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Magnetic Bead and Nanoparticle Based Electrochemiluminescence Amplification Assay for Direct and Sensitive Measuring of Telomerase Activity

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The broad-spectrum expression of telomerase in most malignancies makes it a promising target for a cancer diagnostic and prognostic tool. Conventional polymerase chain reaction (PCR)-based telomerase activity assay is highly sensitive but susceptible to amplification-related errors. Here, we present a novel approach to telomerase activity detection. The detection of telomerase activity is accomplished by the hybridization of electrochemiluminescence (ECL) nanoprobes to telomerase reaction products, subsequent capture by magnetic beads, and in situ measurement of the light signal from ECL nanoprobes. The ECL intensity directly reflects the quantity of telomerase reaction products, thus telomerase activity. The high sensitivity afforded by the current magnetic bead and nanoparticle based ECL detection platform allows measuring of telomerase activity from as little as 500 cultured cancer cells in crude cell extracts without the PCR amplification of telomerase reaction products. In addition, a comparative study of the ECL nanoprobe and linear telomere antisense ECL probe was executed. By the employment of the ECL nanoprobe, a gain of about 100-fold elevation of sensitivity was determined. The method described here is ideal for telomerase activity analysis due to its reliability and high sensitivity.

Human telomerase is a ribonucleoprotein complex that functions as a telomere terminal transferase by adding multiple repeats of the TTAGGG hexamer using its integral RNA as the template. Most human somatic cells repress telomerase expression, and telomeres shorten progressively with each cell division. Eventually a critically short telomere length is reached, and the cell stops dividing and gets into senescence. However, for cancer cells, due to the activation of telomerase, an unlimited replicative capacity is allowed to develop. A screening of nearly all cancer types has found a very strong association between the presence of telomerase activity and malignancy.[1] For its role in carcinogenesis, anticancer chemotherapy, and as a biomarker for the detection of cancer,[2] obtaining new telomerase activity detection methods that are cost-effective, rapid, facile, and applicable to the clinic is an important goal.

Several techniques to assess telomerase activity have been developed. One approach is based on polymerase chain reaction (PCR) which is termed telomere repeat amplification protocol (TRAP).[3] The approach is the most widely used method for monitoring telomerase activity, due to its ultrahigh sensitivity. Modified TRAP assays had also been developed to increase the linearity and sensitivity or to eliminate the use of radioactive nucleotides.[4−6] We recently reported that electrochemiluminescence (ECL) in conjunction with TRAP is a useful tool for detecting and quantifying telomerase activity.[7] Unfortunately, methods based on PCR have some apparent weaknesses: (i) The inhibition of the TaqDNA polymerase by the test compound in the assay mixture may be lead to a false negative result, which is unacceptable in a clinical cancer diagnostic test.[6] (ii) The assay reproducibility and accuracy are compromised by the amplification-related errors. (iii) TRAP assay is inappropriate for the determination of telomerase inhibition; thus, to apply this method for screening telomerase inhibitors is difficult.[8] For these reasons, the direct primer extension-based assay first developed by Morin[9] will be an alternative assay for telomerase activity analysis. However, it is challenging to obtain meaningful quantitative results with conventional primer extension assay due to the fact that telomerase levels are low in cancer cells. Thus, the use of unsafe radioactive material or highly purified samples is customarily needed.[9−13] Recently, a series of technologies for direct telom-

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erase analysis based on optical or spectral detection,[14−20] nanosensors,[21,22] and also magnetomechanical detection,[23] electrochemical detection,[24,25] surface plasmon resonance (SPR),[26] bioluminescent method,[27] and enzyme-linked immunosorbent assay (ELISA)[28] were developed. Although these techniques allow the detection of telomerase activity without the PCR process, each of them still has its shortcomings. For example, some methods mentioned above are costly and lack simplicity or need elaborate instruments and expensive florescent substances. Moreover, little work focuses on the features of high-throughout ability, ease of automation, and compatibility with existing clinical laboratory instrumentation. Thus, the clinical applicability is restricted.

ECL is a general term used to describe a reaction or mechanism that produces light at the surface of an electrode. Because of the high sensitivity and selectivity, simple instrumentation, and low cost, ECL has recently become an important and powerful analytical tool in analytical and clinical application.[29−31] A magnetic bead based ECL method with tris(2,2′-bipyridyl) ruthenium and tripropylamine (TPA) reaction has been demonstrated to be a highly sensitive method for biorelated detection.[32−37]

Recently, the use of nanostructure-based probes in biorelated applications has increased in popularity due to their unique physical and chemical properties.[38,39] Mirkin and co-workers have put forward and developed a new biosensing concept termed biobarcode amplification strategy.[40] The strategy makes use of nanostructure-based probes to improve sensitivity. By employing the strategy, PCR-like signal sensitivity for protein and DNA analysis without enzymatic amplification was achieved.[41−43]

In this paper, we describe a novel and PCR-free telomerase assay without the need of radioactive materials and highly purified telomerase samples. The assay takes advantage of the amplification potential of ECL nanoprobes which carried numerous ECL tags. Streptavidin-coated magnetic beads were used as both the separation tool and the immobilization matrix. ECL detection of magnetic beads enriched telomerase products was executed in situ at the surface of a platinum electrode. The high performance of this assay is related to the determination of telomerase activity from cell extracts equivalent down to 500 cells. The proposed magnetic bead and nanoparticle based ECL detection strategy offers a promise of a reliable method to directly measure of telomerase activity in cancer cells.

MATERIALS AND METHODS

Materials. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), phenylmethylsulfonyl fluoride (PMSF), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), TPA, and the chemicals to synthesize the $Ru(bpy)_3^{2+}$ *N*-hydroxysuccinimide ester (TBR−NHS ester) were purchased from Sigma (St. Louis, MO). RNA secure and RNase were acquired from Ambion. Other chemicals employed were of analytical reagent grade and were used as received. In all instances, high-purity deionized water (18 MΩ) was used throughout. Gold nanoparticles (AuNPs, 30 nm in diameter; stabilized electrostatically with citrate anions) were kindly supplied by Hunan University. Streptavidin-coated magnetic beads (2.8 μm in diameter) were products of Dynal Biotech (Lake Success, NY). All oligonucleotides used in this work were synthesized and HPLC-purified by Sangon Inc. (Shanghai, China). Their sequences are listed in Table 1.

Synthesis of TBR−NHS Ester. Ruthenium bis(2,2′-bipyridine)(2,2′-bipyridine-4,4′-dicarboxylic acid) *N*-hydroxysuccinimide ester (TBR−NHS ester) was prepared according to a previously published paper.[44] To synthesize $Ru(bpy)_2(dcbpy)(PF_6)_2$ first, $Ru(bpy)_2C1_2$ (0.2 g), $NaHCO_3$ (0.2 g), and 2,2′-bipyridine-4,4′-dicarboxylic (0.15 g) were mixed in a 50 mL two-neck round-bottom flask, and 30 mL of 80% MeOH was added. The solution was heated at 80 °C for 10 h. Make sure that the water is flowing through the reflux condenser at this time. The resulted solution was cooled in an ice bath for 2 h. The pH was adjusted with 1 M H_2SO_4 to 4.4. The formed precipitate was filtered and washed three times with 6 mL of MeOH. The filtrate was treated with 2.5 g of $NaPF_6$ in 12.5 mL of H_2O, the resulted mixtures were cooled in an ice bath, and the precipitate was

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Table 1. DNA Sequences and Modifications

DNA	sequence (5′−3′)
biotin−TS primer	5′-biotin−AATCCGTCGAGCAGAGTT-3′
ECL signal probe	5′-SH−$(CH_2)_6$−TTTTTTTGTATC−TBR-3′
telomere capture probe	5′-SH−$(CH_2)_6$−TTTTTTTTTTTTTTT(CTAACC)$_3$-3′
synthesized telomerase product	5′-biotin−AATCCGTCGAGCAGAGTTAG(GGTTAG)$_3$-3′
telomere antisense ECL probe	5′-TBR−CTAACCCTAACCCTAACC-3′
TS primer	5′-AATCCGTCGAGCAGAGTT-3′
ACX primer	5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′

collected by filtration. To synthesize TBR−NHS ester, 0.23 g of dicyclohexylcarbodiimide (DCC) and 0.119 g of *N*-hydroxysuccinimide (NHS) were dissolved in 1.5 mL of dimethylformamide (DMF) with stirring and cooled in an ice bath. An amount of 0.19 g of $Ru(bpy)_2(dcbpy)(PF_6)_2$ was added, and the mixture was stirred for some hours; the formed precipitate was removed by filtration. The formed TBR−NHS ester in the filtrate was kept in a freezer (4 °C) for further use.

DNA−TBR Labeling. DNA−TBR labeling was accomplished by the following procedures. Briefly, amino-modified DNA was dissolved in 100 mM sodium bicarbonate buffer (pH 8.5); then, TBR−NHS ester was added to the solution at 20-fold concentration with respect to the DNA. This mixture was left to react in the dark and was gently shaken during 10 h. Labeled DNA were precipitated by addition of cold absolute ethanol. The mixture was kept 30 min at −20 °C and then centrifuged 20 min at 12 000 rpm. The supernatant was removed, and the pellet was rinsed twice with cold 80% ethanol. The pellet was allowed to dry in vacuum during 10 min, then was dissolved in pure water and stored at −20 °C until use.

Labeling of the ECL Nanoprobe. ECL nanoprobe was prepared according to previously reported DNA−AuNPs conjunction procedures[45,46] with minor modifications. Briefly, 1 μL of 500 mM acetate buffer (pH 5.2) and 1.5 μL of 10 mM TCEP were added to the mixture of 40 pmol of telomere capture probes and 4 nmol of TBR-labeled ECL signal probes, to activate the thiolated DNA. The mixture was incubated for 30 min at room temperature. Then the freshly TCEP-treated probes were added to 1 mL of AuNPs (the concentration of the AuNPs was increased by centrifugation and resuspension to obtain a final concentration of 1 nM) with gentle shaking by hand. The mixture was incubated overnight at room temperature. A solution of 500 mM Tris acetate (pH 8.2) buffer was added dropwise to the sample until a concentration of 5 mM Tris acetate was reached. A solution of 1 M NaCl was added dropwise to reach a final salt concentration of 0.1 M NaCl. The salting buffer should be added as slowly as possible, with frequent tapping of the tube, to avoid possible aggregation of AuNPs. The tube was stored in a drawer for at least another day. Make sure to occasionally shake the mixture gently. The solution was centrifuged at 4 °C for 20 min and 12 000 rpm. The supernatant was discarded, and the precipitate was resuspended in 1 mL of AuNPs washing buffer containing 100 mM NaCl, 25 mM Tris acetate, pH 8.2. The step was repeated three times. The labeled AuNPs were stored at 4 °C. The amount of the ECL signal probes loaded on each AuNP was determined by measuring the ECL signal of the labeled AuNPs to quantify the number of ECL signal probe strands.

Cell Culture and Telomerase Extract Preparation. Cells were prepared as described in our recent paper.[47] Briefly, MCF-7 and HeLa cells were cultured in DMEM medium supplemented with 15% fetal calf serum, and the cells were maintained at 37 °C in a humidified atmosphere (95% air and 5% CO_2). Cells were collected in the exponential phase of growth, and 1×10^6 cells were dispensed in a 1.5 mL EP tube, washed twice with ice-cold PBS, and resuspended in 200 μL of ice-cold CHAPS lysis buffer (10 mM Tris−HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). The CHAPS lysis buffer was pretreated with RNA secure according to the manufacturer's instructions. The lysate was incubated for 30 min on ice and centrifuged 20 min at 14 000 rpm, 4 °C, to pellet insoluble material. Without disturbing the pellet, carefully transfer the cleared lysate to a fresh 1.5 mL EP tube. The lysate was used immediately for telomerase assay or frozen at −80 °C.

Telomerase Extension Reaction. Telomerase extracts were diluted in lysis buffer with respective number of cells; the extracts (5 μL) were added to 45 μL of RNA secure pretreated extension solution containing 1× PCR buffer, 1 mM dATP, dTTP, and dGTP, 1 mM EGTA, and 1 μM 5′-biotinylated TS primer. The solution was incubated at 37 °C for 60 min. For control experiments, telomerase extracts were pretreated with RNase for 15 min at 37 °C or heat-treated (95 °C for 10 min).

Magnetic Bead Based ECL Detection of Telomerase Activity. A custom-built ECL detection system was described in detail in our previous research.[35] For the samples analysis, 50 μL of telomerase reaction products was directly added to 100 μL of bind buffer (10 mM TE, 500 mM NaCl, pH 7.4) containing 20 μL of magnetic beads, and this was incubated in an Eppendorf thermomixer for 30 min at 30 °C. The reaction mixture was separated by using magnetic racks (Dynal, mpc-s) and washed twice with bind buffer to remove the components from the telomerase extract and extension. To the washed magnetic beads−telomerase extension products complexes 50 μL of hybridization buffer (400 mM NaCl in 10 mM phosphate-buffered solution at pH 7.4) and 10 μL of ECL nanoprobes were added. The mixture was vortexed and placed at 40 °C with mixing for 1 h. The mixture was then washed three times using hybridization buffer to remove all unbound ECL nanoprobes and resuspended in 100 μL of ECL assay buffer (200 mM phosphate, 50 μM NaCl, 7 mM NaN_3, 0.8 μM Triton X-100, 0.4 mM Tween 20, 100 mM TPA, pH 8.0) and was injected to the ECL reaction cell. The magnetic beads−telomerase products−ECL nanoprobes com-

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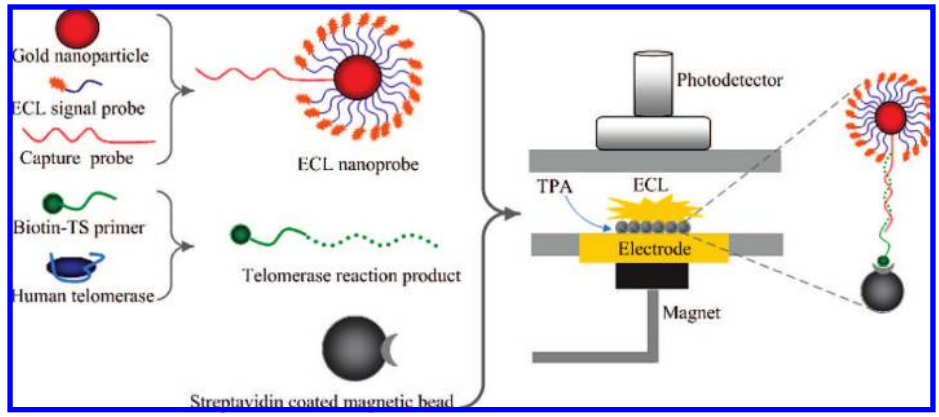
Scheme 1. Strategy for the Magnetic Bead and Nanoparticle Based ECL Amplification Assay of Telomerase Activity



plexes were captured and temporarily immobilized on the working electrode by a magnet under it. The applied potential of the ECL reaction was fixed at 1.25 V, and the photon signal was measured.

For detection of chemically synthesized telomerase product (STP), 5 μL of STP with corresponding concentration was used at each test. All steps were identical to the samples analysis. The procedure of linear telomere antisense ECL probe experiments was all identical to the ECL nanoprobe experiments. Note that the azide ion and surfactant-containing ECL assay buffer employed in the current assay increases the ECL efficiency about 20-fold compared to the azide ion and surfactant-free ECL assay buffer. The mechanism for the improvement was intensively discussed in literatures.[48−50]

Conventional Telomerase Assays. The conventional TRAP experiment was a modification as described previously.[6] Briefly, cell lysate was diluted with designated cell numbers and 2 μL of cell lysate was added to 23 μL of a solution containing 1× PCR buffer, 1 mM EGTA, 0.1 mg mL^{-1} bovine serum albumin, 200 μM dNTPs (50 μM each), and 0.2 μg of TS primer. The solution was incubated at 30 °C for 30 min, then at 90 °C for 2 min to terminate the reaction, followed by the addition of 25 μL of solution which contains 1× PCR buffer, 200 μM dNTPs, 3 U of HS TaqDNA polymerase, and 0.1 μg of ACX primer. PCR was carried out in an Eppendorf AG thermal cycler with the following program: 94 °C for 4 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min; 4 °C hold. PCR products were analyzed on a Bio-Rad (Bio-Rad Laboratories, U.S.A.) slab electrophoresis system. The 10 μL samples were loaded onto a 10% native polyacrylamide gel (29:1 acryl/bisacryl) in 0.5× Tris−borate−EDTA (TBE). Gels were run at room temperature for 1 h at 120 V. The gel was confirmed by silver staining and photographed by a Nikon 4500 digital camera.

RESULTS

Detection Scheme. The experimental concept is schematically shown in Scheme 1. Prior to the test, ECL nanoprobes were synthesized by bifunctioning AuNPs with telomere capture probe and ECL signal probe with a ratio of 1:100. Signal probe is responsible for the ECL detection, and telomere capture probe is used to recognize the telomerase reaction products. For telomerase analysis, biotinylated TS primer was incubated with telomerase extracts from the cancer cells in the presence of the nucleotide mixture dNTPs. TTAGGG repeat units were continuously added to the 3′-end of the primer by telomerase. The telomerization reaction was allowed to proceed for 1 h at 37 °C and stopped by heat denaturing of telomerase. Afterward, streptavidin-coated magnetic beads were added to the products. The telomerase reaction products were selectively captured through biotin−streptavidin linkage. Subsequently, telomerase reaction products were separated and hybridized with ECL nanoprobes. Excess ECL nanoprobes were easily washed off using clean buffer with the assist of a magnetic separator. The resulted magnetic beads−telomerase reaction products−ECL nanoprobes complexes were resuspended in ECL assay buffer for subsequent ECL detection.

Labeling and Assessment of the ECL Nanoprobe. The success of ECL amplification depends on the loading quantity efficiency of the ECL nanoprobe. In this assay, AuNPs were colabeled with two probes, the ECL signal probe and the telomere capture probe. The amount of ECL signal probe on each AuNP directly correlates to the amplification efficiency, thus respect to the sensitivity. In an ideal situation, the signal probe conjugated to the surface of a AuNP should reach saturation while the number of telomere capture probes on a AuNP should be kept minimal. In the current format, we designed a short ECL signal probe (12 bp) and a relatively long telomere capture probe (33 bp). This probe design strategy was based on the following consideration: (i) A short DNA probe leads to lessened repulsive interactions; thus, it will be beneficial to maximizing DNA loading per particle.[51,52] (ii) The telomere capture probe was designed with a linkage region (poly-T spacer, 15 T) and a hybridization recognition region (three telomere antisense repeats). The linkage region is roughly equal to the length of the ECL signal probe, thus moving the hybridization recognition region further away from the AuNPs surface to allow better interaction with the telomerase

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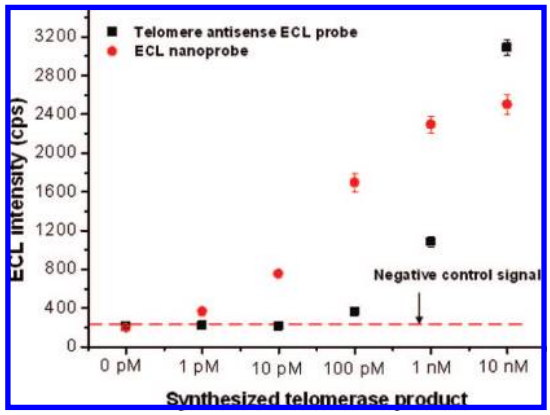

Figure 1. ECL intensities observed upon the analysis of different concentrations of STP using the ECL nanoprobe and telomere antisense ECL probe. The probe details can be found in Table 1.

reaction products and, therefore, facilitating the hybridization process. To estimate the average number of ECL signal probes per AuNP, we directly measured the ECL intensity of the resulting ECL nanoprobes in solutions; the ECL signal intensity was compared with the calibration curve for the pure ECL signal probe. This ECL signal probe concentration divided by the starting molar concentration of AuNPs gives a value of ECL signal probe per AuNP. On the basis of this analysis, there are about 240 ECL signal probes on each AuNP. According to the ratio, about 2 to ~3 telomere capture probes were loaded. The method for determining the surface coverage of thiol-capped ECL signal probes bound to AuNPs is reasonable because of being without an enhanced or quenched effect of AuNPs on the ECL of $Ru(bpy)_3^{2+}$/TPA in the solution under our investigation (data not shown). This is also theoretically corrected because the wavelength range of effective light absorption of AuNPs ranges from 200 to 530 nm, but the light emission of $Ru(bpy)_3^{2+}$* was only detectable at about 620 nm.

Detection of Chemically Synthesized Telomerase Product. To evaluate the performance of the resulting ECL nanoprobe and stimulate the telomerase assay, we employed an STP as the model target DNA sequence. Five different concentrations of STP from 10 nM, 1 nM, 100 pM, 10 pM, and down to 1 pM have been detected, and the ECL intensities were compared to that of a negative control sample (no target). The proposed method offers well-defined concentration dependence. As depicted in Figure 1, the ECL intensity increased almost linearly with the increase of STP concentration to 1 nM. A plateau effect was reached at the STP concentration of 10 nM. We found the signal of 1 pM STP is easy to differentiate from the background (the value is greater than the mean of the negative control plus three times SD). In parallel assays with the same STP concentration range, linear telomere antisense ECL probe (three telomere antisense repeats) was employed to evaluate the amplification efficiency of the ECL nanoprobe. The sequence of the telomere antisense ECL probe was the same as the hybridization region of the telomere capture strand of the ECL nanoprobe and was labeled at its 5′-end with TBR. From Figure 1 we can see until 100 pM the ECL signal can differentiate from the negative control signal. It indicated the sensitivity of the ECL nanoprobe is roughly 2 orders of magnitude higher than the linear telomere antisense ECL probe. We noted that for the ECL nanoprobe there is a decrease in ECL enhancement at higher STP concentration (Figure 1). The decrease in ECL is mostly probably because the ECL nanoprobe will be used up at such a high concentration of STP.

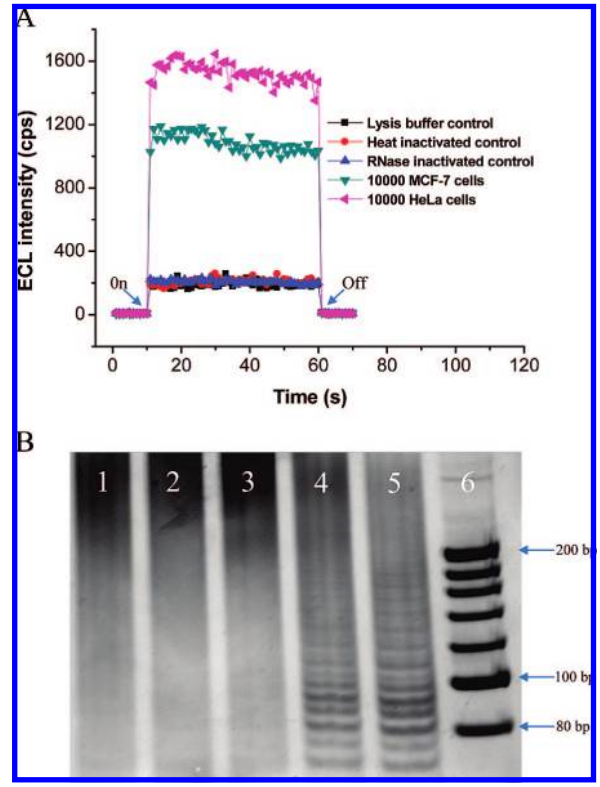

Figure 2. (A) ECL signals corresponding to the analysis of telomerase activity originating from 10 000 HeLa cells, 10 000 MCF-7 cells, lysis buffer control, heat-inactivated control for 10 000 HeLa cells, and RNase-inactivated control for 10 000 HeLa cells. (B) Conventional TRAP−silver-staining assay. Lines 1−6 represent the lysis buffer control, heat-inactivated control for 1000 HeLa cells, RNase-inactivated control for 1000 HeLa cells, 1000 MCF-7 cells, 1000 HeLa cells, and DNA marker, respectively.

Detection of Telomerase Activity from Cultured Cancer Cells. For practicable measuring of telomerase activity, crude telomerase extracts from telomerase-positive HeLa cells and MCF-7 cells were prepared (described in detail in the Materials and Methods section), and a telomeric elongation step on a biotinylated TS primer was performed. Samples were then separated with streptavidin-coated magnetic beads and hybridized with the ECL nanoprobes. Subsequently, the magnetic bead based ECL measurements were performed. Figure 2A shows the ECL intensity emitted upon analyzing the telomerase activity originated from 10 000 HeLa and MCF-7 cells. It shows the ECL emitted upon analysis of an extract from 10 000 HeLa and MCF-7 cells is significantly higher than the ECL signals obtained from lysis buffer control (0 cells). The addition of telomere repeats (TTAGGG) to a known telomerase primer by telomerase is sensitive to RNase and heat, which are known to destroy the essential RNA template and reverse transcriptase protein of telomerase. In control experiments, HeLa cells extract was pretreated by RNase or heat. Results show that in both cases an obvious decrease in the ECL signals was observed and the lysis buffer approached control level. It is clearly confirming the ECL signals were dependent on telomerase activity.

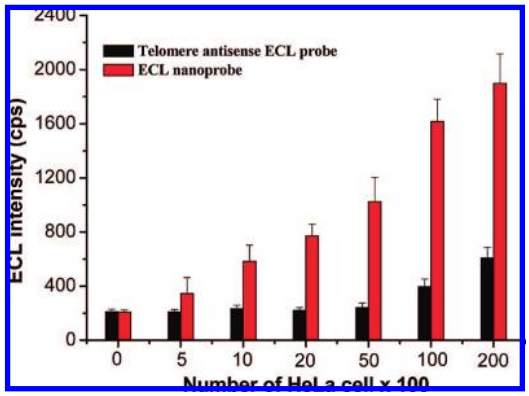

Figure 3. Sensitivity of the current telomerase assay. ECL nanoprobe and telomere antisense ECL probe were used for the analysis of 20 000, 10 000, 5000, 2000, 1000, and 500 HeLa cells and cell-free extract control, respectively.

To further verify the detection accuracy of the new telomerase assay, the relevant cell extracts were analyzed by the conventional TRAP−silver-staining method. As shown in Figure 2B, 6 bp DNA ladder bands indicative of telomerase activity were observed in extracts of 1000 HeLa cell equivalents (line 5) and 1000 MCF-7 cell equivalents (line 4) but not in the control samples (lines 1−3 represent lysis buffer control, heat-inactivated control for 1000 HeLa cells, and RNase-inactivated control for 1000 HeLa cells). We investigated that the HeLa cell line contains a relatively higher level of telomerase than the MCF-7 cell line (Figure 2B). This is consistent with the result obtained from current direct telomerase assay (Figure 2A).

To investigate the precision of the proposed assay, the coefficient of variation (CV) was determined by measuring the ECL signal of 5000 HeLa cells and MCF-7 equivalent with six replicates; CV values for both HeLa and MCF-7 cells were less than 10%.

Sensitivity Assessment. As the telomerization is controlled by the content of telomerase in the cell lysate samples, the amount of hybridized ECL nanoprobes, and the intensity of ECL, should relate to the concentration of cancer cells. To validate the sensitivity of our newly developed telomerase assay, cell extracts were serially diluted with lysis buffer and used as a source for telomerase. Figure 3 shows the ECL intensity emitted from the system analyzing variable numbers of HeLa cells. As expected, the ECL intensity is highly dependent on the number of cells used for the cell extracts. To define if a sample is telomerase-positive, a cutoff value is calculated based on formula 1:

$$V_{\text{cutoff}} = V_{\text{control}} + 3V_{\text{stdev(con)}} \quad (1)$$

where the V_{control} is the average light emission from the lysis buffer control and $V_{\text{stdev(con)}}$ represents the standard deviation ($V_{\text{stdev(con)}}$) of the ECL reading from the lysis buffer control samples. According to this formula, the cutoff level for telomerase-positive samples was set at 245 counts/s. ECL signal less than this value should not be indicated as telomerase-positive under our conditions. With the use of our method, we were able to detect telomerase activity in the HeLa extracts equivalent to 500 cells with a signal value greater than V_{cutoff}. Although the detection of less than 500 cells has been achieved according to the V_{cutoff}, the results were less reproducible for such highly diluted samples (data not shown). To further investigate the signal amplification ability of the current ECL nanoprobes in real samples, we compared the detection sensitivity of the ECL nanoprobe with the linear telomere antisense ECL probe in the current studied range (500−20 000 cells). One can see from Figure 3 that the ECL intensity produced by the use of the linear telomere antisense ECL probe is the same as the blank signal when the HeLa cancer cell numbers are ≤5000. Well-defined ECL signals only can be observed in the cell number of ≥10 000.

DISCUSSION

Cellular immortality is a hallmark of cancer. The identification of telomerase, an enzyme associated with cellular immortality, has created considerable interest in its potential for the early detection of carcinogenesis.[53] Thus, the need for a telomerase assay with greater sensitivity, selectivity, simplicity, cost effectiveness, and throughput in a wide range of applications has provided the driving force for continuous development of new strategies and technologies for analysis of telomerase activity. In this report, we describe a new method for telomerase activity detection, which is characterized by magnetic separation and nanoparticle based ECL signal amplification protocols with high sensitivity and selectivity. The incorporation of magnetic beads and nanoparticles in ECL detection for telomerase assay has resulted in several advantages.

First, streptavidin-coated magnetic beads were specifically chosen for selective immunomagnet capture of biotinylated telomerase reaction products. This binding interaction of streptavidin and biotin is quick, reliable, and strong ($K_d = 10^{-15}$). It dramatically reduces the sample preparation time from tens of hours, for thiol- or phosphothioate-mediated systems,[14,16,21] to just tens of minutes. Otherwise, for ECL detection, the magnetic beads−telomerase products−ECL nanoprobes complexes can readily be collected on the electrode surface by using a magnet, resulting in a construction of a highly condensed $Ru(bpy)_3^{2+}$ domain.[50] The use in the area of telomerase assay seems very promising, since an electrode can be reused by simply washing out the beads from the surface, as a result, leading to the detection process being rapid, and the detection cost is greatly reduced.

Second, in this assay, we designed a high-performance ECL probe for amplified detection telomerase activity. The ECL nanoprobe was designed by coating per AuNP more than 200 short ECL signal probes. This multivalency property makes AuNPs serve as nanoscaffolds to locally concentrate hundreds of ECL signal probes for a target. This facilitates generation of ECL burst signals with much-enhanced sensitivity (~100-fold) compared to the use of a linear telomere antisense ECL probe (Figure 1). The assay permitted easy detection of 1 pM STP. Due to the fact that only 5 μL of STP was used in our assay, a threshold of as low as 5 amol was achieved. We believe that the detection limit in the attomole region may be sufficient to detect the telomerase level without the PCR process, as the reported chemiluminescent assay with a sensitivity of 100 amol,[15] 10 amol of telomere repeats of nanosensors,[22] SPR assay with a sensitivity of 3000 amol,[26] and bioluminescent assay could detect 167 amol of telomeric repeats.[27] In fact, the telomerase level at the concentration of down to 500

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Table 2. Comparsion between the Current Method and Other Reported Techniques for Direct Analysis of Telomerase Activity

method	label	detection modes	sensitivity data	ref
PAGE	radioactive dGTP	autoradiography	200−500 HeLa cells	11
electrochemical assay	label-free	electrochemistry	about 40 cells/μL	14
HPA assay	acridiniumester-labeled DNA probe	chemiluminescence	100 000 HeLa-60 cells	15
catalytic beacons	peroxidase-like DNAzyme	spectroscopy	500 HeLa cells	17
DNAzyme-based assay	DNAzyme-functionalized AuNPs	chemiluminescence	1000 HeLa cells	21
magnetic nanosensor	DNA-functionalized magnetic nanoparticles	magnetic resonance imaging	10 amol of telomeric repeats	22
electrochemical and QCM assay	avidin−alkalinephosphatase conjugate	electrochemistry	1000−5000 HeLa cells	24
bioluminescent method	label-free	bioluminescence	5−10 cells	25
TRE chip-based assay	label-free	SPR	100 tumor cells in 10^5 background cells	26
ELISA	alkaline phosphatase-antidigoxigenin	chemiluminescence	100 amol of telomeric repeats	28
magnetic bead based ECL assay	ECL nanoprobes	electrochemilum-inescence	500 HeLa cells	this work

cells is easy to detect in the current system (S/N > 4), although the sensitivity data of the current telomerase assay was incomparable to the previously developed TRAP-based method, which is sensitive to single-cell samples. However, results were obtained in the current study without relying upon the PCR amplification of telomerase reaction products. This is a significant advance because amplification-related errors can be avoided; thus, more accurate and more reliable telomerase activity data can be obtained.

Finally, with the use of magnetic beads, the current telomerase assay can be easily extended to a high-throughput and automatic screening format. Practically, without significant altering of current protocols is needed when it is associated with a commercial ECL detection platform.[54,55] This capability is indeed a desired feature for future clinical application.

In summary, we have carefully demonstrated a significant sensitivity enhancement in ECL detection of telomerase activity in cancer cells via the introduction of an ECL nanoprobe. The amplified change allows us to reliably determine the telomerase activity down to 500 cells. Moreover, analytical features of different direct telomerase assays were summarized and the sensitivity of the proposed telomerase assay is comparable to these techniques (Table 2). Further applications of the current telomerase assay in clinical samples and the evaluation of telomerase inhibitors are in process in our laboratory.

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