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Telomere elongation-based DNA-Catalytic amplification strategy for sensitive SERS detection of telomerase activity

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ABSTRACT

Telomerase has been regarded as a biomarker for cancer diagnosis as well as the clinical treatment and the reliable detection of intracellular telomerase activity is of great significance. By developing a telomere elongation-based DNA-catalytic amplification strategy, a novel surface-enhanced Raman scattering (SERS) method is proposed for the assay of telomerase activity. In the presence of telomerase and nucleotide mixture dNTPs, the telomerase substrate (TS) primer extended and generated a long single-strand DNA (ssDNA) containing the telomere repeat units (TTAGGG)n, which could catalyze the entropy-driven circuit reaction (EDCR). One of the products of EDCR was ingeniously used as the catalyst of catalytic hairpin assembly (CHA) occured on magnetic beads (MBs). As a result, a large amount of ROX-labeled Raman probes could be anchored on the surface of MBs and used for SERS detection. Using this strategy, the assay can detect telomerase activity from cell extracts equivalent down to single HeLa cell.

1. Introduction

Telomerase is a special ribonucleoprotein that consists of its own RNA template and a reverse transcriptase protein component which can catalyze the synthesis of a repeated DNA sequence (TTAGGG)n at chromosomal ends (Gallardo et al., 2011; Su et al., 2018; J.S. Wang et al., 2017; Greider and Blackburn, 1989). In normal human somatic cells, the telomerase activity is inhibited and the repetitive DNA sequences reduce in length, which triggers cell cycle arrest or cell death ultimately (Qian et al., 2013; Harley, 2008). However, the telomerase becomes active in the high proliferative cell (Stem cells and germ cells) and more than 85% cancer cells in which the telomeres can elongate continuously and the cells are able to divide indefinitely (Hong et al., 2016; H.T. Yang et al., 2017; Ma et al., 2018; Zong et al., 2014). Therefore, telomerase has been regarded as a biomarker for cancer diagnosis as well as the clinical treatment (Ma et al., 2017; Xu et al., 2016; Y.C. Wang et al., 2017) and the reliable detection of intracellular telomerase activity is of great significance.

As a powerful tool, telomere repeat amplification protocol (TRAP) based on the polymerase chain reaction (PCR) is wildly used for detecting the telomerase activity (Kim and Wu, 1997). However, the harmful radioactive fluorescent materials and the complex operation are unavoidable (Xiao et al., 2010). Due to the above drawbacks of the assay, various of PCR-free methods, especially some enzyme-based isothermal amplification strategies have been developed for improving the sensitivity (X.J. Yang et al., 2017). These strategies include rolling circle amplification (RCA) (Zhang et al., 2017), exponential isothermal amplification (EXIA) (Wang et al., 2016), AIEgens and exonuclease III aided quadratic amplification (Min et al., 2017) and so on. Nevertheless, the unstable enzyme activities and harsh experimental conditions such as temperature, pH and specific buffer solution make these amplification techniques unsatisfactory for practical application (Zhang et al., 2014; Huang et al., 2018).

Recently, some enzyme-free methods have been proposed for biomolecule assays. Especially, synthetic DNA has been used as catalyst (Turberfield et al., 2003; Bois et al., 2005; Green et al., 2006; Seelig et al., 2006) for DNA self-assembly, molecular machines and cycle amplification reaction instead of protein enzymes and ribozymes. As a versatile enzyme-free isothermal amplification technique, catalytic hairpin assembly (CHA) permits exponential amplification of a target sequence in 15 min (Walker et al., 1992; Hellyer and Nadeau, 2004) and miscellaneous CHA platforms have been developed for biomolecular analysis combining with other detection techniques (Wen et al., 2016; Zhang et al., 2016; Wu et al., 2017; Shi et al., 2017). In the field

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of DNA logic circuits entropy-driven circuit reaction (EDCR) is a milestone report proposed by Zhang and his co-workers (Zhang et al., 2007; He et al., 2016). Due to the excellent thermo-stability, higher S/B ratio and robust resistance to complex environment, the enzyme-free EDCR strategy is programmed to detect nucleic acids. For example, Ma et al. developed a DNA-programmed nanoparticle disassembly method for microRNA (mRNA) detection based on EDCR (He et al., 2018). Tan et al. developed an entropy-driven three-dimensional DNA amplifier (EDTD) for the specific mRNA assay in the living cells (Gao et al., 2016).

For assisting cancer early diagnosis and overcoming the limitation in the existed methods, a novel DNA-catalytic amplification strategy was developed for the detection of telomerase activity. Coupled with surface-enhanced Raman scattering (SERS) technique, high sensitivity, simplicity and rapidity of telomerase assay in cancer cells was achieved conveniently. In the presence of telomerase and nucleotide mixture dNTPs, the telomerase substrate (TS) primer extended and generated a long single-strand DNA (ssDNA) containing the telomere repeat units (TTAGGG)n, which could catalyze the entropy-driven circuit reaction (EDCR). One of the products of EDCR was ingeniously used as the catalyst of catalytic hairpin assembly (CHA) occured on magnetic beads (MBs). As a result, a large amount of ROX-labeled Raman probes could be anchored on the surface of MBs and used for SERS detection. Using this strategy, the assay can detect telomerase activity from cell extracts equivalent down to 1 HeLa cell.

2. Experimental

2.1. Materials and apparatus

The oligonucleotides used in this experiment were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences are listed in Table S1. The deoxynucleotide solution mixture (dNTPs) was also purchased from Sangon Inc. (Shanghai, China). Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonicacid (CHAPS), phenylmethylsulfonyl fluoride (PMSF), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), glycerol, Chloroauric acid ($HAuCl4{\cdot}3H_2O$), Sodium citrate dihydrate, 3′-Azido-3′-deoxythymidine (AZT) and epigallocatechin gallate (EGCG) were purchased from Sigma-Aldrich (Shanghai) Trading Co. Ltd. RNase A, Klenow polymerase, T7 RNA polymerase and lysozyme were purchased from New England Biolabs (Beijing) LTD. The human telomerase (TE) ELISA Kit was purchased from Shanghai Qiaodu Biotechnology Co., Ltd.

The magnetic beads (MB) modified with the carboxyl group were purchased from the BaseLine ChromTech Research Centre (Tianjin, China); the gold film for the Raman analysis was obtained from the BioNavis Ltd (Finland).

Transmission electron microscopy (TEM) images were taken with a JEM F2100 Field Emission Transmission Electron Microscope (JEOL). UV–vis absorption spectra were obtained with a Cary 50 UV/vis-NIR spectrophotometer (Varian, USA). SERS detection was performed on an inVia Raman microscope (Renishaw, England).

2.2. Preparation of the Raman probe

The Raman probe modified with capture DNA (**S4**) and barcode DNA (**S5**) for Raman signal was prepared as described below. Firstly, 10 μL of 10^{-7} M **S4** and 50 μL of **S5** (10^{-6} M) were added to 1 mL of freshly prepared AuNPs. Then the mixture was shaken gently overnight (approximately 12 h) at 37 °C. Subsequently, the obtained DNA-AuNPs conjugates were aged for 6 h respectively in 200 μL of 0.05 M and 0.1 M NaCl solution. Excess reagents were removed by centrifugation (10000 rpm, 30 min) at 4 °C. 10 μL PBS (0.01 M, pH 7.4) was used for dispersing the red precipitate and the prepared probe was stored at 4 °C in the refrigerator before use.

2.3. Immobilization of hairpin DNA (H2) on the surface of MBs

Haripin DNA (**H2**) was heated to 90 °C for 5 min at the water bath and then allowed to cool to room temperature before use. After washing with imidazol-HCl buffer (pH 6.8, 0.1 M, 200 μL) for three times, the carboxylated MBs was activated in 100 μL of 0.1 M EDC (imidazol-HCl buffer, pH 6.8) at 37 °C for 30 min and used for immobilizing the haripin DNA (**H2**). Then 20 μL of 10^{-6} M amino group modification **H2** was added to the MBs and incubated at 37 °C overnight. Excess Hairpin DNA was removed by magnetic separation and the resulting MBs-H2 was rinsed for three times with PBS (0.01 M, pH 7.4). The precipitate was resuspended in 5 μL of PBS and stored at 4 °C for further use.

2.4. Telomerase extract preparation and extension reaction

The cells were collected during the exponential phase of growth and 5×10^6 cells were dispensed in a 1.5 mL Eppendorf tube (EP tube). After being washed with cold PBS (0.1 M, pH 7.4), 200 μL cold CHAPS lysate buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.5% CHAPS and 10% glycerol) was added and the cells were kept on ice for 30 min. The cell lysate was collected by centrifuging at 16000 rpm for 20 min at 4 °C and then used immediately for telomerase assay or frozen at −80 °C.

For telomere extension reaction, the diluted extract with different concentrations was added to the extended solution containing 0.5 mM dNTPs and 1 nM TS primers (20 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 1 mM EGTA, 63 mM KCl and 0.05% Tween 20). The mixture solution was incubated at 37 °C for 1 h and the elongation reaction was ended by heat denaturing of telomerase (90 °C, 10 min). For control experiments, telomerase extracts need to be pretreated with RNase or heat-treated and the following steps were the same as those mentioned above.

2.5. The catalytic amplification reaction

The three-stranded substrate was firstly prepared as follows: single-stranded (ssDNA) **S1**, **S2** and **S3** (50 μL, 5×10^{-7} M) were mixed together and incubated at 37 °C for 1 h to form the complementary double-stranded DNA (dsDNA). Then the telomerase extension products (**P**), 50 μL of fuel DNA (**F**, 5×10^{-7} M), 100 μL of hairpin DNA **H1** (5×10^{-7} M), the prepared MBs-H2 and Raman probes were added to perform the catalytic amplification reaction. After incubating at 37 °C for 1 h, a large number of Raman probe-anchored MBs complexes were obtained and the excess reagents were removed by magnetic separation. Then the complexes were washed with PBS for three times and redispersed in 10 μL of 0.01 M PBS (pH 7.4).

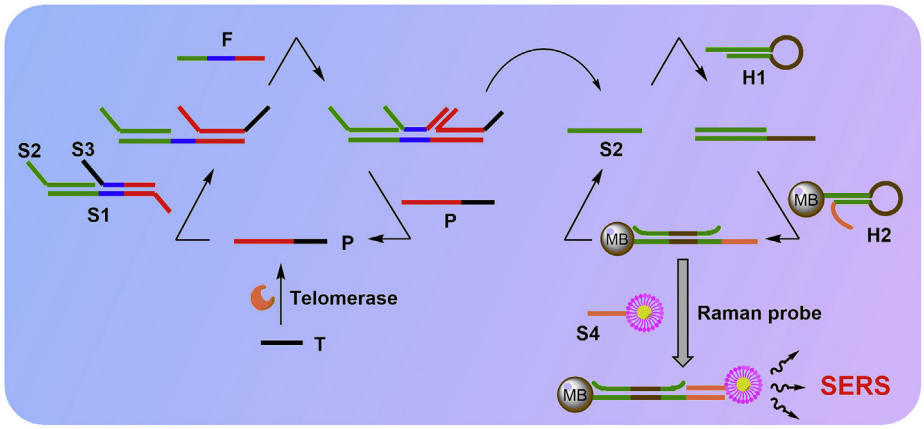
2.6. SERS detection

1.5 μL of the resulted MBs was casted onto the surface of gold film and air-dried at room temperature before Raman analysis. The Raman spectra were measured at 633 nm excitation laser with 50 × objective lens (laser power of 5 mW, and the acquisition time of each spectrum was 5 s). The experiments were carried out in triplicate and the spectra were obtained from three different locations of each sample.

3. Results and discussion

3.1. Principle of the elongation-based DNA-catalytic amplification strategy for telomerase assay

The working principle of the proposed method for telomerase activity detection is shown in Scheme 1. In the presence of telomerase and nucleotide mixture dNTPs, the telomerase substrate (TS) primer (**T**) elongates and generates a long single-strand DNA (ssDNA, **P**) containing the telomere repeat units (TTAGGG)n. As a catalyzer of the entropy-driven circuit reaction (EDCR), ssDNA **P** can recognize the



Scheme 1. Illustration of the elongation-based DNA-catalytic amplification strategy for telomerase assay.

three-strand DNA complex (**S1**, **S2** and **S3**) and bind to the toehold region at 3′-terminus of **S1**, displacing **S3** by toehold-mediatedstrand displacement reaction (TSDR). Meanwhile, a new single-stranded toehold is exposed in the middle of **S1**, which subsequently hybridizes with the single-stranded fuel (**F**) and initiates the second TSDR, releasing **P** and **S2**. Then the ssDNA (**P**) catalyzes another cycle of entropy-driven circuit reaction (EDCR) and **S2** is ingeniously used as the catalyst of MB-assisted catalytic hairpin assembly (CHA). **S2** can hybridize with the hairpin DNA (**H1**) and the stem of **H1** will be unfolded to expose the concealed domain, which is complementary with the 3′-terminal of **H2** immobilized on the surface of MB. The binding between **H1** and **H2** will form specific MB-H2/H1 complexes and replace the hybridized **S2** to trigger a new cycle of MB-assisted CHA. As a result, tremendous MB-H2/H1 complexes will be formed and the Raman probes can be anchored on the surface of MBs by the hybridization of the capture DNA (**S4**) and the exposed sticky end of **H2**. Ultimately, the detection of telomerase activity can be transformed into the SERS intensity of Raman probes.

3.2. Feasibility of the method for the assay of telomerase activity

For assessing the feasibility of our method for the assay of telomerase activity, a series of control experiments were performed based on the Raman signals of Rox reporter on the Raman probes. As could be seen from Scheme 1, in the absence of telomerase extract, the TS primer could not extend to form the long single strand DNA (**P1**), so the cycle amplification reaction could not be initiated and the Raman signal was hardly observed (Fig. 1, curve a). Similarly, the Raman signals were also very low if the telomerase extract was pretreated with RNase or heat which could destroy the essential RNA template and reverse transcriptase protein of telomerase (Fig. 1, curve b and c). On the contrary, the Raman intensity was greatly raised in the presence of telomerase extracts from 500 HeLa cells. The results confirmed that the Raman signals were dependent on telomerase activity and the newly designed approach is feasible. As well known, TS primer is essential for telomere extension reaction. So more control experiments were conducted to further ensure reliability and the corresponding results were shown in Fig. S3 (supplied in Supporting Information).

3.3. SERS detection of telomerase activity in cancer cells

Under the optimal experimental conditions (Optimization of the reaction time was supplied in Supporting Information and the result was shown in Fig. S4), telomerase activity from HeLa cell extracts was detected to validate the sensitivity of the proposed strategy and the Raman signals in response to different number of HeLa cells were evaluated. As shown in Fig. 2A, the characteristic Raman scattering

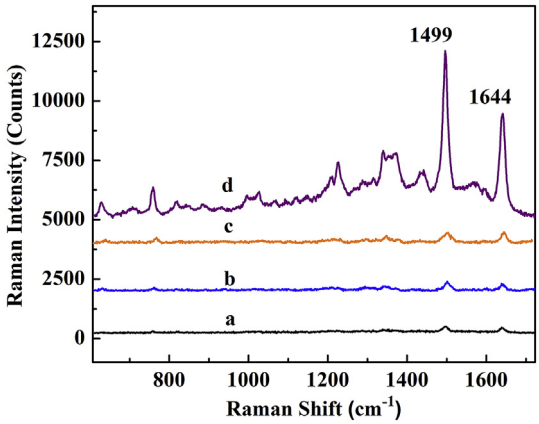


Fig. 1. SERS specra obtained by a series of control experiments: (a) lysis buffer control, (b) RNase-inactivated control for 1000 HeLa cells, (c) heat-inactivated control for 1000 HeLa cells, and (d) telomerase extract originating from 1000 HeLa cells.

peaks of Rox (1499 cm^{-1} and 1644 cm^{-1}) increased with the number of HeLa cells and the integrated peak areas of 1499 cm^{-1} were used for quantifying the number of HeLa cells. As shown in Fig. 2B, the relative peak areas of 1499 cm^{-1} (ΔA_{1499}) display a linear relationship with the logarithm of the number of HeLa cells from 1 to 1000 with a correlation coefficient of 0.994. The regression equation is $\Delta A = 71350 \lg N + 21564$ (ΔA is the integrated peak areas of 1499 cm^{-1} subtracting the blank and N is the number of HeLa cells). The results clearly indicated that the telomerase activity even in one HeLa cell can be sensitively detected. Then a series of eleven repetitive measurements of telomerase activity in 10 HeLa cells were used for estimating the precision, and the relative standard deviation (RSD) was 5.7%, which showed that the enzyme-free method had good reproducibility.

3.4. Specificity of the method

As there are some uncertain interference in the cancer cell extract, the specificity of this method for telomerase activity detection was evaluated and several interferents were used in this experiment including lysis buffer, Klenow DNA polymerase, T7 RNA polymerase and lysozyme. From the results shown in Fig. 3, it coule be seen that a significantly higher Raman signal was observed in the presence of telomerase, Klenow DNA polymerase, T7 RNA polymerase and lysozyme had no obvious Raman enhancement compared with that of the lysis buffer. Even when mixed with Klenow DNA polymerase, T7 RNA polymerase and lysozyme, the Raman signal had no significant change compared with that of telomerase, which indicated that these proteins

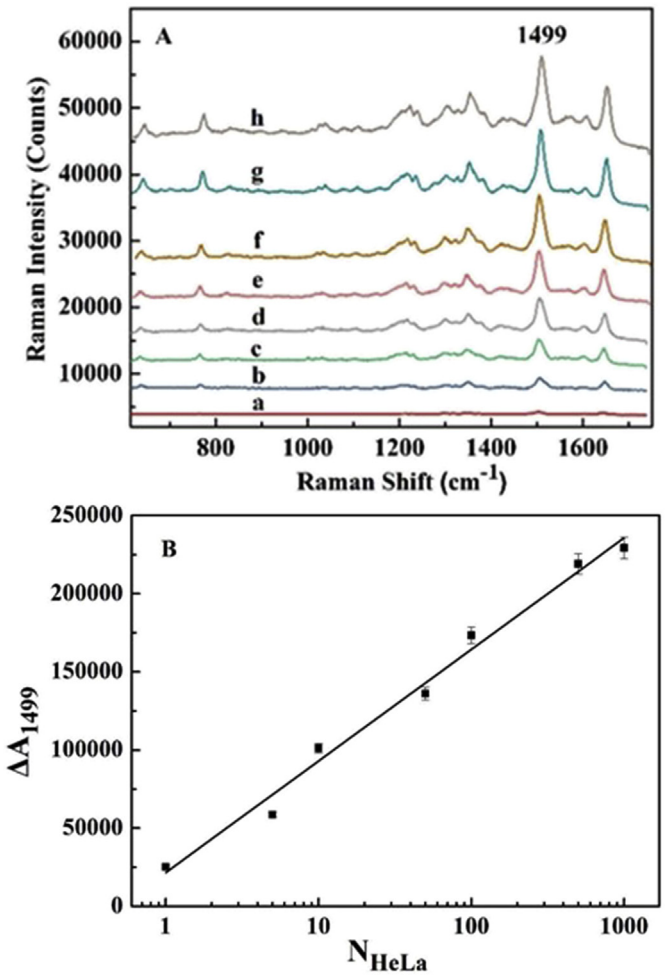


Fig. 2. (A) Raman spectra in response to different number of HeLa cells: (a) 0, (b) 1, (c) 5, (d) 10, (e) 50, (f)100, (g) 500, (h)1000. (B) The calibration curve of the relative integrated peak areas of 1499 cm^{-1} versus the number of HeLa cells. Error bars showed the standard deviation of three experiments.

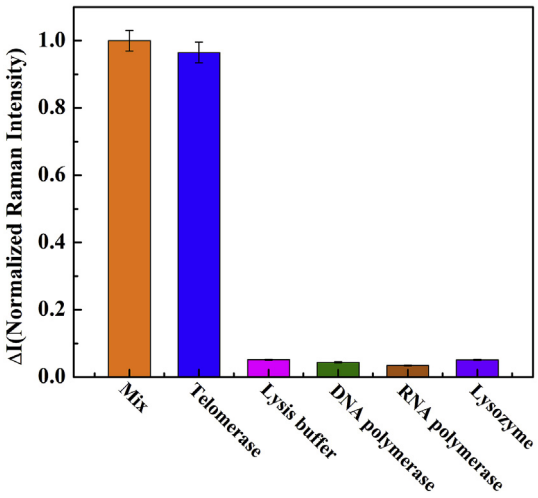


Fig. 3. Specificity of the proposed method for telomerase activity detection.

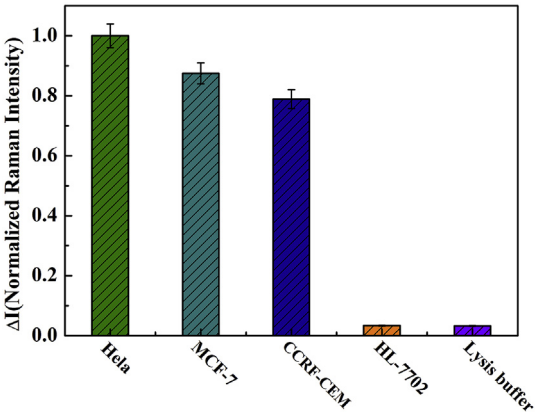


Fig. 4. Comparison of telomerase activity of different cell lines with lysis buffer as the control group.

could not interfere with telomerase analysis and the proposed protocol exhibited satisfactory specificity.

3.5. Inhibition assay

Telomerase plays an important role for cancer cell survival, so it has become an attractive target for cancer therapeutics and screening of inhibitors for telomerase receives more and more interest. In this research, for checking whether our sensitive method can be employed for screening of telomerase inhibitors, some well-characterized telomerase inhibitors, AZT and EGCG8 (Min et al., 2017) were investigated. From Fig. S5 (supplied in Supporting Information), it could be seen that both AZT and EGCG could distinctly inhibit the telomerase activity with the concentration of 1 mM. These results indicate that the proposed method has the potential for screening new inhibitors of telomerase activity.

3.6. Practicability evaluating of telomerase activity

For evaluating the universality of the sensing platform for telomerase detection, we challenged our system with different cell lines such as the human cervical cancer cell line (HeLa), human breast cancer cells (MCF-7), acute lym phoblastic leukemia T-cells line (CCRF-CEM) and human normal liver cell line (HL-7702). As shown in Fig. 4, the cell extract of HeLa, MCF-7 and CCRF-CEM showed positive Raman signal, which was consistent with the previous results of telomerase over-expression in human cancer cells (Blasco, 2005; Wu et al., 2012, 2014). However, normal cell and the heated extract generated very low signals because of the low level of telomerase activity. The above results indicate that this method has great potential for discriminating the telomerase levels in different cell lines.

For assessing the accuracy of this assay, our SERS method was further compared with the ELISA Kit analysis using the telomerase extracted from different cells (HeLa, MCF-7 and HL-7702 cells, 10^4/μL, Commercial telomerase activity detection based on SERS method and Telomerase acivity assay based on commerical ELISA kit were supplied in Supporting Information and the results were shown in Fig. S6 and Fig. S6). As shown in Fig. 5, the results obtained by the SERS assay showed an acceptable agreement with those obtained by ELISA Kit analysis, which indicated that this method holds a great promise for practical applications in telomerase activity detection with great veracity and reliability. The detailed description and data about ELISA Kit analysis were added in the Supporting Information.

4. Conclusions

In summary, a novel DNA-catalytic amplification method has been developed. Coupled with SERS technique, highly sensitive detection of

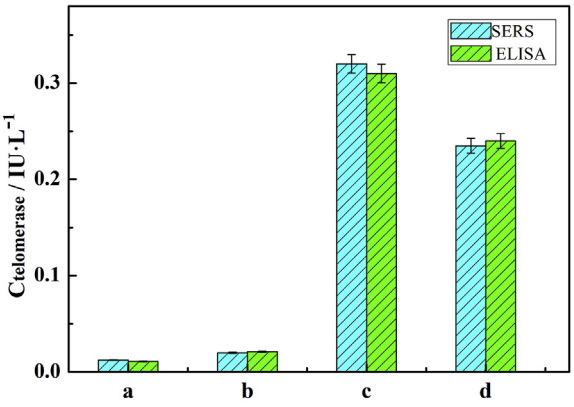


Fig. 5. Concentrations of telomerase in different sample cells measured by SERS and ELISA. (a) Lysis buffer, (b) HL-7702 cell, (c) HeLa cell and (d) MCF-7 cell.

telomerase activity in cancer cells can be detected even at the single-cell level. Compared with other methods, this assay shares several distinct advantages. Firstly, the product of telomere elongation is ingeniously used as the catalyst of entropy-driven circuit reaction (EDCR), which releases another ssDNA and triggers the magnetic beads (MBs) assisted-catalytic hairpin assembly (CHA). Due to the dual cycle amplification, the sensitivity of this method is distinctly improved. Meanwhile, using DNA as catalyst, this approach does not require other protein enzymes and complicated thermal-cycling procedure, making the proposed strategy much facile and cost-effective. Additionally, by the assistance of MBs, the Raman probes can be easily aggregated on the surface of MBs and excess reagents can be removed by magnetic separation, circumventing the high background of this system. Given the attractive analytical characteristics, this mehtod provides a favorable analytical tool for telomerase-related cancer diagnosis and drug screening.

Conflict of interest

This manuscript is solely the work of the authors, and has not been submitted in whole or in part elsewhere.

Declaration of interests

- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111543.

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