μg/mL) for 16 hours and the plasmid DNA (pDNA) was purified using a TIANprep Mini Plasmid Kit following the manufacturer's instructions. Genomic DNA (gDNA) was purified from Hela cells using a TIANamp Genomic DNA kit following the manufacturer's instructions. Total RNA was purified from Hela cells using a RNA simple Total RNA Kit following the manufacturer's instructions. To prepare cell lysates, Hela cells were collected and resuspended in ultrapure water to make homogenate using a sonicator (SCIENTZ, China).

TdT-mediated elongation assay. The typical TdT-mediated elongation experiment was performed in 10 μL of TdT buffer (1×, 0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.01% (v/v) Triton X-100, 1 mM $CoCl_2$, pH 7.2) containing the ssDNA oligo, 1 mM dNTP (10% dTTP, 40% dATP and 50% dGTP), and 4 U of TdT at 37 °C for 2 h, and terminated by heating the solution at 75 °C for 10 min. For TdT-mediated elongation initiated from DSBs, pDNA or gDNA was incubated with DNase I (0.25 U) in reaction buffer (total 10 μL) for 10 min and the reaction was terminated by heating the solution at 80 °C for 20 min. Then 5 μL of the resulting solution containing DNase I-digested pDNA or gDNA was added into 10 μL of TdT buffer to initiate the elongation at 37 °C for 2 h and terminated by heating the solution at 75°C for 10 min.

Circular dichroism experiments. The CD spectra were recorded on MOS-500 spectrophotomer (BioLogic, France) at room temperature. Before the CD measurement, 100 μL of the TdT-elongated ssDNA (0.5 μM), pDNA (1.2 nM) or gDNA (from 2×10^5 Hela cells) samples were mixed with 100 μL Tris buffer (100 mM Tris-HCl, 100 mM KCl, pH 7.4) for 30 min at room temperature. The mixture was transferred to 2 mm path length quartz cuvettes for measurement, scanning from 220 to 340 nm at a rate of 50 nm/min for three times and keeping the slit to 5 nm. Each spectrum was corrected by subtracting the CD data of the Tris buffer alone in the same quartz cell.

Fluorescence measurements. Ten microliter TdT-mediated elongation samples were mixed with 90 μL reaction solution containing 88 μL G-quadruplex dyes buffer (1×, 50 mM Tris-HCl, 50 mM KCl, pH 7.4) and 2 μL 100 μM G-quadreplex specific dyes, including ThT, CV or PPIX, and the total volume was 100 μL. Fluorescence experiments were carried out using a QuantaMaster™ fluorescence spectrophotometer (PTI, Canada). ThT was excited at 425 nm and its emission was recorded from 445 to 600 nm with the maximum emission wavelength at 485 nm. CV was excited at 580 nm and its emission was recorded from 600 to 700 nm with the maximum emission wavelength at 630 nm. PPIX was excited at 410 nm and its emission was recorded from 610 to 750 nm with the maximum emission wavelength at 625 nm. All experiments were performed at least three times.

***In situ* detection of apoptosis in cultured cells.** Hela cell line was purchased from the Cell Bank of KeyGen (Nanjing, China). Hela cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 U/mL penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air. STS was dissolved in dimethyl sulfoxide and used as the apoptosis inducer. The cells were incubated with different concentrations of STS for 40 h. After the treatments, cells were fixed in 4% paraformldehyde for 10 min at 4 °C, washed three times in PBS, permeabilized with 1% Triton X-100 in PBS for 5 min, and then washed three

times again in PBS at room temperature. Then, cell samples were immersed in 50 μL TdT buffer, 60 U of TdT and 1 mM dNTP (10% dTTP, 40% dATP and 50% dGTP) at 37 °C for 60 min in a humidified chamber. Then the cell samples were stained with 2 μM ThT (50 mM Tris-HCl, 50 mM KCl, pH7.4) for 1 min. Stained cells were examined using a confocal laser scanning microscope (CLSM, C1-Si, Nikon, Japan) and the images were acquired and processed using ImageJ (NIH). The number of ThT-positive cells at fluorescent channel and total cells number at DIC channel were quantified and averaged from five randomly chosen microscopic fields (magnification × 200). The apoptosis rates were determined as the percentage of ThT-positive cells of total cells in each condition. Apoptosis rates at various STS concentrations were plotted against STS concentrations. Values are represented as means± SD of 3 independent experiments.

RESULTS AND DISCUSSION

TdT activated *de novo* G-quadruplex synthesis. Prior to apoptotic cell detection, we firstly employed in vitro systems to test whether 3' hydroxyl termini of DSBs can work as primers for TdT polymerization to form G-quadruplex. Since G-rich sequence is the prerequisite for G-quadruplex structure, we envisioned that TdT was able to yield randomly arrayed G-rich ssDNA sequence in the presence of dGTP-rich substrate pool due to its template-independent polymerization, and our previous proof-of-concept work demonstrated that TdT-yielded G-rich sequence can form multiple G-quadruplex structures using ssDNA as primer in a simple aqueous system[34]. To investigate the effect of DSBs as primers, we treated pET-28 plasmid DNA (pDNA) with deoxyribonuclease I (DNase I), an endonuclease that cleaves both double-stranded DNA (dsDNA) and ssDNA. Using DNase I-digested pDNA as primers (1.2 nM), TdT catalysed the polymerization with a previously optimized G-rich dNTP pool (50% dGTP, 40% dATP and 10% dTTP)[34], and the global conformation information of the elongation product was determined using circular dichroism (CD) measurements.

Similar to the result of ssDNA as primer (0.5 μM) (Figure S1a), the DNA product initiated from DSBs had an obvious positive peak near 264 nm and a negative one at 240 nm (Figure 1a), suggesting the formation of G-quadruplex structures with dominant parallel conformers[36]. Furthermore, genomic DNA was digested using DNase I to mimic the generation of apoptotic DSBs as DNase I has been identified as one of the deoxyribonucleases responsible for chromatin cleavage and DNA fragmentation during cell apoptosis[37,38]. Genomic DNA (gDNA) purified from Hela cells (2×10^5) was treated with DNase I digestion followed by TdT-mediated elongation with the G-rich dNTP pool. The DNA products show obviously G-quadruplex structure based on CD spectra observation (Figure 1b). No obvious characteristic peaks of G-quadruplex were observed in genomic DNA incubated with solo DNase I or TdT, proving that endogenous G-quadruplexes are not detectable from genomic DNA. These results indicated that TAGS assay may work as a potent tool for detection of apoptotic DSBs.

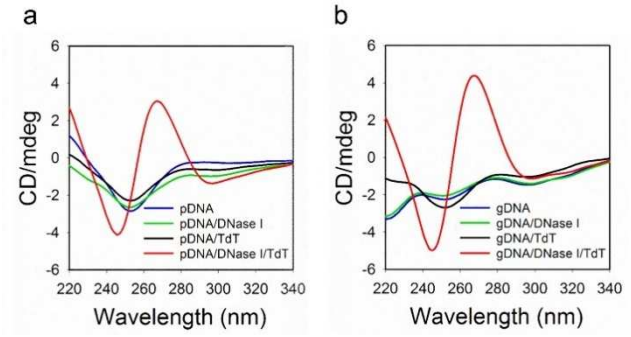


Figure 1. CD spectra characterization of the DNA conformation of TdT-generated G-quadruplexes. (a) Plasmid DNA (pDNA) (1.2 nM) was treated with DNase I (2.5 U) followed by incubation with G-rich dNTP (1 mM) with and without TdT (40 U), respectively. (b) Genomic DNA (gDNA) was purified from 2×10^5 Hela cells and treated with DNase I (2.5 U) followed by incubation with G-rich dNTP (1 mM) with and without TdT (40 U), respectively.

ThT is the optimal probe for staining TdT-generated G-quadruplexes. A number of G-quadruplex-specific dyes have been developed as not only the ligands of G-quadruplex with high affinity but also the selective fluorescent probes for in vitro or in vivo applications[20,34,35,39-41]. To find the best probe for the TAGS assay, we compared the effect of three known selective G-quadruplex dyes, i.e. ThT[35], crystal violet (CV)[42,43], and protoporphyrin IX (PPIX)[44,45]. The ssDNA primer was utilized for TdT-mediated generation of randomly arrayed G-rich sequence. To prove the elongation result, the denaturing gel electrophoresis was used to analyze the TdT-synthesized products and the result showed that the primer was well-elongated to about 400 bases (Figure S1b). The fluorescence enhancements of resulting G-quadruplex upon binding of above probes were evaluated. As shown in Figure 2a, ThT generated negligible background signals without TdT-mediated elongation reaction, whereas the TdT-generated G-rich product caused the significantly enhancement of ThT fluorescence with an ultrahigh signal-to-background (S/B) ratio of 88. However, CV (Figure 2b) and PPIX (Figure 2c) showed obvious fluorescent background and low S/B ratios of 6.2 and 3.0, respectively. We further examined the detection performance of these probes in detail (Figure 2d). All the dyes

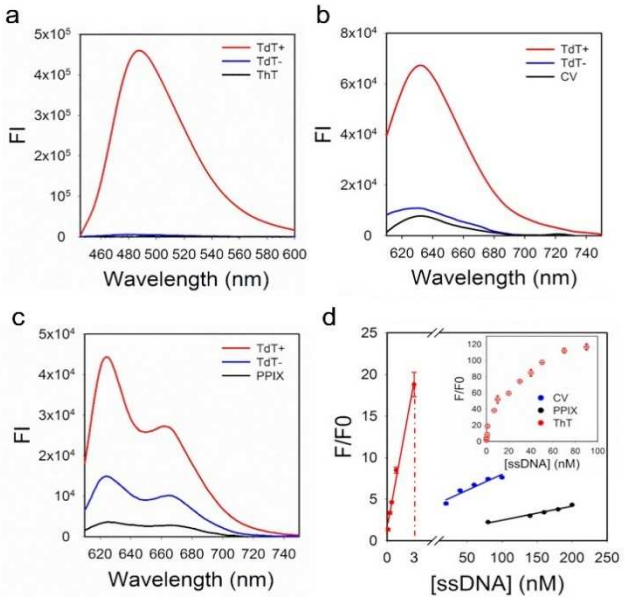


Figure 2. Fluorescence emission spectra of 2 μM ThT (a), CV (b), and PPIX (c) in the presence or absence of TdT-generated G-quadruplex from ssDNA oligo (100 nM). (d) Linear relationship between the fluorescence change and the concentration of ssDNA primer used for TdT-mediated elongation assay followed by addition of ThT (red), CV (blue), and PPIX (black), respectively. Inset: the fluorescent intensities of ThT at 485 nm as a function of the primer concentration.

exhibited a DNA primer-concentration dependent increase in fluorescence intensity (Figure S2a, b, c), but the sensitivity (the gradient value of 5.85 nM^{-1}) of ThT was two orders of magnitude higher than that of CV (the gradient value of 0.04 nM^{-1}) and PPIX (the gradient value of 0.02 nM^{-1}). Moreover, the detection limit (0.01 nM) of ThT was also two or even three orders of magnitude lower than that of CV (5 nM) and PPIX (20 nM) due to the high S/B ratio and low background of ThT (Figure 2d). All these results indicated that ThT is the favourable fluorescent dye for staining the TdT-generated G-quadruplex in our experimental setting. Moreover, to test whether the fluorescent switch-on of ThT really resulted from the TdT-generated G-quadruplex, the cationic porphyrin 5,10,15,20-tetra(N-methyl-4-pyridyl) porphyrin (TMPyP4) (Figure S3a), a potent G-quadruplex-binding ligand, was exploited as the competitive inhibitor for G-quadruplex/ThT complex. As shown in Figure S3b, the fluorescent signal greatly diminished upon addition of TMPyP4 in a dose-dependent manner, suggesting the fluorescent signal specifically comes from ThT upon binding to G-quadruplex. Our results confirmed that ThT is a sensitive and selective fluorogenic dye for lighting-up TdT-yielded G-quadruplex, providing an optimal probe for our TAGS assay[34].

Sensitivity, specificity, and reproducibility of TAGS assay for DSBs detection. To test if TAGS assay sensitively detects DSBs, different concentrations of pET-28 plasmid DNA (pDNA) were digested by DNase I to generate short DNA fragments with DSBs, followed by TAGS assay to generate randomly arrayed G-quadruplexes. Upon ThT addition, the fluorescence intensity gradually increased as pDNA concentration increased (Figure S4a). The fluorescence intensity increased linearly in a pDNA concentration-dependent manner and the limit of detection was about 1.5 pM (Figure S4b). Furthermore, gDNA were isolated from Hela cells and treated with DNase I to generate DSBs. After the TdT-mediated elongation assay with ThT staining, a remarkable fluorescence enhancement was observed within a response range from 0 to 9000 cells (Figure 3a and b).

No significant variation in the fluorescence intensity of ThT was observed when the gDNA was treated with DNase I or TdT alone (Figure 3b), which is in accordance with the results of CD measurement (Figure 1b). This method allowed a quantitative measurement of the DSBs from cellular gDNA with the linear detection range from 9 to 870 cells and the limit of detection is 3 cells (Figure S5).

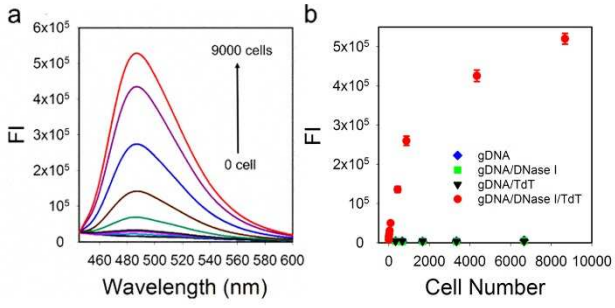


Figure 3. Fluorescent detection of DSBs from DNase I-digested gDNA. gDNA were extracted from Hela cells (2×10^4) and digested by DNase I (0.5 U) followed by TAGS assay with 1 mM G-rich dNTP, 4 U TdT, and 2 μM ThT. (a) The fluorescence emission spectra of ThT versus different number of cells (conresponding to the amount of gDNA for the assay). (b) The fluorescence intensities of ThT at 485 nm were plotted as a function of the number of cells.

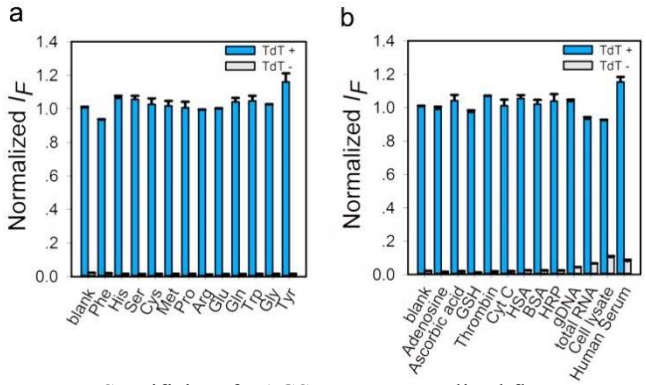


Figure 4. Specificity of TAGS assay. Normalized fluorescence intensity of ThT in reaction mixture, each containing DNase I-treated pDNA (0.24 nM) and G-rich dNTPs pool (1 mM) with or without TdT(4 U), were monitored in the presence of molecules as indicated. (A) Various amino acids including Phe, His, Ser, Cys, Met, Pro, Arg, Glu, Gln, Trp, Gly and Tyr (0.1 mM each) (B) Other cellular molecules: [Adenosine]=1 mM, [Ascorbic acid] =1 mM, [GSH]=1 mM, [Thrombin]=2 U/mL, [Cyt C]=1 μg/L, [HSA]=0.1 mg/L, [BSA]=0.1 mg/L, [HRP]=0.1 mg/L, gDNA from Hela cells (2×10^4), total RNA from Hela cells (2×10^4), cell lysates from Hela cells (2×10^4), and human serum (50 g/L).

Complex cellular environment is composed of various biomolecules, including small molecules, amino acids, peptides, proteins, and nucleic acids, thus their potential effects on the signal of TAGS assay were also evaluated prior to cellular detection. As shown in Figure 4a, the presence of some standard amino acids (Phe, His, Ser, Cys, Met, Pro, Arg, Glu, Gln, Trp, Gly and Tyr) shows no effect on the fluorescent intensity of ThT with or without G-quadruplex produced by TdT-mediated elongation from DNase I-digested pDNA. Similarly, negligible effects on fluorescence response of this detection system were confirmed in the presence of other cellular macromolecules including adenosine, ascorbic acid, GSH, thrombin, Cyt C, HSA, BSA, HRP (Figure 4b). Moreover, cell lysates, gDNA, total RNA (from 2×10^4 Hela cells), and human serum have little influence on the fluorescence intensity of ThT alone or ThT bound by TdT-generated G-quadruplex (Figure 4b). These results suggested that ThT can selectively identify the *de novo* generated G-quadruplex among the background of endogenous DNA/RNA in complex cellular environment. To test the reproducibility of the TAGS assay for DSB detection, we repeated the measurements for 50 times and found that the fluorescent intensity was stable with a relative standard deviation (R.S.D.) of 6.16% (Figure S6). In conclusion, TAGS might provide a feasible and solid approach to analyze apoptotic DSBs at cellular level.

***In situ* detection of apoptosis in cultured cell.** The principle of *in situ* label-free detection of apoptotic cells based on *de novo* G-quadruplex synthesis is shown in Scheme 1. Upon apoptotic signal, caspase-activated deoxyribonucleases are activated to cleave chromosomal DNA preferentially at internucleosomal sections. During DNA fragmentation, a multitude of DSBs generated *in situ* work as favourable primers of TdT-mediated enzymatic elongation in the presence of optimized G-rich dNTP pool. The prolonged randomly arranged G-rich sequence forms G-quadruplexes, which greatly enhances the fluorescence intensity upon ThT addition. The bright fluorescence of ThT indicates the *de novo* synthesis of G-quadruplex in apoptotic cells, allowing a sensitive detection of DNA fragmentation in apoptotic cells at single cell level.

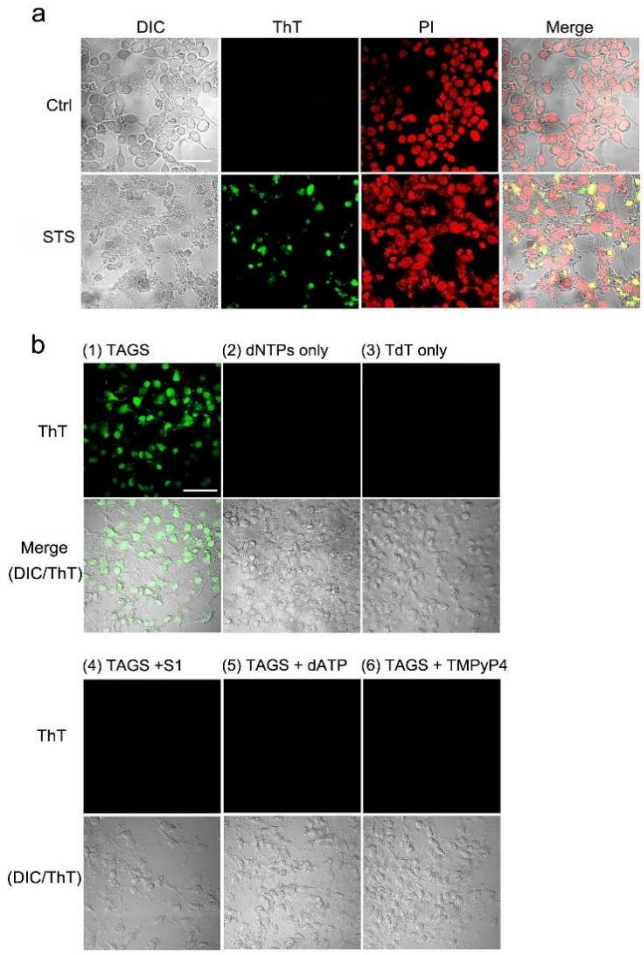


Figure 5. Apoptosis detection in cultured cells using TAGS assay. (a) Hela cells were treated with/without STS (100 nM) for 40 hours to induce apoptosis and the apoptotic cells were incubated with G-rich dNTP pool and TdT, followed by staining with 2 μM ThT. Nuclei were counterstained with PI (5 μg/L). (b) *De novo* synthesized G-quadruplex are essential for fluorescence enhancement of TAGS assay. STS-induced apoptotic cells were subjected to following conditions: (1) TdT-mediated elongation in the presence of G-rich dNTP pool and TdT; (2) G-rich dNTP pool only; (3) TdT only; (4) TdT-mediated elongation in the presence of G-rich dNTP pool followed by incubation with S1 nuclease (10 U) for 2 h; (5) TdT-mediated elongation in the presence of dATP; (6) TdT-mediated elongation in the presence of G-rich dNTP pool followed by addtion of TMPyP4 (6 μM) for 10 min. Scale bar: 50 μm.

We further tested the feasibility of TAGS assay for apoptosis detection at cellular level using apoptotic cells induced with staurosporine (STS), a widely used apoptosis inducer due to its activation of caspase 3 apoptotic pathway[46]. Hela cells were treated with STS (100 nM) for 40 hours and incubated with of TdT (60 U) in the presence of 1 mM unlabeled dNTP pool (10% dTTP, 40% dATP and 50% dGTP) at 37 °C for 1 h. Then, the cells were stained in ThT (2 μM) for 1 minute and then directly visualized using a confocal laser scanning microscope without additional washing steps. The apoptotic cells showed strong fluorescent signal at 488 nm channel (Figure 5a), whereas no signal was observed in control cells (STS untreated), clearly indicating that our method can efficiently distinguish and identify single apoptotic cell from a number of non-apoptotic cells.

To verify that the fluorescence activation response was specific to the DSBs of cleaved chromatin during cell apoptosis, we stained the cell nuclei with propidium Iodide (PI), a specific dye for double strand DNA. We found that activated fluorescence signal derives from ThT co-localized with the signal from nuclei staining by PI, indicating that *de novo* G-quadruplexes were formed within the nuclei (Figure 5a). However, when the STS-induced apoptotic cell was incubated with solo G-rich dNTP pool or TdT, little fluorescence signal was observed (Figure 5b (2) and (3)), suggesting that the signal came from the TdT-mediated polymerization in the apoptotic cells. To confirm that the fluorescence signals were derived from ssDNA synthesized by TdT, the apoptotic cells were subjected to TAGS assay followed by treatment with S1 nuclease that specifically cleaves ssDNA[47,48]. S1 nuclease treatment remarkably eliminated the fluorescent signal (Figure 5b, (4)), implying that the signal came from newly synthesized ssDNA and the interference of cellular RNA and proteins was negligible. To prove the fluorescent signal is specific for G-quadruplex, we replaced the G-rich dNTP pool with 100% dATP in TAGS assay to generate poly-A ssDNA by TdT. In this case, fluorescence signal was totally abolished, indicating that the signal is specifically from *de novo* G-quadruplex (Figure 5b, (5)). Moreover, the addition of TMPyP4 (6 μM) greatly eliminated the fluorescence signal (Figure 5b, (6)), which is consistent with the result from the TMPyP4 competition experiment in solution (Figure S3), further supporting that the fluorescence signal specifically comes from ThT/G-quadruplex complex.

Next, to test whether TAGS assay can identify and quantify apoptotic cell fraction in cultured cell preparations, Hela cells were treated with various concentration of STS and subjected

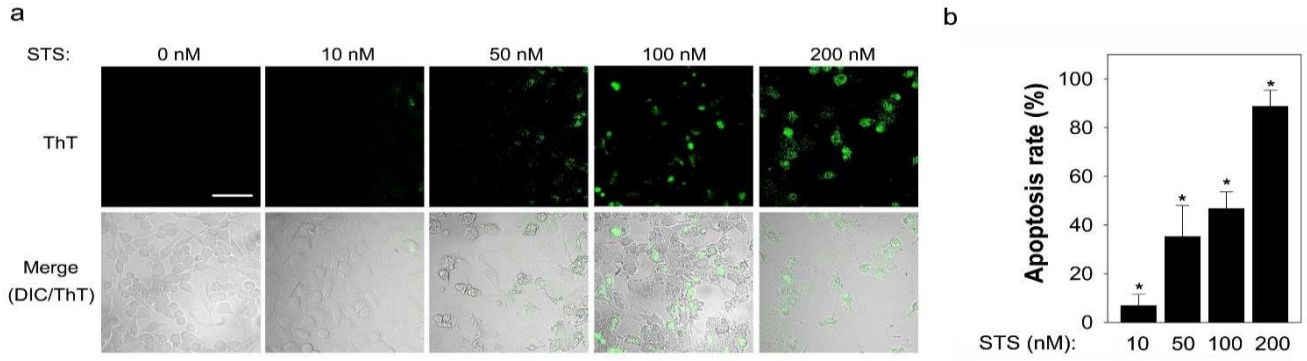


Figure 6. Hela cells were treated with increasing concentrations of STS as indicated for 40 hours followed by TAGS assay. (a) Apoptotic cells were visualized using 2 μM ThT and representative images were shown. Scale bar: 50 μm. (b) The ratios of apoptotic cells (ThT-postive) were quantified and values are represented as means± SD of 3 independent experiments. *P<0.05 vs. control (0 nM STS).

to TAGS assay. As shown in Figure 6, the fluorescent signal of TAGS assay was clearly seen in cells exposed to as low as 10 nM STS and the number of cells with bright fluorescent signal gradually mounted up as STS concentration increased. The DNA fragmentation induced by STS treatment was further validated using traditional DNA ladder assay, and the result exhibited that STS (200 nM) significantly increased DNA fragmentation level in Hela cells (Figure S7). Similarly, we confirmed that active-caspase 3 (cleaved caspase 3) in Hela cells increased as STS concentration went up (Figure S8). According to the cell number ratio of TAGS-positive cell to total cells, the apoptotic ratios in the group of cells treated with 10 nM and 100 nM STS were counted to 6.8% and 46.8%, respectively (Figure 6). We further applied an Annexin V-FITC/PI flow cytometry method for quantitative apoptosis analysis. Similarly, the late apoptosis ratios in the group of cells treated with 10 nM and 100 nM STS were estimated as 5.21% and 44.1% (Figure S9), respectively, well according to the corresponding data of TAGS assay. This result indicates that TAGS assay enables a quantitative analysis of apoptosis comparable to the Annexin V-FITC/PI flow cytometry method.

***In situ* detection of apoptosis at tissue level.** To evaluate the application of TAGS assay for apoptosis detection on tissue sample, we first performed TAGS assay on DNase I-treated kidney tissue sections from adult healthy Sprague-Dawley (SD) rats as positive controls that mimic apoptotic DSBs generation. Tissue sections of the rat kidney were exposed to DNase I followed by TAGS assay. Obvious and bright fluorescent signal at 488 nm channel (green) from individual cell was observed in the section using a confocal microscopy (Figure 7b). Little fluorescent signal was observed from the negative control, a healthy rat kidney section without DNase I treatment. To further test if TAGS assay can be applied for evaluation of apoptosis in a disease animal model, we subcutaneously treated adult SD rats with carbon tetrachloride (CCl_4) at 0.5 mL/kg for three weeks, which is a commonly used experimental model for acute hepatic and renal injury (Figure 7a)[49]. In this acute injury rat model, CCl_4 is metabolized to generate a highly reactive free radical, causing oxidative damage in DNA, proteins and lipids to induce cellular apoptosis[50]. We applied TAGS assay on a kidney section from CCl_4-injured rat and observed strong fluorescent light-up at individual cells in the section (Figure 7b), validating that TAGS assay can be applied for apoptosis detection at tissue level.

Our TAGS assay was further compared with a standard TUNEL assay. However, due to usage of labeled nucleotide (e.g. FITC-dUTP), TUNEL assay requires multiple washing and incubating steps in dark place, which is inconvenient and time-consuming. For comparison, DNase I-treated tissue sections of the rat kidney were used as a model for DSBs detection (Figure 7c). Traditional TUNEL assay shows high fluorescent background without washing with an ultralow S/B

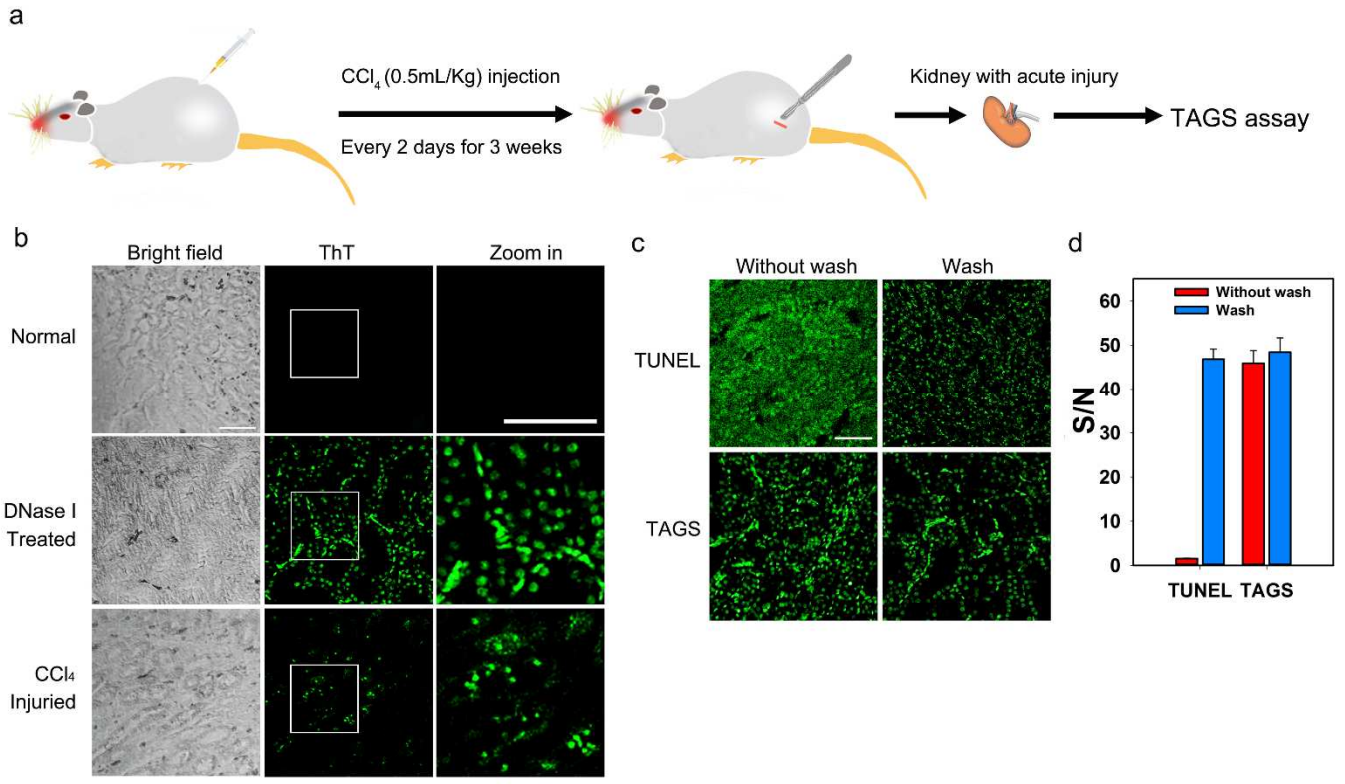


Figure 7. Apoptosis detection in kidney tissue sections using TAGS assay. (a) SD rats were subcutaneously injected with CCl_4 (0.5 ml/kg of body weight) on every other day for 3 weeks to make an experimental model for acute renal injury and the kidneys were subjected to TAGS assay. (b) Normal kidney sections or injured kidney sections were obtained from normal and CCl_4-injured rats, respectively. The positive control tissue sections were incubated with DNase I (3000 U) for generating DSBs. Paraffin sections treated with proteinase K were subjected to TAGS assay followed by ThT staining. The boxed regions in A and C are enlarged (3X) to the right. Scale bar: 100 μm. (c) Tissue sections from normal rat kidney were treated with DNase I (3000 U) and subjected to TUNEL assay or TAGS assay. The fluorescent images were aquired using CLSM. Scale bar: 100 μm. (d) The confocal images from TUNEL assay and TAGS assay with or without washing step were subjected to fluorescent intensity analysis and their S/B values were quantified. jected to fluorescent intensity analysis and their S/B values were quantified.

ratio to be only 1.53, while the acceptable images with an S/B ratio of 46.8 was obtained after three time washing (Figure 7c and d). In opposite, TAGS assay shows obvious and bright clear staining of individual cell, as well as high S/B ratio (45.9 and 48.4, respectively), in the kidney section without or with the washing procedure (Figure 7c and d). Moreover, the photo-stability of FITC used inTUNEL assay was also compared with that of the G-quadruplex/ThT complex employed in TAGS assay. The fluorescent signal of FITC quickly declined 67 % in 30 min while the ThT signal shows negligible decrease (Figure S10). This result indicated that the fluorescent complex of ThT and TdT-generated G-quadruplexes possesses much better photo-stability against photo-bleaching than FITC, which probably resulted from that partial exchange of bound ThT with free ThT in solution prevents the accumulation of photo-bleached complexes. Hence, TAGS possesses several advantages over conventional apoptotic assays as follows: (1) the usage of unmodified dNTP avoids the labeled nucleotides, eliminates non-specific signal, and lowers the cost; (2) its fluorogenic property avoids the time-consuming multi-step washing and incubation and inconvenient light-avoidance operations; (3) due to quick staining of ThT in 1 min, TAGS assay was a "mix and read" procedure ready for microscopic observation; (4) the great photo-stability of fluorescent ThT/G-quadruplex complex implied that TAGS might be more favourable for long term fluorescent microscopic observation.

CONCLUSIONS

In summary, we developed a novel and label-free TAGS assay for in situ "tagging" of apoptotic cell based on the principle of enzyme-mediated G-quadruplex formation. Sensitive and quantitative analysis of apoptosis in cultured cell and tissue section were achieved using TAGS assay. Our method may show potential impacts on multifarious aspects. Firstly, a novel DNA 3'-OH end tagging technique using TAGS presents a promising and feasible method for biochemical analysis. Secondly, the use of TAGS as a signal producer doesn't require modified nucleotides, paving a way for the development of label-free in situ apoptosis assay. Thirdly, TAGS evidenced that G-quadruplex along with specific ligands can be applicable in complex bio-sensing system such as cell or tissue which expanded the application and understanding of G-quadruplex. Moreover, considering the module feature of TAGS by facilely altering G-qaudruplex-targeting fluorogenic probes, TAGS shows great promise for several advanced bio-imaging techniques, including near-infrared or two-photon imaging, by choosing or developing proper probes. Hence, TAGS provides an efficient platform for in situ visualization of apoptosis at single cell level, which holds great potential in biochemical analysis and clinical diagnosis

ASSOCIATED CONTENT

Supporting Information

Please see supporting information (SI) for the experimental details and figures (Figure S1-S10). The Supporting Information is available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Author

*Corresponding author email address: Tel.: +86-731-88821626; Fax: +86-731-88821848. niezhou.hnu@gmail.com; wanghonghui@hnu.edu.cn.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Zhuoliang Liu and Xingyu Luo contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

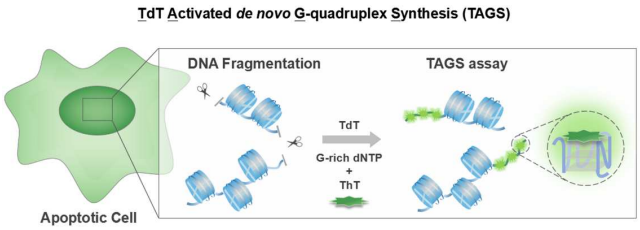
This work was financially supported by the National Natural Science Foundation of China (Nos. 21235002 and 21575038), the Foundation for Innovative Research Groups of NSFC (Grant 21521063), the Young Top-notch Talent for Ten Thousand Talent Program, and the Natural Science Foundation of Hunan Province (No. 2015JJ1005).

REFERENCES

(1) Collie, G. W. and Parkinson, G. N., *Chem. Soc. Rev.*, **2011**, 40, 5867-5892.
(2) Huppert, J. L., *Chem. Soc. Rev.*, **2008**, 37, 1375-1384.
(3) Doluca, O., Withers, J. M. and Filichev, V. V., *Chem. Rev.*, **2013**, 113, 3044-3083.
(4) Golub, E., Albada, H. B., Liao, W. C., Biniuri, Y. and Willner, I., *J. Am. Chem. Soc.*, **2016**, 138, 164-172.
(5) Albada, H. B., Golub, E., and Willner, I.. *Chem. Sci.*, **2016**, 7, 3092-3101.
(6) Mao, X. H., Simon, A. J., Pei, H., Shi, J. Y., Li, J., Huang, Q., Plaxco, K. W. and Fan, C. H.. *Chem. Sci.*, **2015**, 7, 1200-1204.
(7) Jiang, H. X., Zhao, M. Y., Niu, C. D., and Kong, D. M., *Chem. Commun.*, **2015**, 51, 16518-16521.
(8) Kang, H. J., Cui, Y., Yin, H., Scheid, A., Hendricks, W. P., Schmidt, J., Sekulic, A., Kong, D., Trent, J. M., Gokhale, V., Mao, H., and Hurley, L. H., *J. Am. Chem. Soc.*, **2016**, 138, 13673-13692.
(9) Hu, Y., Shen, Q., L, W., Liu, Z., Nie, Z. and Yao, S., *Biosens. Bioelectron.*, **2015**, 63, 331-338.
(10) Kwok, C. K., Marsico, G., Sahakyan, A. B., Chambers, V. S. and Balasubramanian, S., *Nat. Methods*, **2016**, 13, 841-844.
(11) Barlev, A., Sen, D., *J. Am. Chem. Soc*., **2013**, 135, 2596-2603.
(12) Wang, Z. G., Zhan, P., Ding, B., *ACS Nano*, **2013**, 7, 1591-1598.
(13) Biffi, G., Tannahill, D., Mccafferty, J. and Balasubramanian, S., *Nat. Chem.*, **2013**, 5, 182-186.
(14) Guo, J. H., Kong, D. M., Shen, H. X., *Biosens. Bioelectron.*, **2010**,26, 327-332.
(15) Lai, H., Xiao, Y., Yan, S., Tian, F., Zhong, C., Liu, Y., Weng, X. and Zhou, X., *Analyst.*, **2014**, 139, 1834-1388.
(16) Lubitz, I., Zikich, D. and Kotlyar, A., *Biochemistry*, **2010**, 49, 3567-3574.
(17) Li, H., Liu, J., Fang, Y., Qin, Y., Xu, S., Liu, Y. and Wang, E., *Biosens. Bioelectron.*, **2013**, 41, 563-568.
(18) Li, T., Wang, E. and Dong, S., *Anal. Chem.*, **2010**, 82, 7576-7580.
(19) Leung, K. H., He, B., Yang, C., Leung, C. H., Wang, H. M., Ma, D. L., *ACS Appl. Mater. Interfaces*, **2015**, 7, 24046-24052.
(20) Bhasikuttan, A. C. and Mohanty, J., *Chem. Commun*., **2015**, 51, 7581-7597.
(21) Ma, D. L., Lin, S., Leung, K. H., Zhong, H. J., Liu, L. J., and Chan, D. S., *Nanoscale.*, 2014, 15, 8489-8494.
(22) Wang, M., Wang, W., Kang, T. S., Leung, C. H., and Ma, D. L., *Anal*. *Chem*., 2015, 1, 981-987.
(23) Xu, M., Gao, Z., Wei, Q., Chen, G., and Tang, D., *Biosens. Bioelectron.*, 2015, 18, 1-7.
(24) Li, W., Li, Y., Liu.Z, Lin, B., Yi, H., Xu, F., Nie, Z. and Yao, S., *Nucleic Acids Res.* **2016**, 44, 7373-7384.
(25) Fuchs, Y. and Steller, H., *Cell*, **2011**,147, 742-758.
(26) Fesik, S. W., *Nat. Rev. Cancer*, **2005**, 5, 876-885.
(27) Zhang, L., Lei, J. P., Liu, J. T., Ma, F. J. and Ju, H. X., *Chem. Sci.*, **2015**, 6, 3365-3372.
(28) Wlodkowic, D., Telford, W., Skommer, J., Darzynkiewicz, Z., *Methods Cell Biol*., **2011**, 103, 55-98.

(29) Roos, W. P. and Kaina, B., *Trends Mol. Med.*, **2006**, 12, 440-450.
(30) Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A., *J. Cell Biol.*, **1992**, 119, 493-501.
(31) Watanabe, M., Hitomi, M., van der Wee, K., Rothenberg, F., Fisher, S. A., Zucker, R., Svoboda, K. K., Goldsmith, E. C., Heiskanen, K. M. and Nieminen, A. L., *Microsc. Microanal.*, **2002**, 8, 375-391.
(32) Huerta, S., Goulet, E. J., Huerta-Yepez, S. and Livingston, E. H., *J. Surg. Res.*, **2007**, 139, 143-156.
(33) Sloop, G. D., Roa, J. C., Delgado, A. G., Balart, J. T., 3rd Hines, M. O. and Hill, J. M., *Arch. Pathol. Lab. Med.*, **1999**, 123, 529-532.
(34) Liu, Z., Li, W., Nie, Z., Peng, F., Huang, Y. and Yao, S., *Chem. Commun.*, **2014**, 50, 6875-6878.
(35) Mohanty, J., Barooah, N., Dhamodharan, V., Harikrishna, S., Pradeepkumar, P. I. and Bhasikuttan, A. C., *J. Am. Chem. Soc.*, **2013**, 135, 367-376.
(36) Vorlickova, M., Kejnovska, I., Sagi, J., Renciuk, D., Bednarova, K., Motlova, J. and Kypr, J., *Methods*, **2012**, 57, 64-75.
(37) Oliveri, M., Daga, A., Cantoni, C., Lunardi, C., Millo, R. and Puccetti, A., *Eur. J. Immunol.*, **2001**, 31, 743-751.
(38) Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G. and Tschopp, J., *EMBO J.*, **1993**, 12, 371-377.
(39) Chen, S. B., Hu, M.H., Liu, G. C., Wang, J., Ou, T. M., Gu, L. Q., Huang, Z. S. and Tan, J. H., *J. Am. Chem. Soc.*, **2016**, 138, 10382-10385.
(40) Huang, X. X., Zhu, L. N., Wu, B., Huo, Y. F., Duan, N. N. and Kong, D. M., *Nucleic Acids Res.*, **2014**, 42, 8719-8731.
(41) Jin, B., Zhang, X., Zheng, W., Liu, X., Zhou, J., Zhang, N., Wang, F., and Shangguan, D., *Anal. Chem.*, **2014**, 86, 7063-7070.
(42) Li, F., Feng, Y., Zhao, C. and Tang, B., *Chem. Commun.*, **2011**, 47, 11909-11911.
(43) Shen, Q., Zhou, L., Yuan, Y., Huang, Y., Xiang, B., Chen, C., Nie, Z., and Yao, S., *Biosens. Bioelectron.*, **2014**, 55, 187-194.
(44) Xue, Q., Lv, Y., Zhang, Y., Xu, S., Li, R., Yue, Q., Li, H., Wang, L., Gu, X., Zhang, S. *et al.*, *Biosens. Bioelectron.*, **2014**, 61, 351-356.
(45) Li, T., Wang, E. and Dong, S., *Anal. Chem.*, **2010**, 82, 7576-7580.
(46) Chae, H. J., Kang, J. S., Byun, J. O., Han, K. S., Kim, D. U., Oh, S. M., Kim, H. M., Chae, S. W. and Kim, H. R., *Pharmacol. Res.*, **2000**, 42, 373-381.
(47) Zhou, Z., Zhu, J., Zhang, L., Du, Y., Dong, S. and Wang, E., *Anal. Chem.*, **2013**, 85, 2431-2435.
(48) Fuchs, R. P., *Nature*, **1975**, 257, 151-152.
(49) Won, A. J., Kim, S., Kim, Y. G., Kim, K. B., Choi, W. S., Kacew, S., Kim, K. S., Jung, J. H., Lee, B. M., Kim, S. *et al., Mol. Biosyst.*, **2016**, 12, 133-144.
(50) Sahreen, S., Khan, M. R., Khan, R. A. and Alkreathy, H. M., *Food Nutr. Res.*, **2015**, 59, 28438-28448.

for TOC only


TdT Activated de novo G-quadruplex Synthesis (TAGS)
DNA Fragmentation
TAGS assay
TdT
G-rich dNTP
+
ThT
Apoptotic Cell