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Highly sensitive detection of telomerase using a telomere-triggered isothermal exponential amplification-based DNAzyme biosensor†

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A telomere-triggered isothermal exponential amplification-based DNAzyme biosensor is developed for highly sensitive detection of telomerase in cancer cells even at the single-cell level. This biosensor can be further applied for the screening of telomerase inhibitors for anticancer drug development.

Telomerase is a ribonucleoprotein complex consisting of a functional RNA (hTR) that serves as a template for telomeric DNA synthesis[1] and a reverse transcriptase protein component (hTERT) that catalyzes the addition of telomeric repeats $(TTAGGG)_n$ onto the 3′-ends of chromosomes.[2] Telomerase activity is prominent in highly proliferative cells (such as stem cells and germ cells) and in 85% of all known human cancers. However, in most normal human cells, telomerase activity is either repressed or absent.[3] Thus, telomerase has been regarded as both a biomarker and a target for the development of cancer diagnostics, prognostics, and therapeutics.[3b,4]

A polymerase chain reaction (PCR)-based telomere repeat amplification protocol (TRAP) has served as a powerful method for the telomerase activity assay.[5] Even though some TRAP-based methods might provide quantitative results, they involve the use of harmful radioactive, expensive florescent substances,[6] and are usually time-consuming and laborious.[7] Recently, a number of PCR-free methods have been developed, such as optical fiber sensing using DNA beacons[8] and two-color coincidence,[9] surface plasmon resonance,[10] biocatalytic precipitation-based electrochemical method,[11] magnetic bead-based electrochemiluminescent method,[12] magneto-mechanical method,[13] and bio-barcode assay.[14] Although these methods do not involve the thermal cycling protocol and polymerase, most of them still require time-consuming protocols and rely on expensive instruments.

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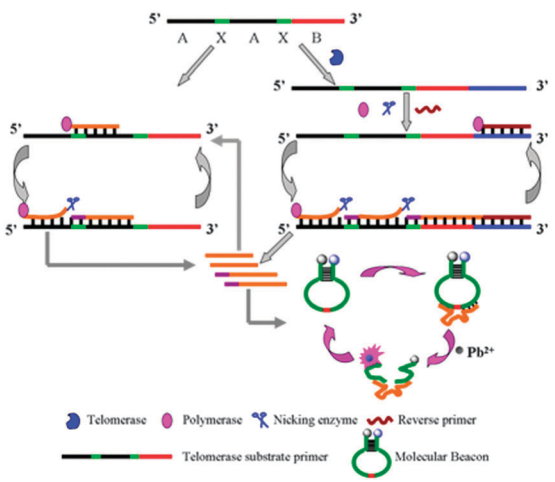
DNAzymes (also called catalytic DNA or deoxyribozymes) are DNA-based biocatalysts.[15] The interest in 8–17 DNAzyme[16,17] can be attributed to both its minimal size and its ability to catalyze RNA cleavage with high activity and specificity in the presence of specific metal ions as cofactors.[18] Recently, 8–17 DNAzyme has been employed for the detection of metal ions, DNAs, and a number of biomolecules due to its easy synthetic preparation and the reduced nonspecific adsorption.[19] Herein, we design a new method for sensitive detection of telomerase activity by integrating two-step signal amplification: one by a protein enzyme and the other by a DNA enzyme (DNAzyme).

As shown in Scheme 1, our strategy for telomerase activity assay involves the following three steps: (1) telomere extension reaction, (2) polymerase-mediated exponential amplification reaction (EXPAR),[20] (3) 8–17 DNAzyme-mediated signal amplification.[19] In the first step, the TS primer sequences (region B) are recognized by telomerase, which adds a number of telomeric repeats $(TTAGGG)_n$ onto the 3′-end of the primer to form a longer single stranded DNA.



Scheme 1 Schematic illustration for the detection of telomerase activity using a telomere-triggered isothermal exponential amplification-based DNAzyme biosensor.

In the second step, the reverse primers can hybridize with their complementary sequence at the 3′-terminus of these extended products and initiate the polymerization in the presence of Bst 2.0 WarmStart DNA polymerase and dNTPs to form the double-stranded DNA (dsDNA) with two recognition sites (region X) for nicking enzyme Nt.BspQI. Subsequent single-stranded nicking generates a new replication site for the polymerase, and two short single-stranded 8–17 DNAzyme sequences (complementary to region A) will be displaced and released. These DNAzymes can bind other free probes to initiate a new cycle of polymerization, nicking and displacement, leading to an exponential amplification and consequently the generation of a large amount of DNAzymes. The process of telomere-induced EXPAR is monitored by real-time fluorescence measurement and the amplification products are confirmed by non-denaturating polyacrylamide gel electrophoresis (PAGE) (see ESI,† Fig. S1). In the third step, the 8–17 DNAzyme can be hybridized with a hairpin-structured molecular beacon (MB) substrate to form a catalytic and molecular beacon (CAMB) system. Upon the addition of the cofactor Pb^{2+}, the DNAzyme is activated to catalyze the cleavage of MB substrate into two pieces at the rA position, causing the separation of the fluorophore–quencher pair from each other and consequently the increase of the fluorescent signal and the release of a free DNAzyme.[19] The released DNAzyme can then hybridize with another MB to trigger the second cycle of cleavage. Eventually, each released 8–17 DNAzyme can undergo a number of cycles to trigger the cleavage of abundant MB substrates, generating an amplified signal for the telomerase activity assay.

To evaluate the sensitivity of the proposed method, we detect both the synthetic telomerase product of TPC5 at various concentrations and a series of diluted HEK293T cell extracts under the optimal experimental conditions (see ESI,† Fig. S2). As shown in Fig. 1A, the fluorescence intensity gradually increases with increasing TPC5 concentration. This is in accordance with the fact that high concentration of TPC5 can generate abundant 8–17 DNAzymes, which can catalyze the cleavage of abundant MB substrates to induce the improved fluorescence signal. Notably, the value of F/F_0 exhibits a linear correlation with the logarithm (log) of the concentration of TPC5 over a range of 11 orders of magnitude from 0.1 aM to 10 nM (inset of Fig. 1A). The correlation equation is $F/F_0 = 21.54 + 1.08 \log_{10} C$ ($R^2 = 0.9940$), where F and F_0 are the fluorescence intensity in the presence of TPC5 and in the absence of TPC5, respectively, and C is the concentration of TPC5. Notably, the dynamic range of current assay is about 1 order of magnitude larger than that of MB-based real-time EXPIATR assay,[21] and is comparable to the telomere-induced two-stage isothermal amplification-mediated chemiluminescence assay.[22] Fig. 1B shows that the fluorescence intensity gradually increases with increasing number of HEK293T cells, suggesting that the amount of DNAzyme and the intensity of the fluorescence signal are associated with the number of cells.[23] The fluorescence intensity has a linear relationship with the number of HEK293T cells in the range from 1 cell to 10^5 cells. The correlation equation is $F/F_0 = 2.59 + 0.62 \log_{10} N$ ($R^2 = 0.9897$), where F and F_0 are the fluorescence intensity in the presence and in the absence of HEK293T cell extracts, respectively, and N is the number of cells (inset of Fig. 1B). Notably, the telomerase activity in even one HEK293T cell can be sensitively detected. Our method is more sensitive than the reported PCR-based telomeric repeat amplification protocol (10 cells)[5b] and PCR-free methods, such as DNA beacon-based optical fiber sensing (500 cells),[8] magnetic bead-based electrochemiluminescent methods (500 cells),[12] magneto-mechanical methods (100 cells)[13] and bio-barcode methods (10 cells).[14] The high sensitivity of the proposed method can be attributed to the following two factors: (1) the high amplification efficiency of EXPAR allows a single probe to generate abundant DNAzymes;[20] (2) the 8–17 DNAzyme can undergo a number of cycles to trigger the cleavage of abundant MB substrates, achieving an amplified signal for the telomerase activity assay.[19]

To evaluate the potentiality of the proposed method for clinical diagnosis, the telomerase activities of another cancer cell line HeLa and a normal cell line MRC-5 were further investigated. As expected, HeLa cancer cells display a positive telomerase activity (Fig. 2), which is consistent with the over-expression of telomerase in most known human tumors.[3] In contrast, the MRC-5 cells cannot generate any improved fluorescence signal (Fig. 2) due to the lack of telomerase activity in normal cells. These results clearly demonstrate the feasibility of the proposed method for the detection of telomerase activity in clinical samples.

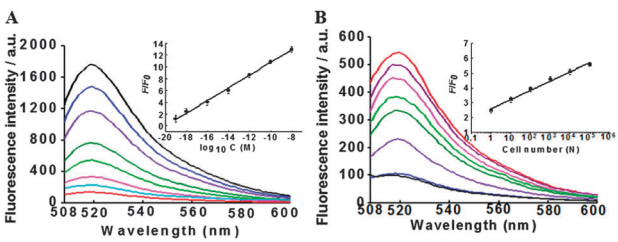


Fig. 1 (A) Fluorescence emission spectra in response to different concentrations of TPC5: 1×10^{-8} M, 1×10^{-10} M, 1×10^{-12} M, 1×10^{-14} M, 1×10^{-16} M, 1×10^{-18} M, 1×10^{-19} M and in the absence of TPC5 from top to bottom. The inset shows the log-linear correlation between the fluorescence ratio (F/F_0) at 518 nm and the TPC5 concentration. (B) Fluorescence emission spectra in response to various HEK293T cell extracts equivalent to 100 000 cells, 10 000 cells, 1000 cells, 10 cells, 1 cell and heat-inactivated cell extracts equivalent to 1000 cells, the control without cell extracts from top to bottom. The inset shows the log-linear correlation between the fluorescence ratio (F/F_0) at 518 nm and the cell numbers. Error bars show the standard deviations of three experiments.

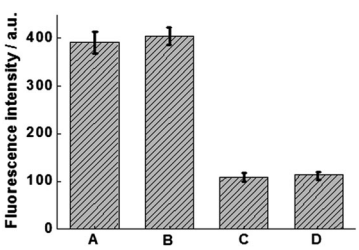


Fig. 2 Fluorescence intensity in response to cell extracts equivalent to 100 cells in HEK293T (A), HeLa (B), MRC-5 (C), and the control group with only lysis buffer (D). Error bars show the standard deviations of three experiments.

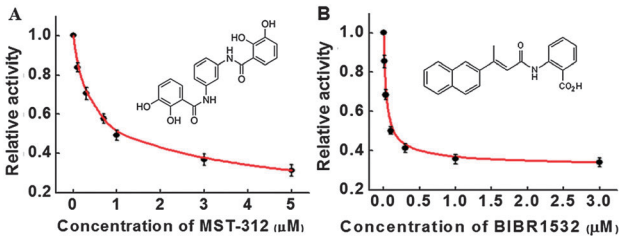


Fig. 3 (A) Dose-dependent inhibition of telomerase activity in HEK293T cells by MST-312. Inset: the chemical structure of MST-312. (B) Dose-dependent inhibition of telomerase activity in HEK293T cells by BIBR1532. Inset: the chemical structure of BIBR1532. Error bars show the standard deviation of three experiments.

About 85% of cancer cells exhibit elevated amounts of telomerase, which leads to the stabilization of telomeres and the unlimited growth potentiality.[3] Most cancer cells are reliant on telomerase for their survival, thus telomerase has become an attractive target for the development of new cancer therapeutics.[4] Recently, a variety of small compounds with the capability of inhibiting telomerase activity have been discovered, including 2-[(*E*)-3-naphthalen-2-yl-but-2-enoylamino]benzoic acid (BIBR 1532)[24] and telomerase inhibitor IX, *N*,*N*-bis(2,3-dihydroxybenzoyl)-1,2-phenylenediamine (MST-31).[25] As shown in Fig. 3, both MST-312 and BIBR1532 inhibit telomerase activity in HEK293T cells in a dose-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) of 0.77 ± 0.08 μM for MST-312 (Fig. 3A) and a IC_{50} of 0.205 ± 0.03 μM for BIBR1532 (Fig. 3B). These results demonstrate that the proposed method might provide a new approach for screening new inhibitors of telomerase activity.

In summary, we have developed a telomere-triggered isothermal exponential amplification-based DNAzyme biosensor for highly sensitive detection of telomerase. The EXPAR can be initiated only in the presence of telomeres, producing abundant copies of DNAzyme. Each DNAzyme can undergo a number of cycles to trigger the cleavage of excess MB substrates, providing an amplified signal for the telomerase activity assay. With two-step signal amplification, the proposed method can sensitively detect the synthetic telomerase product of TPC5 with a large dynamic range of 11 orders of magnitude from 0.1 aM to 10 nM, and can even detect the telomerase activity of a single HEK293 cell. More importantly, the proposed method can be further applied for the screening of telomerase inhibitors for anticancer drug development.

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