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Real-Time, Quantitative Lighting-up Detection of Telomerase in Urines of Bladder Cancer Patients by AIEgens

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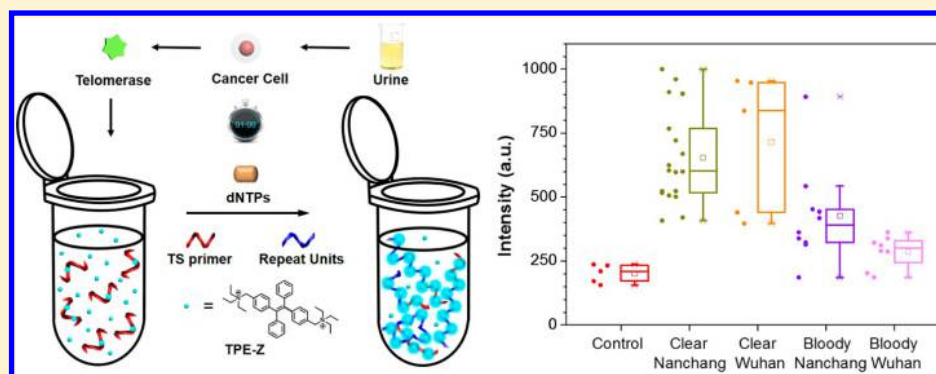
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Supporting Information



ABSTRACT: As a biomarker for early cancer diagnosis, telomerase are one of the promising targets for cancer therapeutics. Inspired by the fluorescent emission principle of aggregation-induced emission fluorogens, we creatively designed an AIE-based turn-on method to detect telomerase activity from cell extracts. A positively charged fluorogen (TPE-Z) is not fluorescent when freely diffused in solution. The fluorescence of TPE-Z is enhanced with the elongation of the DNA strand which could light up telomere elongation process. By exploitation of it, we can detect telomerase activity from different cell lines (E-J, HeLa, MCF-7, and HLF) with high sensitivity and specificity. Moreover, our method is successfully employed to demonstrate the applications in bladder cancer diagnosis (41 urine specimens from bladder cancer patients and 15 urine specimens from normal people are detected). The AIE-based method provides a simple one-pot technique for quantification and monitoring of the telomerase activity and shows great potential for future use in clinical tests.

Detection of biomarkers for cancers with high sensitivity and selectivity is an essential step for the diagnosis, prevention and treatment of cancers as well as for the understanding of cancer pathogenesis. Over the past few decades, telomerase has gained increasing interest as one of the most common biomarkers for cancers.^{1–3} Telomerase is a ribonucleoprotein which is responsible for telomere maintenance by adding the tandem repeats of (TTAGGG)_n to the ends of chromosomes, which is associated with cell immortality and thus cancers. In most normal somatic cells, telomerase activity is highly depressed,^{4–6} while the up-regulation or reactivation of telomerase activity is observed in approximately 90% of human cancer cells. This suggests that methods for the detection of telomerase activity could potentially be used for

cancer diagnosis, screening of anticancer drugs, and evaluation of cancer therapy.^{7–10}

Currently, there are mainly two types of assays available for telomerase activity detection: the amplification-based and the direct assays.¹¹ Telomere repeat amplification protocol (TRAP) is one of the most popular amplification-based assays for detecting telomerase activity.^{12–15} The original TRAP, however, is time-consuming and relied on PCR method, which could inherently generate false positive/negative artifacts and, thus, is not suitable for quantitative analysis and screening

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compounds for telomerase inhibition.^{16–20} Alternatively, direct detection methods based on optical detection, array, and biosensor chip, electrochemical strategies or telomerase purification have been reported.^{21–28} Among them, fluorescence methods have received much attention because of the simplicity, sensitivity, and capacity to high-throughput screening.^{29–38} For example, fluorescence biosensors based on “molecular beacons” have been developed to detect telomerase activity.³⁹ This process, however, requires prelabeling of oligonucleotides with a fluorogen and a quencher by chemical reactions. Furthermore, the working units in the bioprobes are always hydrophobic aromatic rings and other p-conjugated chromophores.^{40–44} The fluorogen tends to aggregate when absorbed onto strand surfaces owing to the incompatibility of the dyes with the hydrophilic media (aqueous buffer solutions), leading to severe emission self-quenching, namely, aggregation-caused quenching (ACQ), which limits the label-to-analyte ratio and forces researchers to use very dilute solutions of fluorogens.^{45–48}

To address this concern, we are interested in a group of fluorogens that are nonemissive when molecularly dissolved but highly luminescent when supramolecularly aggregated.⁴⁹

Tang's group coined this phenomenon as “aggregation-induced emission” (AIE) and proposed restriction of intramolecular motions (RIM) as the mechanism.^{50–56} In this work, we make use of the AIE property and develop a simple fluorescent assay for the detection of telomerase activity with superior sensitivity, and demonstrate their applications in clinical cancer sample due to the high specificity. Tetraphenylethene (TPE) is an iconic AIE dye, which can be easily prepared and readily functionalized. A positively charged quaternized tetraphenylethene salt (TPE-Z) prepared according to the previous literature⁵⁷ is chosen in this study. Owing to its amphiphilic nature, TPE-Z is soluble in water. Thus, it is nearly nonfluorescent in buffers, which provides low background for the sensing system. The fluorescence of TPE-Z remains weak in the presence of short DNA oligonucleotides such as Ex-0 (18-nt; Figure 1a, left). In the presence of longer single-stranded DNA, such as Ex-6 (54-nt; Figure 1a, right), the emission of TPE-Z is turned on with the peak at 478 nm. Longer ssDNA results in more significant fluorescence increase (Figure S1a–c). Attracted by electrostatic force, the positively charged TPE-Z can spontaneously bind to the negatively charged DNA backbone. Once bound to the backbone of the DNA strand, the RIM process is occurred, which thus blocks the radiationless relaxation pathways of TPE-Z and activates its fluorescence.⁵⁸ As a longer DNA strand can provide more negative charges, it offers more binding sites for more TPE-Z molecules to bind and aggregate, resulting in stronger fluorescence signals. In a parallel experiment, SYBR Green I, a conventional nucleic acid dye, is chosen for comparison (Figure S1d–f). Different from TPE-Z, SYBR Green I exhibits slightly lower fluorescence intensity in the presence of longer single-stranded DNA rather than shorter single-stranded DNA, which implies that SYBR Green I (ACQ dye) is not a suitable telomerase activity probe in this system.

EXPERIMENTAL SECTION

Materials. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under nitrogen immediately prior to use. Water is purified by a Millipore filtration system. Other chemicals were purchased from Aldrich, Alfa Aesar, and used as received without further purification. The 1× CHAPS

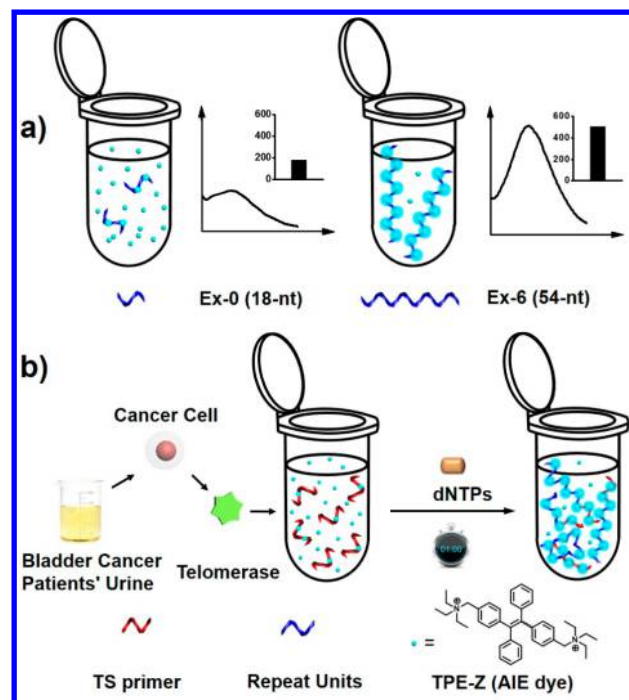


Figure 1. (a) AIE-based turn-on technique for different length of single-stranded DNA. The longer single-stranded DNA results in more significant fluorescence increase. (b) Schematic illustration of AIE-based simple one-pot technique for telomerase activity detection. In the presence of active telomerase extracted from urine specimens of bladder cancer patients, TS primer is extended by adding TTAGGG repeat units to the 3'-end to generate telomerase product, the fluorescence emission of TPE-Z probe will experience off-to-on change (from left to right), which can be monitored by FL spectra.

Lysis Buffer was purchased from Millipore (Bedford, MA). The deoxynucleotide solution mixture (dNTPs), RNase inhibitor, and DEPC-treated water were purchased from TaKaRa Bio Inc. (Dalian, China; DEPC = diethylpyrocarbonate). Oligonucleotides were synthesized by TaKaRa Bio Inc. (Dalian, China). E-J cells, MCF-7 cells, and HeLa cells were obtained from Xiangya Central Experiment Laboratory. HLF cells were obtained from China Center for Type Culture Collection. Patient samples were donated by Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Cell Culture. E-J and HeLa cancer cells were cultured in 1640 (GIBCO) medium with 10% fetal calf serum and 1% penicillin streptomycin (PS, 10000 IU penicillin and 10000 $\mu\text{g}/\text{mL}$ streptomycin, MULTICELL) in a culture flask at 37 °C in a humidified atmosphere containing 5% CO_2 . MCF-7 cancer cells were cultured similarly in Dulbecco's modified Eagle's medium (DMEM, MULTICELL), and HLF cells were cultured similarly in Minimum Essential Medium (MEM/EBSS, 1×, HyClone).

Telomerase Extracted from Cultured Cells. Cancer cells were first suspended in 1× CHAPS lysis buffer (lysis buffer, Millipore) to make the concentrate 5000 cells/ μL and incubated on ice for 30 min. Then the mixture was centrifuged at 12000 g for 20 min at 4 °C, and the supernatant was transferred, aliquoted, and stored at –80 °C.

Telomerase Extracted from Urine Samples. Fresh urine samples were collected and centrifuged at 850 g for 10 min at 4 °C, washed once using phosphate-buffered saline (PBS, Multicell). Centrifuged above samples at 2300 g for 5 min at 4 °C. Resuspended the precipitate in 200 mL of ice-cold 1×

CHAPS lysis buffer and then incubated on ice for 30 min. Centrifuged the mixture at 10000 g for 20 min at 4 °C. The supernatant was transferred, aliquoted, and stored at −80 °C.

Telomerase Extension Reaction and Detection by TPE-Z. Telomerase extracts from cells were first diluted in 1× CHAPS lysis buffer. Then appropriate volume of diluted telomerase extracts equivalent to a series of respective number of cells and 7.8 μM TPE-Z were added into the telomerase extension reaction buffer containing dNTPs, TS primer, and RNase inhibitor, with the total volume of 200 μL. The solution was incubated at 37 °C for 60 min, then transferred to 94 °C for 10 min to end the reaction. For the heat-inactivated experiment, telomerase extracts from cancer cells were heat-treated at 95 °C for 20 min before use.

RESULTS AND DISCUSSION

In the above experiment, we observe that the fluorescence of TPE-Z is enhanced with the length of the DNA strand. We thus speculate that this property could be useful for measuring the activity of telomerase. The principle of the AIE-based turn-on method for the telomerase activity assay is illustrated in Figure 1b. In the absence of telomerase, the solution containing TPE-Z and telomerase substrate oligonucleotides (TS primer) is weakly fluorescent (Figure 1b, left). The probe is at its “off” state due to the less negative charge sites of TS primer (18-nt). In the presence of active telomerase, TS primer could be extended with dNTPs in the solution, producing single strand of telomeric repeated sequence at its 3'-end. Once the DNA chain becomes longer, the fluorescence of TPE-Z will be enhanced owing to the added negatively charged sites for the dye molecules to bind and aggregate (Figure 1b, right). On the other hand, if there is no telomerase or the telomerase is in an inactive state, the emission of TPE-Z will remain weak. By using this method, the relationship between emission intensity and analyte concentration is investigated and established, which could facilitate the detection of telomerase activity in aqueous buffers or even body fluids.

To find out the optimized conditions for telomerase activity detection, several experiments are conducted. As shown in Figure S2, the fluorescence intensities of TPE-Z increase rapidly in the presence of TS primer and active telomerase during the first 40 min and reached an equilibration step at 1 h. The concentration of either TS primer or dNTPs is varied accordingly. Finally, we define the concentrations of TS primer and dNTPs, respectively (Figures S3 and S4). Under the optimized conditions, a more than 11× increase in fluorescence intensity is observed when we perform this assay with a sample of ~25000 telomerase-positive MCF-7 cells (Figures S5–8). These results suggest the potential of TPE-Z to work as an in vitro bioprobe for detection of the activity of the telomerase extract.

In order to test the response of the probe to telomerase, extracts from telomerase-positive human bladder cancer cells (E-J) are serially diluted and used as a source of telomerase. A TS primer is incubated with the cell extract at 37 °C for 1 h, and then the fluorescence intensity was recorded. As shown in Figure 2a, the fluorescent intensity is enhanced gradually when the number of the E-J cells was raised from 0 to 10000. These results demonstrate the positive correlation between the fluorescence increment at 478 nm and the cell numbers (Figure 2b). In terms of sensitivity, this method can measure telomerase activity down to 10 E-J cells with broad detection range. To our knowledge, the sensitivity is comparable or even

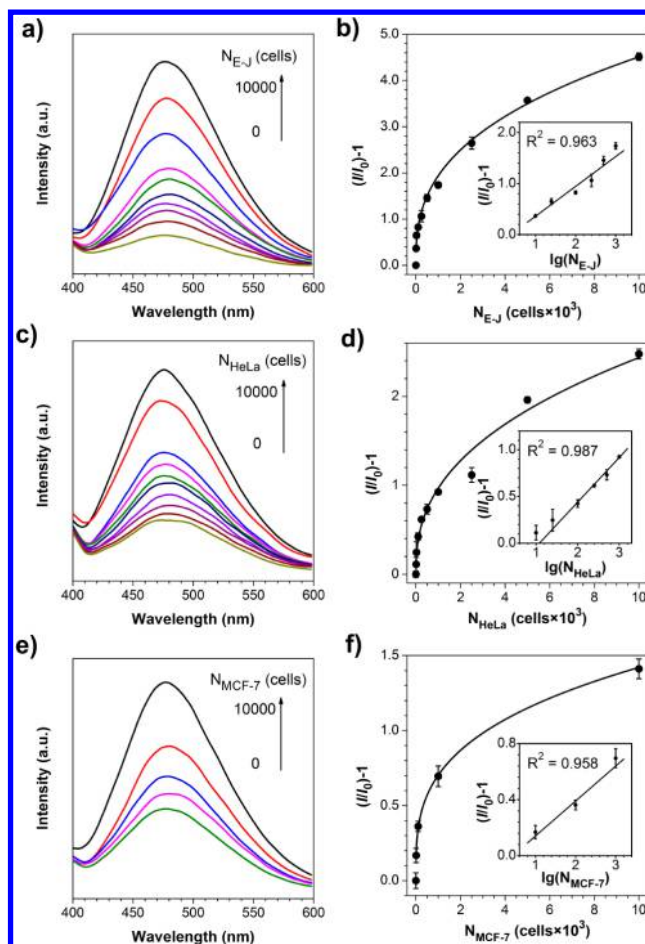


Figure 2. Activity detection of telomerase extracts from three types of cancer cells. Emission spectra of TPE-Z in response to telomerase extracts from different numbers of bladder cancer cells (E-J, a), cervical cancer cells (Hela, c), and breast cancer cells (MCF-7, e). The relationship between fluorescence enhancement and the cell numbers of E-J cells (b), Hela cells (d), and MCF-7 cells (f). Inset: linear relationship between the fluorescence enhancement and the logarithm of cell numbers. The fluorescent intensity is enhanced gradually when the number of the cancer cells is raised from 0 to 10000. Error bars indicate standard deviation of triplicate tests.

superior to most previously reported optical methods (shown in Table S1). Table S1 summarizes the performance of several typical colorimetry assays, chemiluminescence and fluorescence assays for detection of telomerase. However, most assays above need complex sample pretreatments or several kinds of enzymes, which leading to the critical conditions of detection. For example, the fluorescence method applying FAM as fluorophore and EB as quencher require prelabeling the TS primer with FAM.⁵⁹ Moreover, to demonstrate that it is a general and reliable method for telomerase detection, other cancer cell lines, including breast cancer cell (MCF-7) and cervical cancer cell (HeLa) are tested (Figure 2c–f). As expected, the method using cell extracts from MCF-7 and HeLa cells also generate positive telomerase activity.

The mechanism of aggregation via telomerase-triggered TS primer elongation could be verified in the following experiments. First, telomere-mimicking fragments of different lengths are chosen to study how the length of the DNA strands would affect the result (Table S2). The inset of Figure 3a displays the plot of the fluorescence intensity (monitored at 478 nm) versus

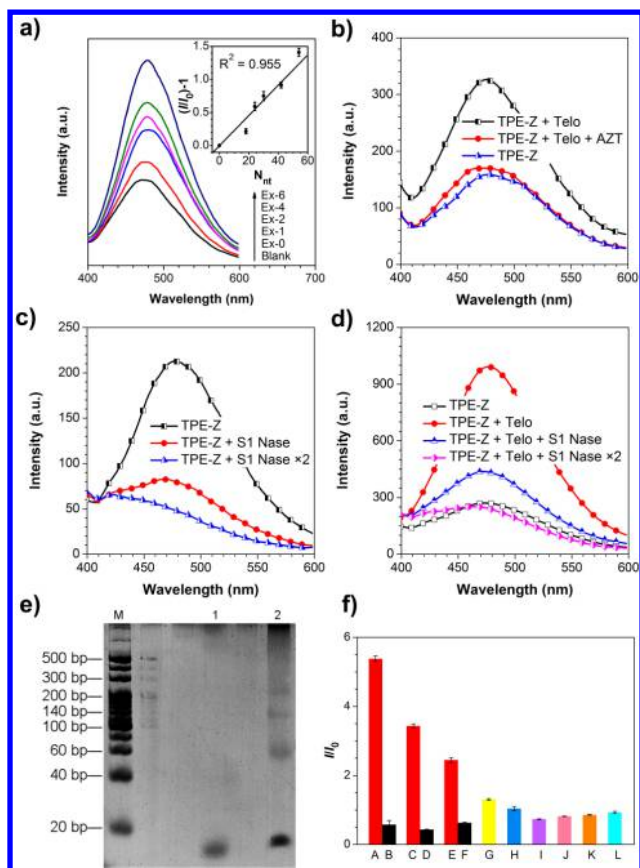


Figure 3. Working mechanism interpretation of the AIE-based telomerase activity assay. (a) Fluorescence emission spectra of TPE-Z and telomerase extracts from 10000 MCF-7 cancer cells in response to different oligonucleotides (blank, Ex-0, Ex-1, Ex-2, Ex-4, and Ex-6). Inset: linear relationship between $(I/I_0) - 1$ and lengths of oligonucleotides. Error bars indicate standard deviation of triplicate tests. (b) Inhibition of telomerase activity in extracts from 10000 MCF-7 cancer cells by AZT. (c) Fluorescence emission spectra of TPE-Z (square), and TPE-Z in the presence of elongated TS primer without (circle) and with (triangle) 18 U S1 Nase and with twice (diamond) 18 U S1 Nase. (d) Fluorescence emission spectra of TPE-Z in the presence of TS primer without (square) and with (circle) 18 U S1 Nase and with twice (triangle) 18 U S1 Nase. (e) Nondenaturing PAGE analysis of AIE-based assay: DNA ladder marker (lane M), TS primer in the absence (lane 1) and presence (lane 2) of telomerase extracts from 75000 MCF-7 cells. (f) Fluorescence responses of this sensing system to telomerase extracts from 10000 cancer cells E-J (A), HeLa (C), and MCF-7 (E), human lung fibroblast cells (HLF, G), heat-inactivated telomerase extracts from 25000 cancer cells E-J (B), HeLa (D), and MCF-7 (F), thrombin (H), trypsin (I), BSA (J), lysis buffer (K), and Bst DNA polymerase (L), respectively.

lengths of DNA, which indicates that longer single-stranded DNA results in more significant fluorescence in detection medium (Figure S9). Second, the inhibition of telomerase activity is also measured by using 3'-azido-3'- deoxythymidine (AZT) as a model inhibitor, into the mixture for telomerase extension reaction. The fluorescence intensity decreases after adding AZT, suggesting the potential application of the assay for screening telomerase inhibitors and telomerase-targeted drugs (Figure 3b). S1 Nuclease (S1 Nase), which is known to be a single-strand-specific endonuclease and hydrolyzes primarily single-stranded DNA and RNA to yield 5-nucleoside monophosphates and a tiny amount of dinucleotides,⁶⁰ is selected to digest the DNA strand. The TS primer (Figure 3c)

and its elongation product (Figure 3d) were selected as the substrate for nuclease S1. By conducting cleaved reaction, the fluorescence intensity gradually decreased, which is simply due to the DNA fragmentation, and as a result, the aggregation of TPE-Z becomes less efficient. Finally, we employ non-denaturing polyacrylamide gel electrophoresis (PAGE) analysis to monitor the resultant DNA in the above experiments. We observe a strong band at 60 bp indicative of telomerase products but cannot observe any telomerase products in control samples (Figures 3e and S10), which is inconsistent with the obtained fluorescence results.

In order to confirm the fluorescence enhancement observed in the above experiments is dependent on telomerase activity only, control experiments are performed by inactive telomerase or other proteins. To prove the activity of cancer cell extracts and the credibility of our method, a commercial ELISA Kit is used. From Figure S11, we can see the telomerase activity of E-J cancer cells extract is 11.50 IU/L (8000 E-J cells in 50 μ L detection system), which demonstrate the high activity of the telomerase we used. The telomerase extracted from three clear and two bloody clear urine specimens also possess activity in the same order of activity magnitude compared of our method, showing the credibility of our method (Table S3). As telomerase is sensitive to heat, heating to 95 $^{\circ}$ C is used to inactivate telomerase for the control experiment.⁶¹ When three kinds of telomerase-positive cancer cells extracts are pretreated with higher temperature, no detectable increase in fluorescence intensity over background are recorded. We also do not observe notable signal change when extracts of human lung fibroblast cells (HLF, a human normal cell line) are employed. To test the interference, we test the samples containing lysis buffer, Bst DNA polymerase, BSA, and thrombin, respectively. The results shown in Figure 3f confirm there is no process of extension on the TS primer if the solution contained no telomerase or the telomerase has been preheated. Moreover, we challenge this sensing platform to extracts from 10000 HeLa cancer cells which are treated with 100 μ M AZT. The result shows that it is the TS primer elongation rather than microviscosity that cause the fluorescent enhancement (Figure S12). Thus, the proposed method possesses broad applicability for monitoring telomerase activity and distinguishing different cells.

We demonstrate the clinical potentials of the AIE-based turn on method for telomerase activity detection. Bladder cancer is one of most common genitourinary malignancy that has the highest recurrence rate.^{62–64} We collect urine specimens from 41 bladder cancer patients and 15 normal people to evaluate the applicability in bladder cancer diagnosis (Figure 4, Tables S4 and S5, and Figure S13). As shown in Figure 4a, most of the clinical samples are above the threshold level according to the definition of $I_0 + 3\sigma$ (the horizontal dashed line; I_0 , average fluorescence intensity at 478 nm of 5 blank samples; σ , standard deviation of 5 blank samples). The relative deviations (RSD) are less than 9%. We applied this method in the determination of bladder cancer in clear sample (urine specimens of bladder cancer patients whose urine is clear) and bloody sample (gross hematuria, urine specimens of bladder cancer patients whose urines contain more than 4 mL blood in 1000 mL urines and are bloody macroscopically) without any treatment, to verify the detection capability, and demonstrate the proof of principle. The bloody urine of some bladder cancer patients is mainly due to rupture of renal blood vessel or rise of permeability of renal capillary wall. The photograph of bladder cancer urine samples is shown in Figure S14. The ratios of positive results to total

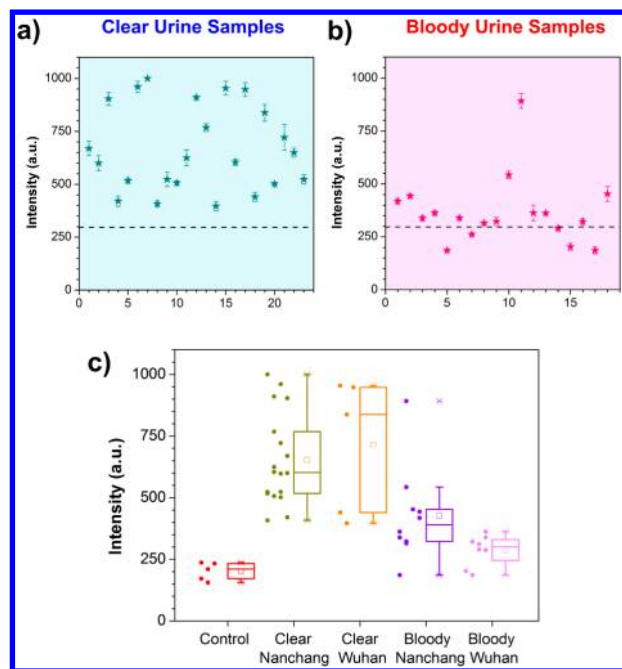


Figure 4. Results of AIE-based telomerase activity detection in real urine samples. Fluorescence intensity at 478 nm of detection system in response to telomerase extracts from urine specimens of 23 bladder cancer patients whose urine are clear (a) and 18 patients whose urine is bloody (b). The horizontal dashed line represents the threshold level according to the definition of $I_0 + 3\sigma$. (c) Box plot representation of telomerase activity detection by using AIE-based turn on technique. Error bars indicate standard deviation of triplicate tests.

samples are found out to be 23/23 (100%) for clear samples and 13/18 (72%) for blood samples, respectively. It indicates that the presence of blood would negatively affect the results because the interference occurs more in bloody samples. Where the patients come from are illustrated in Figure 4c. There is no significant difference between the patients from Wuhan (nearly the Yangtze River) and Nanchang (far from the Yangtze River, the distance of two city is 400 km), which is observed for the positive ratios (Figure S15). Moreover, we detect urine of 15 normal people using this system (Figure S13). The intensity of normal samples is nearly around the control and almost invariable. All the experiments confirm the possibility that the proposed AIE-based method can be applied as a novel strategy for bladder cancer detection in untreated real urine.

CONCLUSION

In conclusion, we have used a positively charged fluorogen (TPE-Z) for the construction of a novel method for detection of telomerase activity from primary cell samples. In comparison with the methods that require for elaborate instruments and expensive fluorescent-labeled nucleotides, our proposed method is more simple (label free), highly sensitive (down to 10 cells), and rapid (within 1 h). The practicality of this method is demonstrated by quantifying the intracellular telomerase activity in different cell lines (E-J, HeLa, MCF-7, and HLF). We have proven its clinical tests capabilities by 41 urine specimens (bloody samples and clear samples) from bladder cancer patients and 15 urine specimens from normal people. Thus, by providing a simple, noninvasive method with high applicability, we anticipate that it will accelerate the understanding of telomerase involved biological processes and

provide a promising tool for detection of telomerase activity and clinical diagnostics.

ASSOCIATED CONTENT

Supporting Information

Detailed description of the experimental procedures, DNA sequences, and additional figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01099.

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Notes

The authors declare no competing financial interest.

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