



A conformational switch-based fluorescent biosensor for homogeneous detection of telomerase activity

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ABSTRACT

As a universal tumor biomarker, research on the activity and inhibition of telomerase is of great importance for cancer diagnosis and therapy. Herein, we demonstrate the conformational switch-based fluorescence detection of telomerase activity using a redesigned RNA aptamer Spinach. Briefly, the original Spinach aptamer was extended at its 5' end and folded into an inactive conformation, where association with the small molecule fluorophore, 5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) was prevented. Only in the presence of telomerase, (TTAGGG)_n repeats were added to the 3' end of the telomerase substrate primer, and the elongation products hybridized with inactive Spinach molecules, triggering its conformational switch and refolding it into the active, DFHBI-binding conformation. Moreover, the fluorescence signal was further amplified through a target recycling circuit, where Ribonuclease H (RNase H) specifically hydrolyzed the phosphodiester bonds of RNA in the DNA-RNA hybrid. The released telomere products could then hybridize to new inactive Spinach molecules and initiate multiple amplification cycles. The proposed fluorescent biosensor presented great performance for telomerase activity detection from 100 to 5×10^4 HeLa cells with a detection limit of 100 cells. Besides, this new assay offers a good biosensing platform for differentiation of cancer cell lines from normal cell line and evaluation the inhibition efficiency of telomere-binding ligand, which is of great importance for telomerase-related cancer diagnosis and therapy.

1. Introduction

Telomerase is a unique ribonucleoprotein complex which adds (TTAGGG)_n repeats on the telomeric end of chromosomal DNA using the telomerase RNA component of the complex as a template [1,2]. Telomerase activity is up-regulated in over 85% of human tumor cells and telomerase has potential to be used as both a cancer marker for early diagnosis and a therapeutic target [3,4]. Due to the importance and universality of telomerase, it is of significantly theoretical and practical importance to reliably and sensitively detect telomerase activity. Considerable efforts have been put forth to detect telomerase activity over the past decades using techniques such as a polymerase chain reaction-based (PCR)-based telomerase repeat amplification protocol (TRAP) [5,6], optical-based assays with nucleic acid beacons and a telomeric G-quadruplex structure [7], surface plasmon resonance [8], fluorescence [9], electrochemistry [10,11] and nanotechnology-based methods [12,13]. Despite this progress, the reliable, point-of-care detection of telomerase activity remains a central challenge for clinical cancer diagnosis.

RNA aptamers [14] have been described that bind and enhance the fluorescence of small molecule dyes such as triphenylmethane and

cyanine dyes [15,16]. Spinach is a 98-nt-long RNA aptamer that binds to and switches on the fluorescence of 5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), a small molecule that resembles the chromophore of green fluorescent protein (GFP) [17]. In contrast to other fluorophores which are susceptible to nonspecific fluorescence upon incubation with cells or biological materials, DFHBI does not exhibit nonspecific fluorescence activation, making background fluorescence from the unbound fluorophore minimal [18,19]. Inspired by its excellent performance, several Spinach-based RNA sensors have been developed in biochemical assays [20–26], such as in the fluorescence imaging of small molecules and metabolites via a stem sequence that functions as a transducer [20,21], or protein quantification via fusion formation between the aptamer and Spinach [22].

Herein, we established an efficient detection method for telomerase activity based on the conformational switch of RNA Spinach. From the perspective of structure, Spinach contains three stem loops encircling a central loop, and the stability of stem loop 3 plays an essential role in Spinach fluorescence [20]. Therefore, redesign of original Spinach aptamer in stem loop 3 was utilized as the entry point in our method. Briefly, its original 5' and 3' ends were linked and stem loop 3 was split

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into two arms (split branch, split loop). The 5' end (split loop) was programmed as a relatively stable molecular beacon with 12 bp in the stem and 14 bp in the loop, thus interdicting intramolecular hybridization with the 3' end (split branch). This inactive conformation could not associate with DFHBI to emit fluorescence. Ingeniously, the telomerase reaction products (TRP) hybridized with inactive Spinach molecules, triggering its conformational switch and refolding it into the active, DFHBI-binding conformation and thus emitting fluorescence. What's more, RNase H hydrolyzed the phosphodiester bonds of RNA in the DNA-RNA hybrid, resulting in a sequence recycling circuit and a drastic increase in sensitivity. Therefore, the above-proposed homogeneous fluorescent strategy based on RNase H-aided amplification via the sequential recycling of the telomerase extended DNA strand is a promising candidate for the highly sensitive detection of the activity of telomerase extracted from cancer cells.

2. Experimental section

2.1. Chemicals and reagents

All oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). DFHBI was purchased from Lucerna Technologies (New York, NY). Deoxynucleotides (dNTPs), RNase H, DNase I, and T7 high scribe kit were obtained from TaKaRa (Dalian, China). PCR Cleanup Kit was purchased from Axygen (Union City, CA). TMPyT4 was bought from APExBio (Houston, TX). Bovine serum albumin (BSA) was provided by Aizite Biotechnology Corp. (Shanghai, China). Other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All of the solutions were prepared with ultrapure water (Millipore purification system, 18.2 M Ω cm), treated with diethyl pyrocarbonate (DEPC) and sterilized with high-pressure steam.

2.2. Preparation and analysis of Spinach probe

Redesigned RNA aptamers with different split branches and loops were created by employing a single stranded DNA template, a series of forward and reverse primers. (All the sequences used in preparation of Spinach probe were shown in [Supporting information, Table S1](#)). The forward primer contained a 5' T7 promoter sequence to generate double-stranded DNA templates. After purification with a PCR Cleanup Kit, PCR products were used as templates for in vitro T7 transcription reactions and then DNase I was utilized to degrade the residual DNA template (2 U, 37 °C, 1 h). RNAs were purified via precipitation using 0.3 M ammonium acetate, and then the depositing RNA pellets were washed twice in 70% ethanol, and quantified using the absorbance at 260 and 280 nm (U-2900, Hitachi).

2.3. Preparation of telomerase extracts

Human cervical carcinoma cells (HeLa), human hepatoma cells (Huh7), breast cancer cells (MCF-7) and the normal liver cells (HL-7702) were cultured in the Dulbecco's Modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal calf serum and incubated at 37 °C in a humidified atmosphere (95% air and 5% CO₂). Cells were collected at 70–80% confluence, and washed twice with 6 mL ice-cold phosphate buffered saline (PBS) solution, the cell densities were determined using a hemocytometer. After centrifugation, the corresponding number of cells was suspended in ice-cold CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM ethylenediamine tetraacetic acid (EGTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM mercaptoethanol, 0.5% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 10% glycerol) at a concentration of 1×10^7 cells/mL and incubated on ice for 30 min. Then the lysate was centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was collected carefully and transferred into

clean microcentrifuge tubes. The collected lysate was used immediately for the telomerase assay or was frozen at –80 °C.

2.4. Telomerase extension

Telomerase extracts were diluted in extension buffer with the respective number of cells. The extracts (1 μ L) were added to a 19 μ L extension reaction mixture (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 0.1 mg/mL BSA, 1 mM dNTP, and 0.5 μ M TS primer). The extension solution was incubated at 37 °C for 1 h, mixed with the RNA probe in 85 °C for 5 min, and then cooled to room temperature to form a stable structure. For producing the negative control, cell extracts were heated at 85 °C for 5 min prior to the extension reaction to inactive telomerase.

2.5. TRPs-initiated Spinach reconstruction

TRP-assisted Spinach reconstruction was performed in 20 μ L of $1 \times$ RNase H buffer (40 mM Tris-HCl, pH = 7.7, 4 mM MgCl₂, 1 mM dithiothreitol (DTT), 4% Glycerol, 0.003% BSA) containing 75 pmol Spinach probe, 15 U RNase H, and varying concentrations of the telomerase extension solution or synthetic target sequence at 37 °C for 90 min before the fluorescence measurements.

2.6. Fluorescence detection of telomerase activity

The above solution (20 μ L) was heated at 85 °C for 5 min to inactive the enzyme, mixed with 80 μ L DFHBI solution (20 μ M DFHBI, 8 mM HEPES, 25 mM KCl, 1 mM MgCl₂) and incubated in a dark at room temperature for 30 min. Fluorescence measurements were performed using an F7000 fluorescence spectrometer with the following instrument parameters: excitation wavelength, 460 nm; emission wavelength, 505 nm; slit widths, 5 nm.

3. Results and discussion

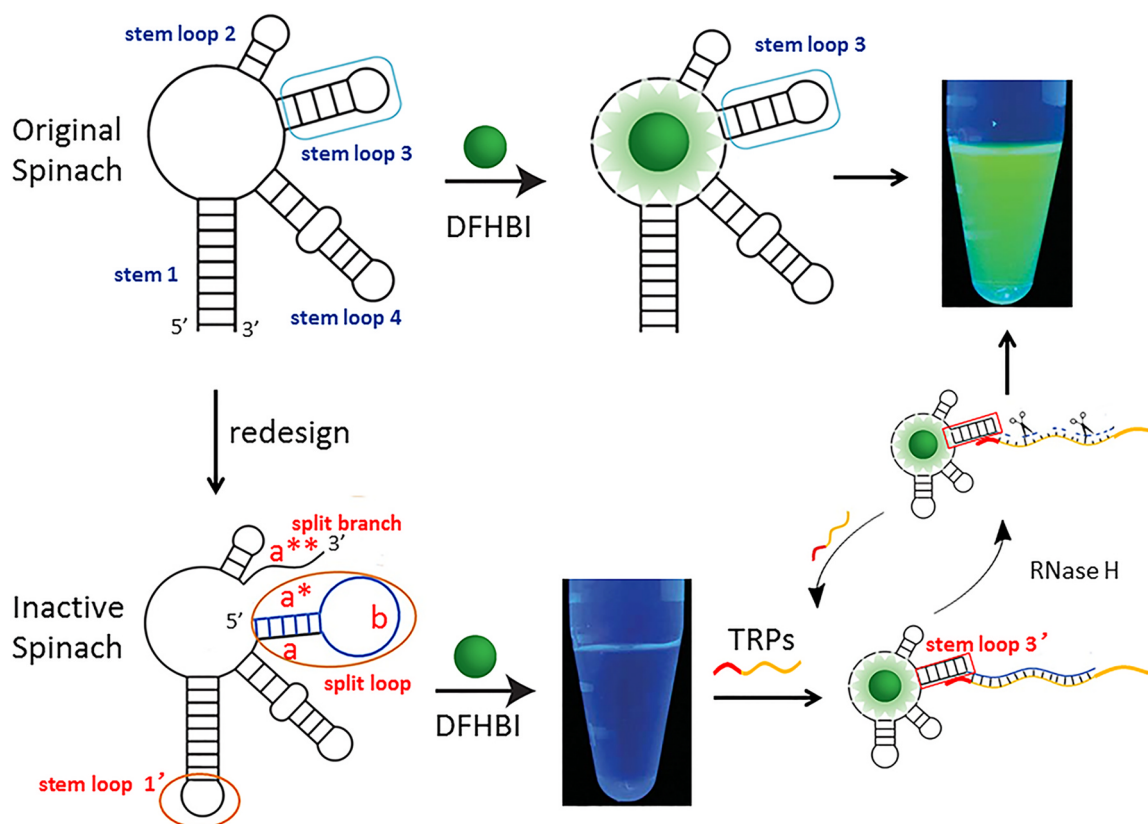
3.1. Principle of assay

The principle of sequence-initiated Spinach reconstruction is illustrated in [Scheme 1](#). Original Spinach was redesigned in a sequence-active manner, where its 5' and 3' termini were linked; the newly formed stem 1 was named as stem loop 1'. Meanwhile, original stem loop 3 was split into two arms, where the new extended 5'-end could fold into a new hairpin structure (a/b/a*), thus preventing hybridization with the split branch on the 3' end (a**). Most importantly, this newly designed inactive conformation hardly associated with DFHBI, and low fluorescence was observed. After the addition of telomerase, the (TTAGGG)_n repeats were elongated on the telomere primer. The elongation region was comprised of the complementary pairs within the blue region in the new split loop of inactive Spinach, thus leading to a TRP-initiated Spinach reconstruction, i.e., triggering its conformational switch and refolding into the active DFHBI-binding conformation. The reconstructed DNA-RNA complex could be recognized by RNase H, leading to the digestion of the blue region (a*/b). This digestion process released the extended telomerase extended DNA strand, which then hybridized with fresh Spinach sensors to initiate the recycling digestion processes. As such, the sensitive label-free and immobilization-free fluorescence detection of telomerase activity was achieved.

3.2. Feasibility of fluorescence aptasensor

3.2.1. Redesign of original Spinach

To redesign original Spinach to be activated in a TRP-initiated manner, two committed steps should be considered: 1) the redesigned split loop ([Scheme 1](#), a/b/a*) should be stable in the absence of TRP to ensure a low background signal. The more base-pairs the stem region (a



Scheme 1. Principle of the telomerase reaction products-initiated Spinach reconstruction.

+ a*) contains, the less the noise will occur. 2) The split loop could only be opened after the addition of TRPs, thus allowing its hybridization with the 3' end (a**), and refolding it into the active, DFHBI-binding conformation and leading to a strong fluorescence signal.

In this design, the complementary base pairs of a/a* and a/a** could influence the sequence displacement reaction in a synergistic way: a panel of Spinach-based sensors should be investigated in regards to the fluorescence signal as a function of the synthetic telomerase reaction product (Table S1, TRP). To achieve this objective, we synthesized different versions of Spinach probes enzymatically. In general, a series of forward and reverse primers attached to the same template was designed, producing the corresponding double-stranded DNA by PCR. Since all forward primers contained the T7 promoter, the PCR products could be utilized as a new template for T7 RNA polymerase-driven transcription. All sequences used in this study and corresponding details are provided as Supporting information. By selecting appropriate forward/reverse combinations, we added several nucleotides at the 5' and 3' ends of the Spinach.

Referring to the secondary construction of the original Spinach [18], various versions of Spinach were produced and the corresponding fluorescence in response to the synthetic target sequence is shown in Fig. 1. We fixed the complementary base pairs of stem loop 3' to 8 bp (a + a**) and investigated the structure of the split loop (a + a*) (Fig. 1A). A better performance was demonstrated when it had 14 stem base pairs (a/a*) in the split loop. If the stem base pairs contained more than 14 bp, it was too stable for the target sequence to open the hairpin structure; when there were less than 12 bp, the split branch (a**) could hybridize with region a, resulting in some background noise. Therefore, we determined that the split loop should have 14 base pairs complementary in the stem part. Next, we investigated the length of the stem loop 3' (Fig. 1B) in a similar way. When 8 complementary base pairs were present in stem loop 3', Spinach exhibited the optimum

performance; if the stem loop 3' was too long, a higher noise was observed, whereas if it was too short, a lower signal was observed.

Finally, we determined that Spinach aptamer which contained 14 complementary base pairs in the stem of the split loop (a/a*) and 8 base pairs in stem loop 3' (a/a**) was the optimal aptamer for the detection of telomere activity (Fig. 2A, named as TRP Spinach: AGC ACC CUA ACC CUA ACC CUA ACC CUA AAG GGU UAG GGU GCU UGU UGA GUA GAG UGU GAG CUC CGU AAC UAG UCG CGU CGA AAG ACG CGA CUG AAU GAA AUG GUG AAG GAC GGG UCC AGC ACC CU). Fig. 2B represents different fluorescence signals with and without the target sequence, confirming the feasibility of the design.

3.2.2. Optimization of experimental conditions

To achieve optimal analytical performance, several experimental parameters were investigated systematically. Firstly, the effect of probe amount on the fluorescence intensity was explored. Owing to the split-hybridization-reconstruction balance in our design, the split Spinach itself could result in a low background signal, so the amount of probe was of crucial importance. We investigated the changes in fluorescence intensity with 5 pmol, 25 pmol, 50 pmol, 75 pmol and 100 pmol of the probe. As shown in Fig. S1, the fluorescence intensity increased as the amount of template increased. When the amount was 75 pmol, the fluorescence intensity reached a maximum. Therefore, 75 pmol was chosen as the optimum probe amount. Furthermore, since the concentration and the reaction time of RNase H have significant influence on the amplification reaction, the systems with different RNase H concentrations and different reaction time were investigated, respectively. As shown in Fig. S2, the fluorescence intensity reached a maximum when the amount of RNase H was 15 U, and declined slightly with incubation time after 90 min. Therefore, 15 U and 90 min were chosen for the enzyme-assisted target recycling aptamer reconstruction. In addition, the amount of DFHBI was also optimized. As shown in Fig. S3, 20 μ M of DFHBI was employed in the subsequent detection.

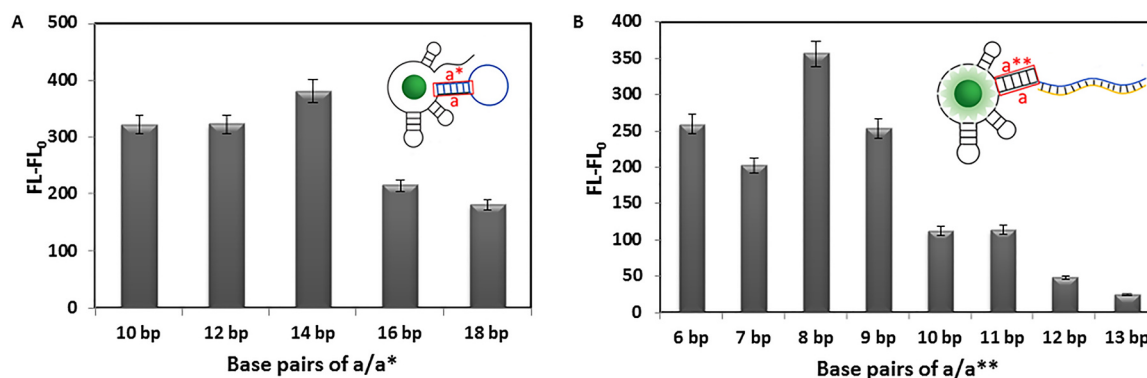


Fig. 1. A) Fluorescence intensity vs. length of complementary base pairs of a/a* (split loop). B) Fluorescence intensity vs. length of complementary base pairs of a/a** (stem loop 3'). Experimental conditions: 75 pmol of RNA sensor, 100 fmol of TRP, 15 U of RNase H, 90 min RNase H reaction and 20 μ M DFHBI in 100 μ L mixture buffer. Incubation in the dark at room temperature for 30 min after denaturation at 85 $^{\circ}$ C for 5 min. The fluorescence intensity was determined at 505 nm with an excitation at 460 nm. Error bars show the standard deviation of three experiments.

Considering that the reaction time of telomerase may affect the length of extension products and the fluorescence signal, we further examined the extension time. Fig. S4 showed that fluorescence intensity increased slightly and reached a constant after 60 min. Thus, the extension time was selected as 60 min.

3.2.3. Detection of telomerase activity

Under the optimized experimental conditions, the sensitivity and dynamic range were assessed using synthetic target, yielding a limit of detection (LOD) as low as 20 fM ($S/N = 3$) and a dynamic range of 100 fM – 5 nM by monitoring the dependence of the fluorescence intensity on the concentration of TRP (Fig. S5). To verify the promising capability of this method for real sample assay, we applied the fluorescent aptasensor to test the telomerase activity of HeLa cells. A calibration graph of 100–50000 HeLa cells showed an approximately linear correlation ($R^2 = 0.9868$, Fig. 3) between the amount of cell extract and

fluorescence intensity (represented by $Lg (FL - FL_0) = 0.5627 \lg (N) + 0.0557$, where FL is the fluorescence intensity of HeLa cells and FL_0 is the fluorescence intensity of the corresponding heated HeLa, N is the number of HeLa cells).

To demonstrate the broad application capability of this method, besides HeLa cells, we further tested telomerase extracts from different cancer cell lines (Huh7 and MCF-7) and human normal liver cells (HL-7702). Fig. 4A clearly showed that the heat-inactivated HeLa cells extracts had a low fluorescence intensity and only a slight fluorescence enhancement was seen for the HL-7702 cells extracts, however the cancer cells extracts gave a significant fluorescence enhancement, indicating that the fluorescence signal was due to the high active telomerase in these cancer cell lines. To further verify the detection accuracy of this method, the relevant cell extracts were analyzed using conventional TRAP-polyacrylamide gel electrophoresis (TRAP-PAGE) assay. As shown in Fig. 4B, multiple bands in ladder-like patterns were

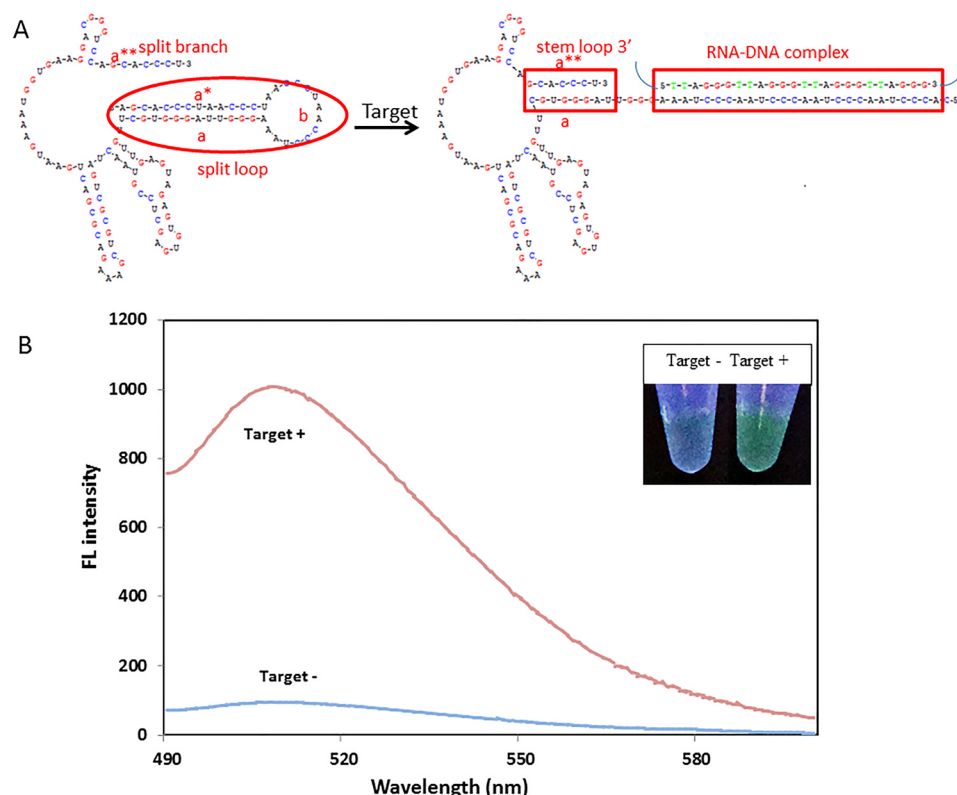


Fig. 2. A) Secondary structure prediction of TRP Spinach with and without synthetic TRP sequence. B) Fluorescence intensity of Spinach samples and their photograph upon excitation with UV lamp. Control samples were as follows: target+ (100 pmol of TRP Spinach, 10 pmol of TRP, 5 U of RNase H and 10 μ M DFHBI), target- (100 pmol of TRP Spinach, 5 U of RNase H and 10 μ M DFHBI). Incubation in the dark at room temperature for 30 min after denaturation at 85 $^{\circ}$ C for 5 min, the fluorescence intensity was determined with an excitation at 460 nm.

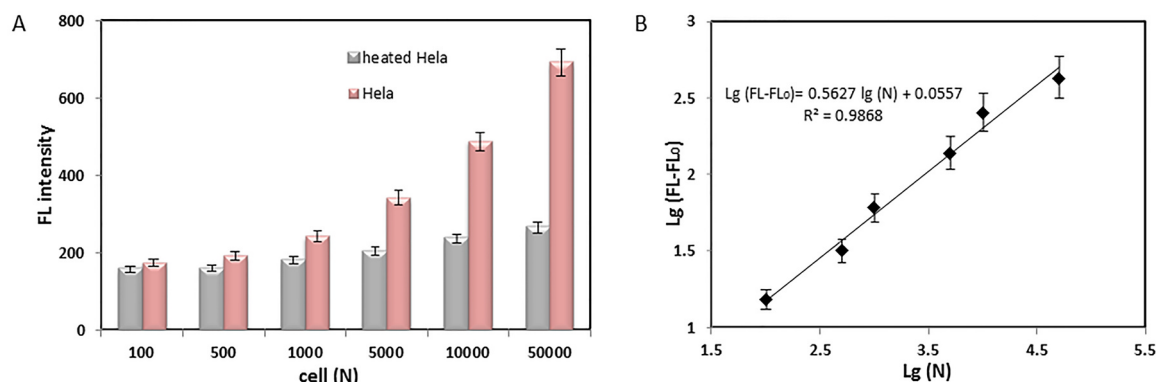


Fig. 3. A) Fluorescence intensity change as a function of number of HeLa cells. B) Calibration curve corresponding to the fluorescence intensity change as a function of number of HeLa cells. Fluorescence intensity was detected as described in the Experimental section. Error bars show the standard deviation of three experiments.

observed in cancer cell extracts while the bands of normal cells and heated HeLa cells were less and shallower, this was in accordance with our fluorescence results. On the other hand, no obvious difference of telomerase activity was observed among these different cancer cells. This may be due to the reason that PCR hid the difference of telomerase activity in cancer cells. Therefore, the proposed strategy exhibits excellent selectivity for telomerase activity detection and has great potential to be applied in clinical tests.

3.3. Detection of inhibition efficiency of inhibitors

Shortening of telomeres along with an up-regulation in telomerase is implicated in the immortality of tumor cells. Targeting either telomeres or telomerase with specific compounds has been proposed as an anticancer strategy with tumor specificity. Herein, 5,10,15,20-tetra-(N-methyl-4-pyridyl) porphine (TMPyP4), an effective G-quadruplex-interacting ligand was used as an inhibitor to investigate the feasibility of proposed method. Different concentration of TMPyP4 was mixed with extracts from 20000 HeLa cells for 30 min at room temperature before performing the telomerase extension reaction. The inhibition efficiency (%) was given by the following formula:

$$\text{Inhibition (\%)} = \frac{\Delta I_3 - \Delta I_2}{\Delta I_3 - \Delta I_1} \times 100\%$$

where ΔI_1 meant the fluorescence intensity of TRP Spinach without telomerase, ΔI_2 meant the fluorescence intensity treated with 20000

HeLa cells incubated with inhibitor, and ΔI_3 meant the fluorescence intensity treated with 20000 HeLa cells without inhibitor. The IC₅₀ (Fig. 5) for TMPyP4 was obtained as 6.1 μM by using the software “graphad prism”, which was in agreement with the reported results [27,28]. Thus, the proposed method had a potential application in discovery of new telomerase inhibitors and screening of telomerase-targeted anticancer drugs.

4. Conclusion

Spinach is a 98-nt-long RNA aptamer that binds to and switches on the fluorescence of DFHBI. In particular, both Spinach and DFHBI are essentially non-fluorescent when unbound, whereas the Spinach-DFHBI complex is shown to lead to a large increase in green fluorescence emission. In this study, we redesigned the Spinach aptamer to detect telomerase activity. In the presence of telomerase, (TTAGGG)_n repeats were added to the 3' end of the telomerase substrate primer, and the enzyme-assisted Spinach reconstruction was induced for the detection of telomerase activity with a low detection limit of 100 HeLa cells. Besides this, this RNA Spinach would allow this technology to be further implemented as an expressible signaling system. Therefore, the assay showed its potential to be applied in biological analysis, which might be significant in disease diagnosis in the future.

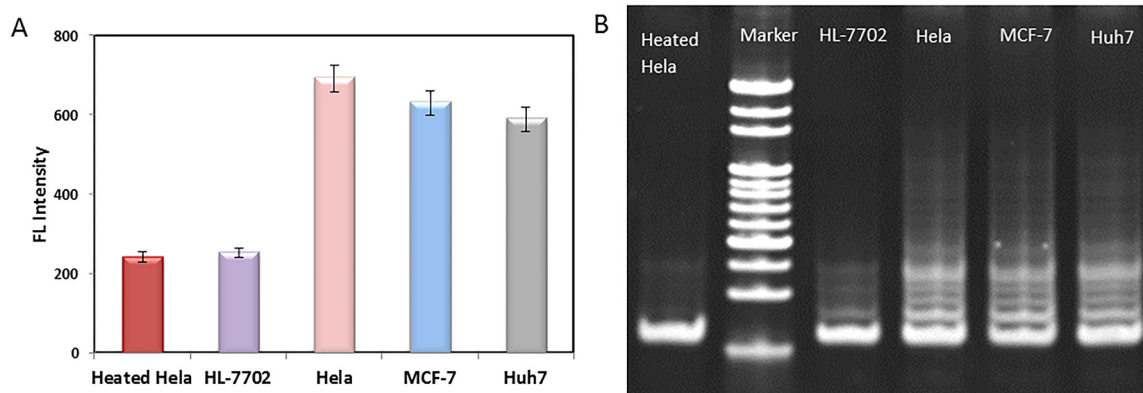


Fig. 4. A) Fluorescence intensity of different cell lines. Experimental conditions: 75 pmol of TRP Spinach, 15 U RNase H and extension solution of 20000 different cells in 20 μL 1 $\times$ RNase H buffer. After 90 min of RNase H reaction, fluorescence intensity was detected as described in the Experimental section. Error bars show the standard deviation of three experiments. B) TRAP assay for the telomerase activity of different cell lines. From the bottom up, the marker's size is 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 300, 400 and 500 bp.

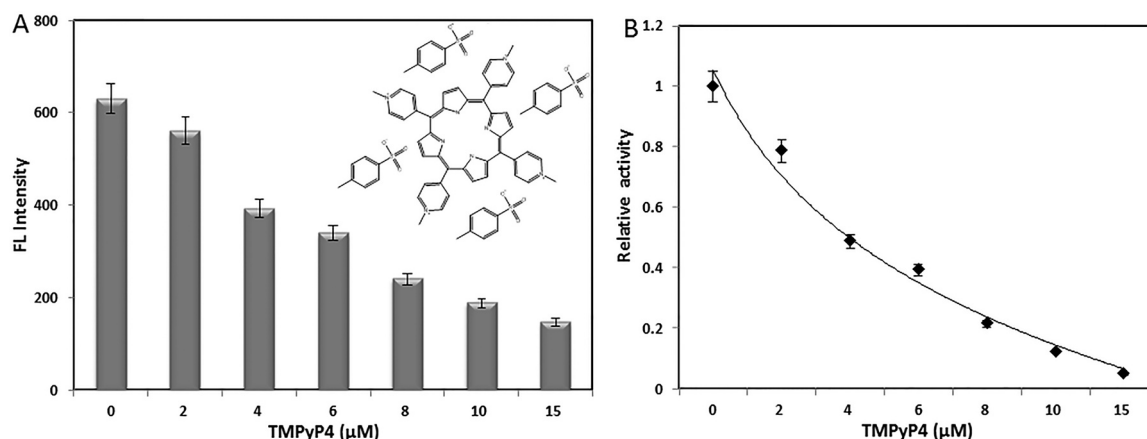


Fig. 5. A) Inhibition of telomerase activity by TMPyP4 with the fluorescence assay. B) The relative activity (1-Inhibition %) of telomerase versus TMPyP4. A total of 20000 HeLa cells were used. Error bars showed the standard deviation of three samples.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.talanta.2019.02.018](https://doi.org/10.1016/j.talanta.2019.02.018).

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