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Analysis of telomerase activity based on a spired DNA tetrahedron TS primer

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ABSTRACT

The development of sensitive telomerase biosensors is hindered by the restricted accessibility of telomere strand (TS) primer and the limited enzyme reaction space, which is mainly confined by the vertical distance. In this work, we designed an electrochemical telomerase biosensor based on a spired DNA tetrahedron TS primer (STTS). By adding a rigid dsDNA spire onto the top of the DNA tetrahedron, we successfully regulated the distance between the TS primer and the surface, and thus greatly facilitated the telomerase elongation on surface. The signal-to-noise ratio was 2 times higher than TSP without the spire structure. The limit of detection was calculated to be lower than 10 HeLa cells, which is at least 2 magnitudes lower than other surface extension-based electrochemical telomerase sensors without amplification. The practicability of STTS sensor was also demonstrated by analysing various other cell lines including cancer cells, stem cells of high telomerase activity and somatic cells of low telomerase activity.

1. Introduction

Telomeres (Blackburn, 1991), located at the end of the eukaryotic chromosome, are identified as stabilizers for the chromosomal terminuses against degradation and processing. Telomerase is a unique ribonucleoprotein reverse transcriptase which maintains the telomeres during the cell doubling, by adding single-stranded telomeric repeats to the 3′ ends of the telomeres (Greider and Blackburn, 1989; Yu et al., 1990; Blackburn, 1994).

Telomerase (Lee and Blackburn, 1993) catalyzes elongation of the telomere to compensate the natural shorten (Harley et al., 1990) of the chromosomes, thus make the cell immortal. The telomerase activity is repressed in most of normal healthy somatic cells, but about 85–90% human cancer cells carry high telomerase activity. Therefore, considerable research enthusiasm has been attracted to the development of the telomerase assay, which is playing a key role in confirming the relationship between telomerase activity and disease pathologies, such as cancer, age-related diseases, and premature ageing syndromes (Blasco, 2005; Harley, 2008).

Although the telomere repeat amplification protocol (TRAP) (Kim et al., 1994) has been developed as a sensitive telomerase assay based on PCR, it is time-consuming and still often inhibited by poorly defined interferents present in cell extracts. Fortunately, electrochemical biosensors, with their advantages of low cost (Liu et al., 2011), high-sensitivity, and high compatibility with other technologies (Liu et al., 2010, 2012), offer us great opportunities for advanced telomerase assays (Pavlov et al., 2004; Yang et al., 2011). Recently, a series of electrochemical telomerase biosensors (ETBs) have been developed based on G-rich tetraplex DNA binder (Sato et al., 2005), bio-barcode method (Li et al., 2010), alkaline phosphatase label (Pavlov et al., 2004) and guanine oxidation signals (Eskiocak et al., 2007) etc. The advances of these works indicated an exciting potential of electrochemical telomerase assays (Yi et al., 2014) in cancer research and drug screening.

Nevertheless, the sensitivity and reliability of ETBs is still often limited (Zhou and Xing, 2012). A possible reason is that the accessibility of the TS primer and the activity of telomerase are reduced in the locally crowding surface-assembled monolayer (SAM), where neither the orientation nor the density of the TS primers is well controlled. In order to circumvent these problems, we designed a novel DNA-nanostructure scaffold for TS primers based on a DNA tetrahedral-structured probe (TSP) (Pei et al., 2010). TSP is a tetrahedral DNA nano structure with a thiol at each

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bottom vertex and a pendent capture probe on its top vertex. Excellent TSP-related electrochemical biosensors for cocaine (Wen et al., 2011) and microRNA (Lin et al., 2014) have been developed, showing TSP's advantages of stable and ordered self-assembling and resistance of unspecific absorbance (Wen et al., 2013).

For sensitive telomerase analysis, we replaced the pendent capture probe with a TS primer, and most importantly, we added a double-strand fragment between the DNA tetrahedron and TS, which raised the TS like a spire on top of the tetrahedron. The novel spired DNA tetrahedron TS primer (STTS) critically increased the vertical distance between the telomerase and the gold surface, and thus provided the telomerase reaction a necessary bulk-solution-like phase for enzyme combination and elongation. Meanwhile, the rigid spire-structure anchored the TS primer with higher controllability and accessibility. Our STTS assay remarkably improved the signal-to-noise ratio and sensitivity, and successfully analyzed the telomerase activity in a wide range of HeLa cell numbers.

2. Experimental

2.1. Reagents

All oligonucleotides were synthesized and purified by Invitrogen (CA, USA), and the sequences are listed in Table 1. The 3,3′,5,5′ tetramethylbenzidine (TMB) substrate was purchased from Neogen (Lexington, KY) in the format of a ready-to-use reagent (K-blue low activity substrate, H_2O_2 included). Horseradish peroxidase-conjugated avidin (avidin–HRP) was from eBioscience Inc. (Santiago CA). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and diethyl pyrocarbonate (DEPC) were from Sigma. All other chemicals were of analytical grade. All solutions were prepared with Milli-Q water (18 MΩ cm resistivity) and treated with 0.1% DEPC to ensure a RNA-free analysis environment.

2.2. Construction of TSP and STTS

Normal TSP was constructed following our previous work (Wen et al., 2013). Four strands TSP-A, B, C, D were dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with a final concentration of 50 μM. The TSP-A contained a tetrahedron fragment and a TS primer fragment (Table 1). 1 μL of each strand was mixed with 1 μL of TCEP (500 mM) and 45 μL of TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0). The resulting mixture was heated to 95 °C for 10 min, and then cooled to 4 °C for 30 min to form homogeneous and stable TSP. The temperature program was performed on a thermal cycler S1000TM (MJ Research Inc., SA).

For STTS construction, a novel STTS-A was designed to replace the TSP-A by inserting a spire-hybridization fragment between the tetrahedron fragment and the TS primer (STTS-A in Table 1). All other reaction conditions were the same as TSP.

2.3. Modification of gold surface with STTS, TSP and ssP

Step 1: 3 μL of 1 μM TS primer (STTS, TSP or single-strand TS primer (ssP)) was pipetted to gold electrode surface followed by an overnight incubation at room temperature. The resulted electrode was rinsed with 0.01 M phosphate buffered saline (PBS, prepared with RNase-free water) and dried lightly with N_2. Step 2: for STTS, 10 μL of 1 μM Spire-Complementary DNA (SC-DNA) were pipetted to the electrode and incubated for 1 h at 37 °C to form a rigid spire. For ssP, 10 μL of 2 mM 6-mercapto-1-hexanol (MCH) were pipetted to the surface of gold electrode and incubated for 1 h at room temprature. For TSP, just go to step 3. Step 3: the electrode was rinsed with 0.01 M PBS and dried lightly with N_2 before telomerase extension.

2.4. Telomerase elongation

Telomerase extracts were firstly diluted with lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) to achieve a series of concentrations, and then 4 μL of the resulted solution was mixed with 6 μL of the telomerase extension reaction buffer (20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 1 mM EGTA, 63 mM KCl, 0.05% Tween 20, 0.2 mM biotin-dATP, 0.2 mM dGTP, 0.2 mM dTTP). The mixture was pipetted to the surface of modified gold electrode and incubated for 2 h at 30 °C. For negative control, telomerase extracts were heat-treated at 95 °C for 10 min.

2.5. Electrochemical impedance spectroscopy (EIS) measurement

Electrochemical impedance spectroscopy (EIS) measurements were performed using a μAUTOLAB III electrochemical workstation (Metrohm Autolab, Switzerland) and a three-electrode system with an Ag/AgCl reference electrode, a Pt counter electrode, and a gold working electrode. The EIS analysis solution was 10 mM PBS (pH 7.4) containing 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and 0.1 M KCl.

2.6. Electrochemical detection of telomerase activity

After the telomerase elongation, the electrode was rinsed with 0.01 M PBS buffer, dried lightly with N_2, and then incubated with 3 μL of avidin–HRP (0.5 U/mL) for 45 min at room temperature. The sensor was then extensively rinsed with 0.01 M PBS, dried lightly with N_2, and then subjected to electrochemical measurements.

Electrochemical measurements were performed with a 16-channel PM3000 multichannel potentiostat (Genefluidics, Duarte, CA) on a bare gold sensor chip (SC1000-16X, GENE fluidics). The chip contains 16 electrode units which consist of one gold work electrode, one gold auxiliary electrode and one gold reference electrode (Liu et al., 2008). After rinsed with 0.01 M PBS and dried lightly with N_2, the chip surface was covered with 50 μL TMB co-substrate. Amperometry detection was performed at −200 mV (VS Au reference electrode) and the electrocatalytic reduction current was measured at 60 s after the HRP redox reaction reached the steady state.

Table 1
Sequences of oligonucleotides used in this work.

DNA	Sequences (5′–3′)
single-strand TS primer	SH-TTTTTTTTTTAATCCGTCGAGCAGAGTT
TSP-A	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTATTTTTTTTTT**AATCCGTCGAGCAGAGTT**
TSP-B	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
TSP-C	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC
TSP-D	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
STTS-A	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTATTTTTTTTTT***CATCTTGCCT*****AATCCGTCGAGCAGAGTT**
Spire-Complementary DNA (SC-DNA)	***AGGCAAGATGAAAAAAAAAA***

2.7. Cell culture

Human cervix adenocarcinoma HeLa cell line, human breast carcinoma cell lines MCF-7, rat adrenal pheochromocytoma cell line PC-12, mouse pluripotent embryonic stem cell ES-E14TG2a and mouse fibroblast cell line NIH/3T3 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The PC-12 cells cultured in RPMI-1640 (GIBICO) medium, NIH/3T3 in DMEM (GIBICO), MCF-7 and Hela in EMEM (GIBICO) were supplemented with 15% fetal calf serum (Invitrogen), 100 U/ml penicillin and 100 U/ml gentamicin, ES-E14TG2a cells were cultured in GMEM medium (Invitrogen) supplemented with 15% FBS (v/v) (Biochrom), 1% (v/v) L-glutamine (Invitrogen), 1% (v/v) NEAA (Inviteogen), 1000 U/ml ESGRO supplement (Millipore), 0.1 mM 2-mercaptoethanol. All the cells were maintained at 37 °C in humidified atmosphere (95% air and 5% CO_2).

2.8. Preparation of telomerase extracts

The telomerase extracts were prepared according to a previously reported protocol (Xiao et al., 2010). Firstly, the cells were collected and counted in the exponential phase of growth. Then 1×10^6 cells were transferred into an RNase-free 1.5 ml EP tube and washed twice with ice-cold PBS by centrifugation at 1800 rpm for 5 min. After discarding the supernatant carefully, the cells were resuspended in 200 μL of ice-cold lysis buffer, and incubated for 30 min on ice, and then centrifuged at 12,000 rpm for 20 min at 4 °C. Finally, the supernatant was collected carefully and transferred to microcentrifuge tube and stored at −80 °C before analysis.

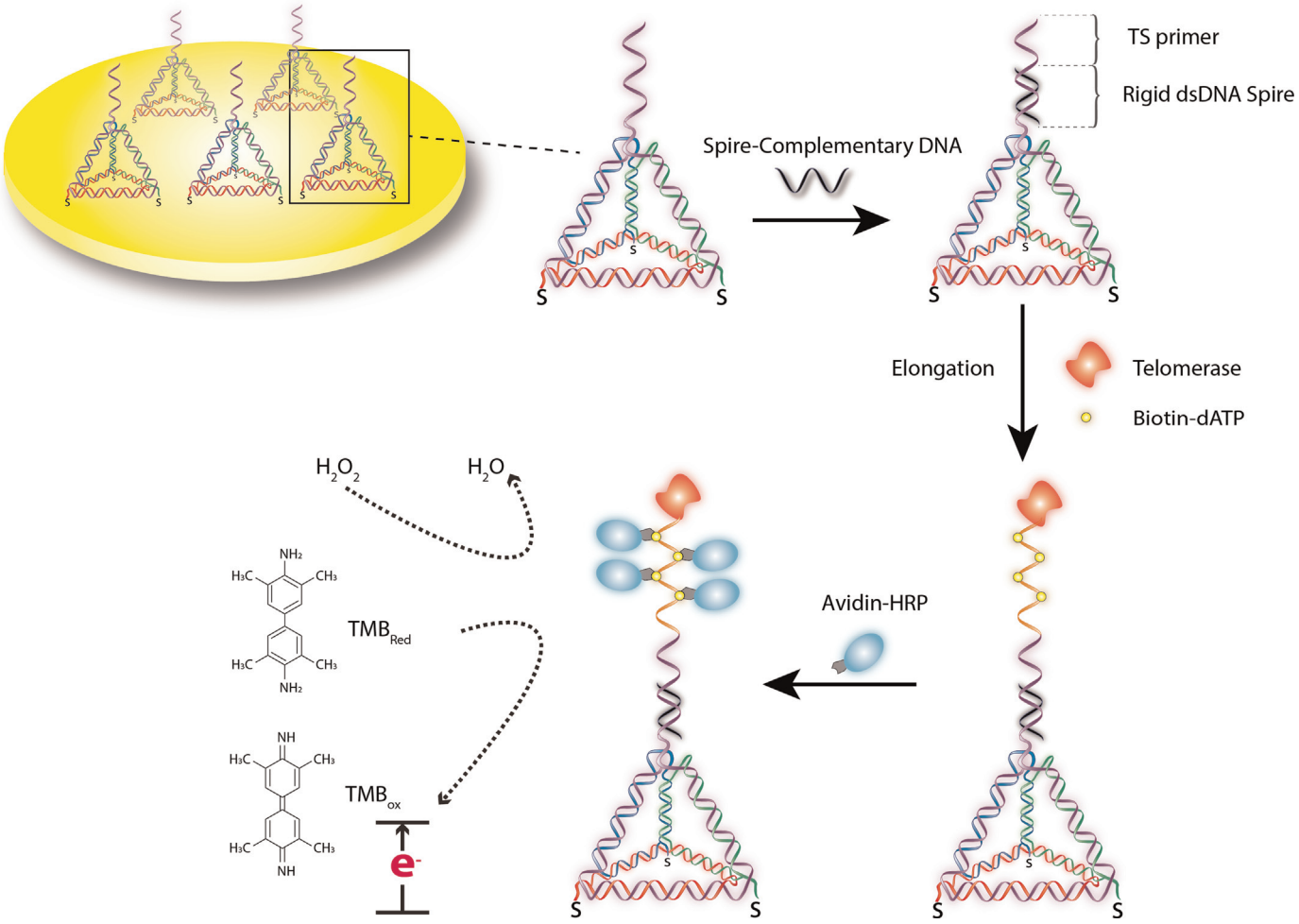
2.9. Real-time PCR of the TRAP assays

25 μL TRAP reaction solution consisted of 1 × TRAP buffer, 5 mM dNTPs, 0.05 μg TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 0.05 μg ACX return primer (5′-GCGCGG[CTTACC]3CTAACC-3′), 1 U taq polymerase, 1 × SYBR Green I Nucleic Acid Gel Stain (Invitrogen, CA), 1 μL CHAPS cell extract or CHAPS lysis buffer and RNAase free water. The thermal program was started with an incubation at 30 °C for 10 min, then 33 repeated cycles of 94 °C for 30 s and 60 °C for 30 s were performed.

3. Result and discussion

3.1. Strategy of the STTS analysis

In this work, we designed a spired DNA tetrahedron TS primer (STTS). A double-strand DNA (dsDNA) was designed on top of a DNA tetrahedron like a tower spire, and the TS primer for telomerase extension was at the top end of the spire (Scheme 1). The DNA tetrahedral-structure with a long pendent DNA was firstly constructed by four DNA strands (STTS-A, and TSP-B, C, D, Table 1) and then immobilized onto the gold surface through three thiols on the vertices. A SC-DNA was then added to hybridize with the bottom part of the pendent DNA. In this way, the top TS primer



Scheme 1. Schematic illustration of our STTS-based analysis of telomerase activity.

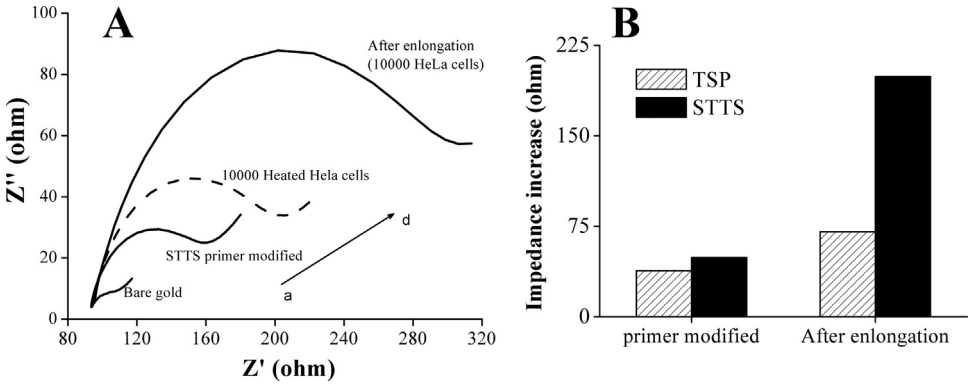


Fig. 1. EIS characterization of the assemble process: (A) EIS of STTS modified electrodes at different analysis stages: (a) bare gold, (b) gold electrode modified with STTP primer, (c) STTS modified gold electrode incubated with heated extract from HeLa cells, (d) STTS modified gold electrode incubated with extract from HeLa cells. (B) Comparison of the EIS results between TSP and STTS. The number of HeLa cells was 10,000.

was stably raised upright, giving it a controllable distance from the surface and the DNA tetrahedron.

With the presence of telomerase, the TS primer was elongated using mixed dNTPs including biotin labelled dATP (Weizmann et al., 2004; Rothacker et al., 2007). The subsequently added avidin–HRP would be captured by the biotin, and then catalyzed the electrochemical reduction of TMB to consequently generate an amplified current signal.

3.2. EIS characterization of the assembling process

Electrochemical impedance spectroscopy (EIS) was performed to characterize the establishing process of the STTS telomerase sensor. 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ was used as the probe electrons. At the beginning, the impedance of the bare gold was very low, and then the self-assembling of the STTS primer (Fig. 1A, b) and the following telomerase extension (Fig. 1A, d) blocked the transfer of the electrons to the electrode surface, thus dramatically increased the impedance value. However, when we incubated the STTS modified electrode with heated HeLa cells as a negative control, only an obviously limited impedance increase was found, which might came from only the unspecific absorption in the cell extract (Fig. 1A, c). TSP assembling and telomerase extension was also characterized. As shown in Fig. 1B, the impedance increase of TSP primer modified electrode was less than STTS primer modified electrode, presumably because of the double-strand on top of the STTS increased the blocking effect. After the telomerase extension of same number of HeLa cells, the impedance increase of STTS modified electrode was obviously higher, which demonstrated the improvement of the telomerase combination and the extension reaction on the STTS primer than TSP primer.

3.3. Comparison of different sensing strategies

The aim of ETBs is to sensitively and directly detect the telomerase activity on the surface without any pre-elongation in bulk solution. Not like other surface-based biosensors such as electrochemical hybridization or immunoassay which pursue only a biological recognition, the ETBs need to realize not only the specific enzyme combination but also the telomere surface elongation. That brings higher requirement to the design of the sensing surface.

Three telomerase sensors with different TS primers were investigated for the analysis of telomerase, including single-strand TS primer (ssP), normal DNA tetrahedral-structure TS primer (TSP) and STTS (this work). Extracts from 5000 Hela cells were analyzed by both ssP and TSP sensor, while extract from only 3000 Hela cells was analyzed by STTS. Meanwhile, lysis buffer was analyzed

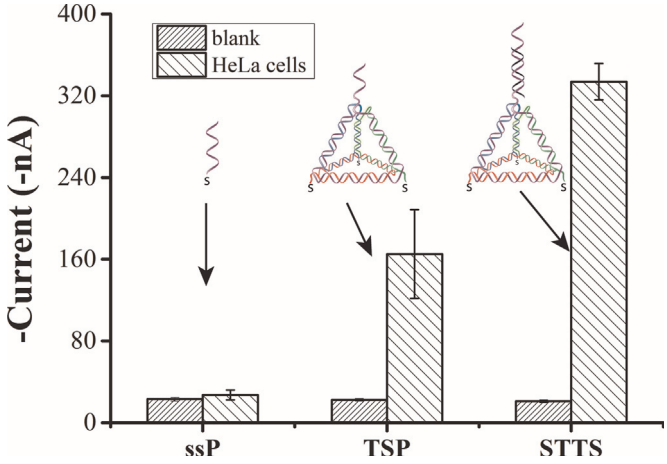


Fig. 2. Telomerase analysis results from three different sensors: ssP, TSP and STTS. HeLa cell number for ssP and TSP was 5000, while it was only 3000 for STTS. A lysis buffer was analysis as the blank. The error bars represent standard deviations for measurements taken from at least three independent experiments.

as blank by all three sensors. For ssP sensor, MCH was utilized to occupy the exposed surface space and force the TS primer to an upright orientation for the surface telomerase elongation. Thus the blank signal was relatively low. However, even under the optimal condition, the signal gain of ssP sensor was almost undetectable, which was probably attributed to the limited distance between the TS primer and the surface.

Benefiting from the DNA tetrahedron-structure, both the TSP and STTS sensor achieved lower background and higher signal gain than ssP sensor, because the DNA tetrahedron helped to resist the protein absorption and to regulate the TS primer as reported in previous works (Pei et al., 2010). Significantly, the signal gain from STTS was about 2 times higher than TSP sensor, even though the cell number for STTS is only 3000 (Fig. 2). The remarkable increase of the signal gain obviously demonstrated the improved telomerase combination and elongation on STTS surface. That is presumably because the dsDNA spire stably supported the soft TS primer with about 6.8 nm (20 base pairs) longer vertical distance, which greatly facilitated the telomerase elongation. Overall, the results of the sensor investigation were exactly consistent with our prediction.

3.4. Telomerase elongation time

After the STTS was modified on the electrode surface, the extract from 6000 HeLa cells was analyzed, and the current signal

was continuously monitored during the following 4 h (Fig. 3). We interestingly found that, in the initial 3 h, the current signal quickly increased as the elongation time went on, clearly showing the successfully telomerase elongation on the STTS surface, but after 3 h, the signal increase slowed down to a platform stage, presumably because the telomerase activity and the dNTPs were depleted. Therefore, we employed 3 h as an optimized telomerase elongation time in the assay.

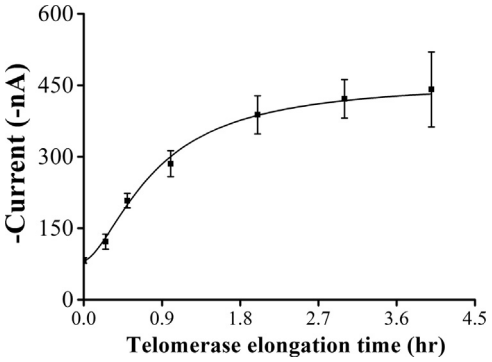


Fig. 3. Relationship between amperometric current signal and the elongation time for STTS-based electrochemical telomerase sensor. Extracts from 6000 HeLa cells was analyzed after different telomerase elongation times (0 min, 15 min, 30 min, 1 h, 2 h, 3 h and 4 h).

3.5. Electrochemical analysis of telomerase

The amperometry analysis was performed on a multi-channel potentiostat under a potential of −200 mV. The resulted decay *I–t* curve was recorded until it reached a plateau (steady-state current) at 60 s. Firstly, we detected the telomerase activity of two extracts from 50,000 HeLa cells: one is freshly prepared, the other is heat treated to inactivate most of the telomerase. The resulted *I–t* curve was presented in Fig. 4A. HeLa cells generated a dramatically high current signal (~1600 nA), while the heated sample only generated a negligible signal (~86 nA).

Next, cell extracts from a broad range of Hela cell numbers were analyzed by STTS sensor. In order to clearly show the increasing amperometric signals as the cell number was increased from 0–20,000 cells, we zoomed in the *I–t* curves to 1000 nA range, thus the *I–t* curve for 50,000 cells was too high to be shown in the same figure (Fig. 4B).

We found that the amperometric signal increased monotonically with the logarithm no. of HeLa cells (Fig. 4C). Significantly, the signal for 45 (~90 nA) could be easily distinguished from the background (~21 nA) (Fig. 4C inset). The detection limit was calculated to be lower than 10 HeLa cells (> 3 standard deviations

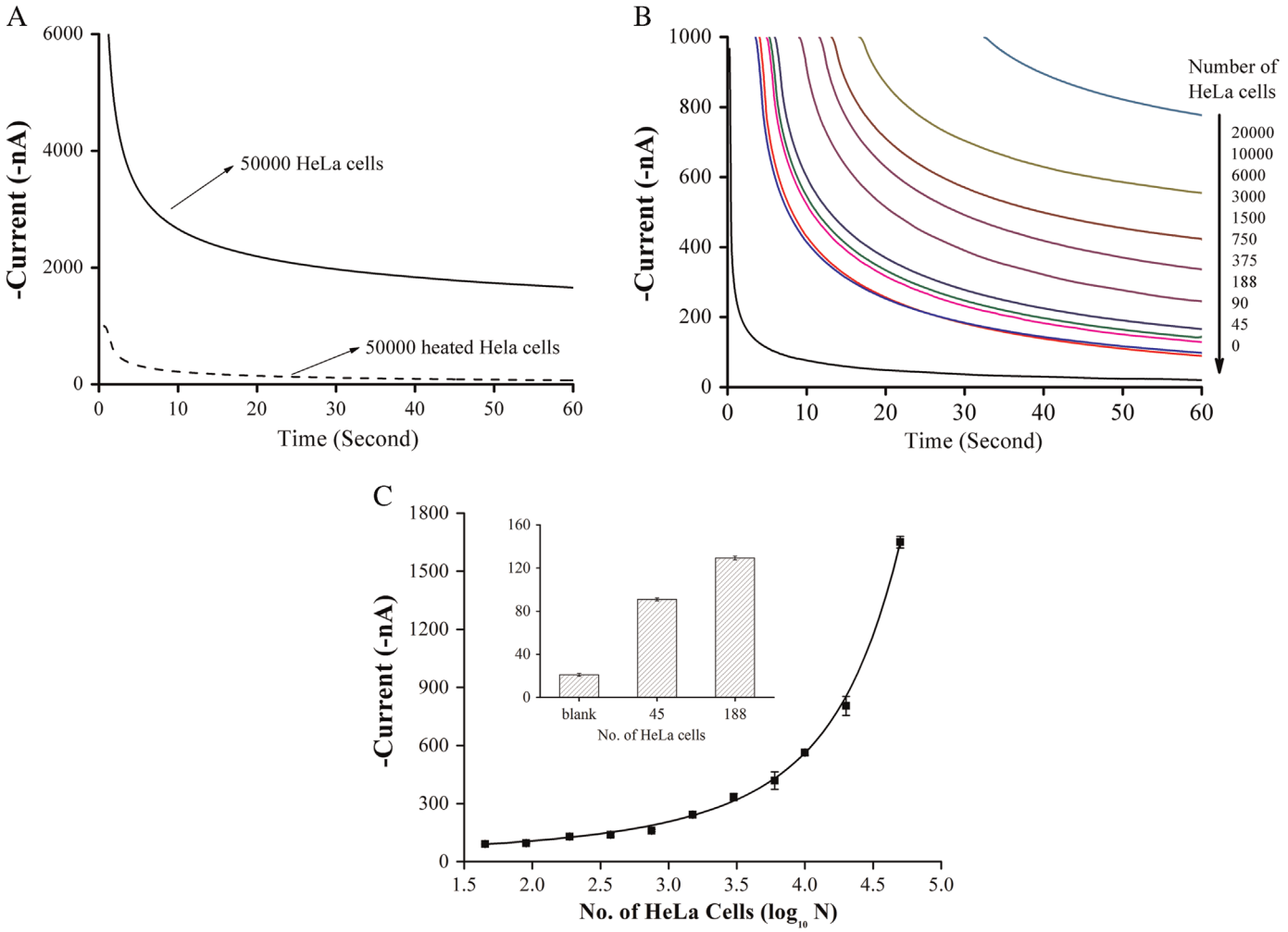


Fig. 4. (A) *I–t* curves for 50,000 HeLa cells (solid line) and 50,000 heat inactivated HeLa cells (dashed line). (B) *I–t* curves for a range of HeLa cell numbers. From top to bottom: 0, 45, 90, 188, 375, 750, 1500, 3000, 6000, 10,000, 20,000. The potential for *I–t* analysis was −200 mV. (C) Logarithmic plot of amperometric current vs HeLa cell numbers (0–50,000 cells). Inset shows the current signal from blank, 45 and 188 HeLa cells. The error bars represent standard deviations for measurements taken from at least three independent experiments.

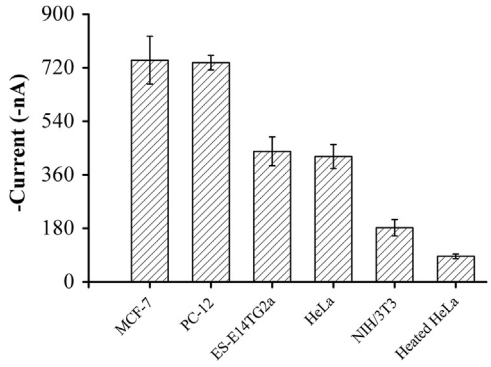


Fig. 5. Analysis results of telomerase activity of extracts from different cell lines. (From left to right) MCF7, PC-12, embryonic stem cell ES-E14TG2a, HeLa cells, normal cell line NIH/3T3. Number of all the cell lines was 6000. Meanwhile, 50,000 heat inactivated HeLa cells were analyzed as a negative control.

(SDs)), and the dynamic range covered 4 orders of magnitude (0–50,000 cells). The sensitivity of STTS sensor is at least 2 magnitudes lower than other surface extension-based electrochemical telomerase sensors without amplification (Pavlov et al., 2004; Shao et al., 2008; Li et al., 2011; Yang et al., 2011; Kim et al., 2013), and even comparable with to those strategies combined complex amplification to the surface primer strategies (Zheng et al., 2008; Li et al., 2010).

3.6. Analysis of different cell lines

To ensure the practicability, we challenged our system with different cell lines. Consisted with reported research results, relatively high telomerase activity was successfully detected in extracts of two human cancer cells (MCF-7 (Xiao et al., 2010), HeLa), one rat adrenal pheochromocytoma cell line PC-12 (Fu et al., 1999) and one mouse pluripotent embryonic stem cell ES-E14TG2a. On the contrary, only low current signal was achieved for mouse fibroblast cell line NIH/3T3 (Takahashi et al., 2003; Ak et al., 2011). Extract of 60,000 heat inactivated HeLa cells was simultaneously analyzed as a negative control (Fig. 5). The results demonstrated the capability of STTS sensor to quantificationally monitor the telomerase activity in various cell lines. The telomerase activity of HeLa cells adopted in this work was further checked by TRAP assay (Fig. S1).

4. Conclusions

In summary, we have developed a high sensitive telomerase sensor based on a spired DNA tetrahedron TS primer without traditional PCR amplification. The DNA tetrahedron greatly restrained the unspecific adsorption and regulated the density of the surface probes. Furthermore, the rigid dsDNA spire controlled the distance between the TS primer and the electrode surface. In this way, more than 2 times higher signal gain was achieved compared with normal TSP without the spire-structure (~1590% signal increase for 3000 cells by STTS vs ~740% signal increase for 5000 cells by TSP). We believe the design of the spire structure on top of the DNA tetrahedron, could be applied in various kinds of surface primer based biosensors by conveniently change the length to match different target molecular dimensions.

Acknowledgments

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.08.056.

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