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Non-invasive diagnosis of bladder cancer by detecting telomerase activity in human urine using hybridization chain reaction and dynamic light scattering

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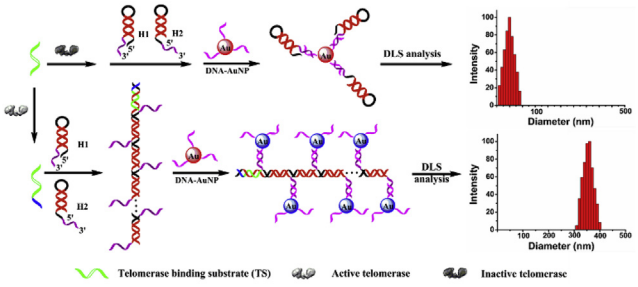
HIGHLIGHTS

- A non-invasive method for the diagnosis of bladder cancer was developed by determinating the telomerase activity in human urine.
- The telomerase activity was estimated based upon hybridization chain reaction and dynamic light scattering.
- The proposed method had a dynamic range of 10–1000 cells, and a detection limit of 4 MCF-7 cells.
- The proposed method had a high specificity for non-invasive diagnosis of bladder cancer.

GRAPHICAL ABSTRACT



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ABSTRACT

Cystoscopy and histology are the gold standards for detection of bladder cancer. However, these methods are highly subjective, expensive, and invasive. We have developed a non-invasive method for the diagnosis of bladder cancer by detecting telomerase activity in human urine. Telomerase substrate (TS) primer is elongated with repeating sequences of (TTAGGG)n in the presence of telomerase. The elongated primer can trigger hybridization chain reaction between two hairpins H1 and H2, result in the aggregation of AuNPs due to the hybridization between the tail sequence on H1 (or H2) and DNA-AuNPs probe, and accompany with the increase of hydrodynamic diameter of AuNPs, which can be measured with dynamic light scattering (DLS). The biosensor displayed a detection limit of 4 MCF-7 cells (a signal-to-noise ratio of 3) and a dynamic range of 10–1000 cells. Moreover, only urine specimens from bladder cancer patients induced a significant change in the average hydrodynamic diameter, indicating its specificity for the non-invasive diagnosis of bladder cancer.

1. Introduction

Bladder cancer, which is a very aggressive malignant tumor, is the fourth most common malignancy in men and the ninth in women [1]. Urine cytology, cystoscopy, and histology are the

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commonly used approaches for detecting and monitoring of bladder cancer [2,3]. However, these methods are highly subjective, expensive, and invasive and are often unable to reveal low-grade urothelial bladder carcinomas (UBC) at the first stage of transformation (urine cytology) or bladder cancer which remain below the mucosal surface (cystoscopy) [4]. Therefore, development of an inexpensive, non-invasive, reliable, and simple test for diagnosis of bladder cancer is of utmost importance. In recent years, there are many research in cancer diagnostics [5], and some non-invasive tests performed on voided urine have been developed, these tests include estimation of telomerase activity levels by telomeric repeat amplification protocol (TRAP) assay [6,7], detection of DNA damage in exfoliate urinary cells by comet assay [8], and analysis of urinary DNA methylation [9]. However, these biomarkers are yet not to be validated in subjects who are at risk of developing bladder cancer.

Telomerase is a ribonucleoprotein complex which maintains the length of the telomere by adding telomeric repeats (TTAGGG)n onto the end of telomere using its intrinsic RNA template, reverse transcriptase, and associated protein [10,11]. Telomerase activity is inhibited in normal human cells and telomeres are progressively shortened after each replication cycle, resulting in cell senescence and death [12]. However, telomerase is reactivated and overexpressed in over 85% of malignant cancer cells, allowing them to survive and proliferate uncontrollably [13,14]. Therefore, telomerase has been considered as a potential biomarker and as a promising therapeutic target for cancer [15]. Sensitive methods for the detection of telomerase activity is significant for cancer diagnosis, screening of anticancer drugs, and evaluation of cancer therapy [16]. Currently, PCR-based TRAP is the most widely used method for the detection of telomerase activity [17]. However, TRAP is a tedious process which involves sophisticated manipulation and may produce false signal [18]. To address these issues, alternative methods like colorimetry [19–21], fluorescence [22], chemiluminescence [23], fluorescence imaging [24], electrochemistry [25,26], and surface plasmon resonance have been developed [27]. Although these methods have shown varying extent of success, the development of a PCR-free amplification strategy for further improvement of sensitivity is still an urgent need.

Among a variety of strategies used for nucleic acid amplification, hybridization chain reaction (HCR) has attracted considerable attention due to its excellent capability for isothermal amplification [28,29]. HCR is an enzyme-free process in which the polymerization of oligonucleotides can form nicked double helices as a result of a cascade of hybridization events triggered by an initiator. Using various probes, HCR has been applied in developing colorimetric [30], fluorescence [31], chemiluminescence [32], and electrochemical biosensor [33]. Au nanoparticles (AuNPs), one of the most commonly used nanomaterials, have been employed to construct a sensing platform, due to their large specific surface area, unique optical properties and good biocompatibility [34]. Lately, HCR-based optical [35,36] and electrochemical [37,38] sensors were developed with unmodified gold nanoparticles because of its high speed, better sensitivity, and low cost. DNA-functionalized AuNPs probes are immune to interference. Hence, these probes have been used to establish HCR-based colorimetric sensor for DNA, microRNA, ATP and acetylcholinesterase activity [39,40].

Dynamic light scattering (DLS) is a common optical technique for measuring the size of particles with diameters ranging from 0.5 nm to 6 μm [41]. The range of size of most nanomaterials is within the ideal detection range of DLS. Hence, it has become an important tool in nanotechnology research [42]. Moreover, AuNPs have a strong light scattering property, which makes it an ideal probe for the development of biosensors. Thus, a series of DLS sensor for DNA [43], biomarker [44], protein [45,46], glucose [47], and Pb^{2+} [48] were established. Here, we propose a new AuNP-based DLS assay for sensitive detection of telomerase activity in human urine using HCR. On addition of telomerase, the telomerase primer sequence (TS) is catalyzed and elongated to form telomeric repeats, which can act as the initiator and trigger the HCR process between two hairpin probes (H1 and H2) to form long nicked dsDNA. The HCR products thus obtained can hybridize with DNA-AuNPs probe, causing an aggregation of AuNPs, resulting in a dramatic increase in particle size, which can be measured using DLS.

2. Experimental

2.1. Materials

DNA oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The deoxynucleotide solution mixtures (dNTPs), BSA, RNase inhibitor, and 1 × CHAPS lysis buffer were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Fresh urine samples were collected from Sun Yat-Sen University Cancer Center, Guangzhou China. PBS (10 mM, pH 7.4, with 0.1 M NaCl) was used in this experiment.

2.2. Instrumentation

The transmission electron microscopy (TEM) images of the AuNPs were captured using JEM-3010 transmission electron microscope. Samples for TEM characterization were prepared by adding a drop of colloidal solution on a carbon-coated copper grid and drying at room temperature. Dynamic light scattering (DLS) tests were performed on EliteSizer nanoparticle/Zeta potentiometer (Anton Paar Ltd., Austria) at room temperature. The images of gel electrophoresis were scanned by the Gel Image Analysis System (JY02S, Beijing, China). pH values were measured by a pHs-3E meter.

2.3. Cell culture and telomerase extraction

The way of telomerase extraction from MCF-7 cells and Urine Samples was according to the literation [19]. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The cells were harvested with trypsin and 7×10^6 cells were collected. The cells were suspended in a 1.5 mL EP tube, washed twice with ice-cold PBS by centrifugation at 1000 rpm for 5 min, and resuspended in 200 μL of ice-cold CHAPS lysis buffer. The lysate was incubated on ice for 30 min and was centrifuged at 12000 rpm for 20 min at 4 °C. The extract was stored at −80 °C until further use.

Fresh urine samples were collected, centrifuged at 850 rpm for 10 min at 4 °C, and washed once using PBS. The samples were centrifuged at 2300 rpm for 5 min at 4 °C. The precipitate was resuspended in 200 μL of ice-cold CHAPS lysis buffer, incubated on ice for 30 min and was centrifuged at 12000 rpm for 20 min at 4 °C, and the supernatant was also stored at −80 °C for use.

2.4. Preparation of DNA-functionalized gold nanoparticles

The DNA-AuNPs probe was prepared according to the literature and purified with centrifugation [39]. Firstly, AuNPs were prepared by reducing 1.0 mM HAuCl4 (20 mL) with 38.8 mM sodium citrate (2.0 mL). The DNA-AuNPs probe was prepared as explained below. Thiolated DNA was dissolved in 10 mM acetate buffer solution (60 μL, pH 5.0), Then, 20 mM TCEP (12 μL) was added to the solution, and the mixtures was incubated for 1.5 h. Subsequently, 10 mL AuNPs (10.6 nM) was added and the resultant mixture was stored at 25 °C for 16 h. The mixture was aged for 44 h with a gradual addition of NaCl until a final concentration of 0.1 M was reached.

Finally, the solution was centrifuged thrice at 13800 rpm for 30 min each to remove excess reagents and free DNA, and the red oily precipitate that contained the AuNPs-DNA probe was washed with 10 mM of PBS (pH 7.4, with 0.1 M NaCl) each time. The AuNPs-DNA precipitates were dissolved in the same buffer and stored at 4 °C before use.

2.5. Telomerase substrate extension

The cell extracts were first diluted to form suspensions with different cell densities using lysis buffer. The solution that contained telomerase extract, 1.5 μM TS primer, 1.0 mM dNTPs, 0.2 mg/mL BSA, and 0.4 U/μL RNase inhibitor was incubated at 37 °C for 3 h, and was further inactivated by heating at 95 °C for 5 min.

2.6. HCR and gel electrophoresis

H1 and H2 were heated to 95 °C for 5 min and then allowed to cool to room temperature before use. The solution that contained H1 (1.0 μM), H2 (1.0 μM), and telomerase extension products was incubated at 37 °C overnight. Agarose gel (12%) was prepared using 0.5 × Tris-borate-EDTA (TBE) buffer. The electrophoresis was performed at 120 V for 70 min in 0.5 × TBE buffer and was then scanned using the gel image analysis system.

2.7. DLS measurements

HCR mixture (5 μL) was added to 45 μL of PBS containing 4 nM AuNPs-DNA probes and incubated at 30 °C for 4 h. The mixture was detected by DLS at 25 °C with an equilibration time of 10 s and measurement angle of 90°. All sizes reported here were based on the intensity of particle size distributions, and each reported particle size was the average of three times measurements.

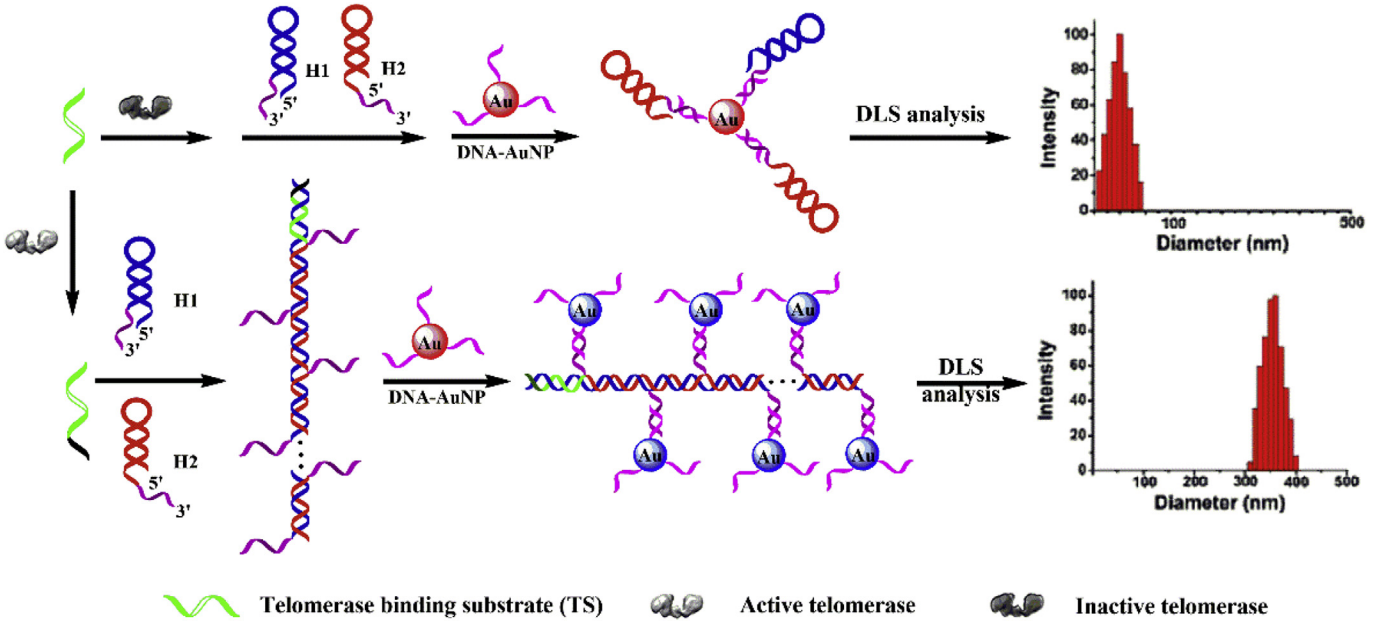
3. Results and discussion

3.1. The principle of telomerase activity assay

The strategy for detection of telomerase activity is illustrated in Scheme 1. HS-5′-AAA AAA AAA AAA AAA AAA AA was designed to modify the AuNPs and act as DNA-AuNPs probe. Oligonucleotide 5′—CCC TAA CCC TAA CTC TGC TCGA AAG AGA TCG AGC AGA GTT AGGG TT TTT TTT TTT TTT-3′ (H1) and 5′-TCG AGC AGA GTT AGGG TTA GGG CCC TAA CTC TGC TCGA TCT CTT TT TTT TTT TTT TTT-3′ (H2) were designed for the study. These oligonucleotides had two parts-the sequences that are underlined were designed for HCR while the remaining 14 bases were designed to hybridize with DNA-AuNPs probe. Oligonucleotide 5′-AAT CCG TCG AGC AGA GTT-3′ was used as TS primer, and it does not trigger the HCR between H1 and H2 directly. Hence, DNA-AuNP probe remains detached irrespective of its hybridization to H1 (and H2). The TS primer is elongated in the presence of telomerase and dNTPs. The elongated primer initiates HCR between H1 and H2 to form long nicked dsDNA. The products of HCR can hybridize with AuNPs-DNA probe and lead to an aggregation of gold nanoparticles, which can be measured by DLS technique.

3.2. Feasibility of telomerase activity assay

To demonstrate the practicability of telomerase activity assay, proof-of-principle experiments were carried out. Firstly, this method was based on the occurrence of HCR process. To gain clarity on this, we used polyacrylamide gel electrophoresis (PAGE) to investigate the HCR process. As shown in Fig. 1A, in the absence of telomerase, the TS primer could not be elongated and no HCR product was formed (Fig. 1A, lane 2). In the presence of telomerase, the TS primer was elongated and prolonged DNA products were observed (Fig. 1A, lane 3), accompanied by an attenuation of the band for TS primer, indicating the occurrence of HCR. The color of the solution containing a mixture of AuNPs, TS primer, H1 and H2 was red, indicating that no HCR had occurred and that the AuNPs were dispersed well. While it changed to purple with the addition of telomerase, revealing the occurrence of HCR between H1 and H2 (Fig. 1B). The change in size of AuNPs was measured with DLS as shown in Fig. 1C/D. The average size of AuNPs in the control sample (without active telomerase) was approximately 56.4 nm, while it increased to 347.5 nm upon addition of active telomerase that was extracted from 10000 MCF 7 cells, revealing that the aggregation of



Scheme 1. Schematic illustration of telomerase activity assay by DLS.

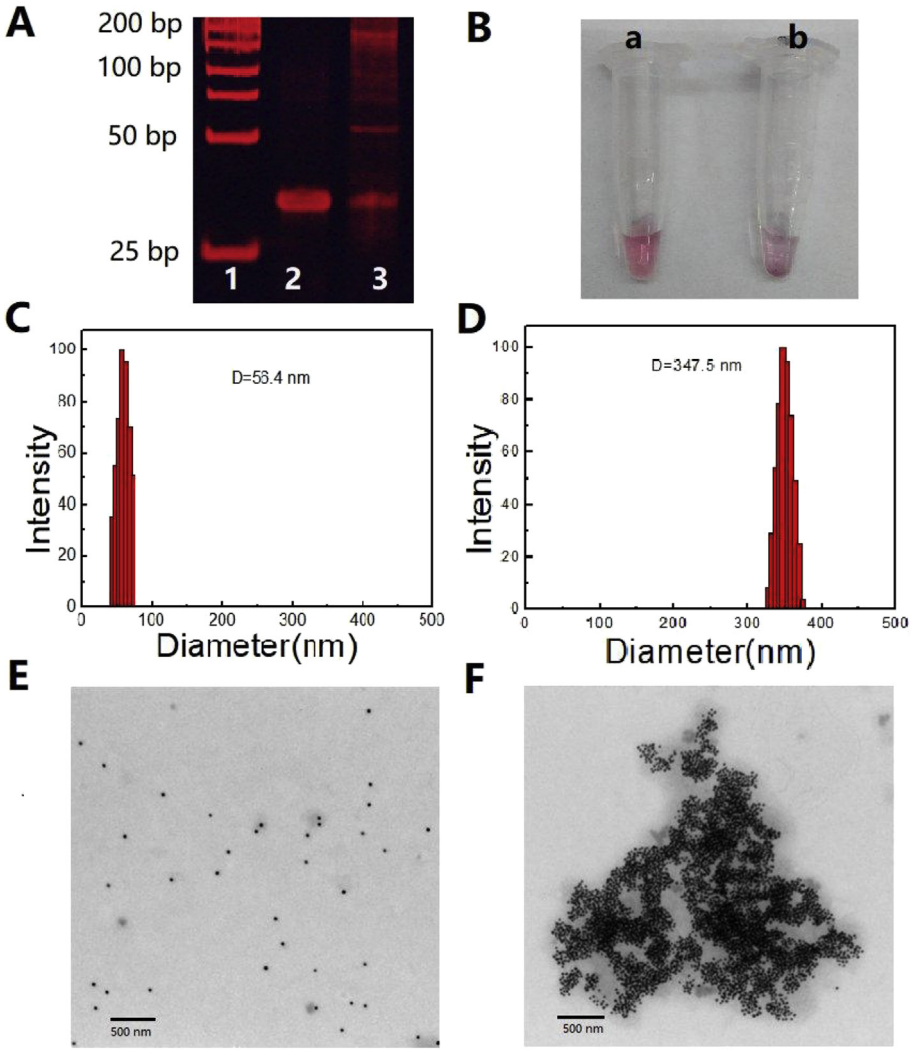


Fig. 1. (A) Polyacrylamide gel electrophoresis images of HCR products. Lane 1: the DNA marker; Lane 2: TS primer + H1 + H2; Lane 3: elongated TS primer + H1 + H2. (B)Color change of AuNPs in the presence of inactive (a)/active(b) MCF-7 cells. Diameter and TEM image of AuNPs in the presence of inactive (C, E)/active (D, F) MCF-7 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

AuNPs was triggered by the addition of active telomerase. TEM images in Fig. 1E/F further confirmed that the aggregation of AuNPs was induced by telomerase, and that it was in tune with the change in size distribution. These results demonstrated that the TS primer was extended by telomerase, which triggered the occurrence of HCR between H1 and H2, resulting in the aggregation of AuNPs, causing an increase in the average hydrodynamic diameter.

3.3. Optimization of detection conditions

In order to achieve high accuracy and sensitivity, some main factors should be strictly controlled at this experiment, so the parameters that have a direct influence to the results of the experiment have been optimized. As shown in Fig. 1A, the temperature and time for telomerase catalyzed extension were optimized, the diameter of the particles by detection system obviously changed with the increase of incubation temperature. Considering the change of background, 37 °C was selected as the incubation temperature for the telomerase catalyzed extension. Then the reaction time for telomerase catalyzed extension was investigated, as shown in Fig. 2 B, with the prolongation of the incubation time, the diameter of the particles was increased at first and then reached a plateau at 3 h, which was chosen as the optimal incubation time. HCR could also significantly affect the detection results of DLS, as shown in Fig. 2C, the best efficiency was obtained at 37 °C, so 37 °C of HCR temperature was selected. Fig. 2 D showed the effect of HCR time on the detection results, when the incubation time exceeded 12 h, the diameter of the particles was gradually levelled off, then 12 h was selected as HCR time. Moreover, aggregation temperature and time of AuNPs affect the particle size as well. When the temperature increased as presented in Fig. 2 E, the diameter of the particles was increased, but there is a high background signal at 40 °C, so 30 °C can be the appropriate aggregation temperature. And the diameter of the particles was increased with the aggregation time, which was shown in Fig. 2 F, in order to get a higher signal and lower background, 4 h was confirmed as the aggregation time. The length of H1 and H2 were optimized (Fig. S1), 58 bases of each hairpin were used for the subsequent research.

3.4. Telomerase activity assay by DLS

Fig. 3 shows the response curves of the DLS sensing system using cell extracts from different number of MCF-7 cells. It indicated that the average hydrodynamic diameters of AuNPs increased

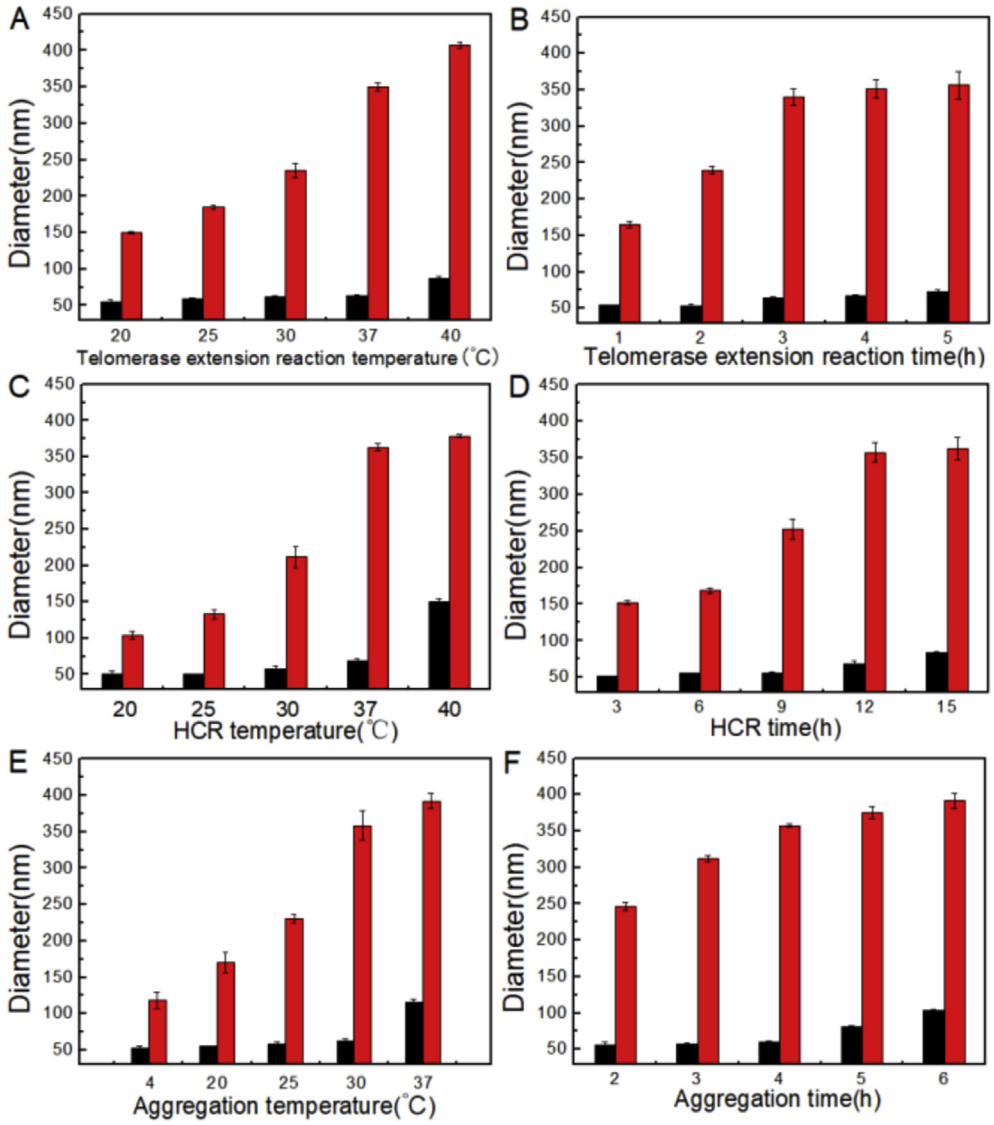


Fig. 2. Effects of (A) the telomerase extension reaction temperature, (B) the telomerase extension reaction time, (C) the HCR temperature, (D) the HCR time, (E) the aggregation temperature, (F) the aggregation time on the performance of the sensing system. Black columns: control experiments; Red columns: with 10000 MCF-7 cells. Error bars show the standard deviations of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

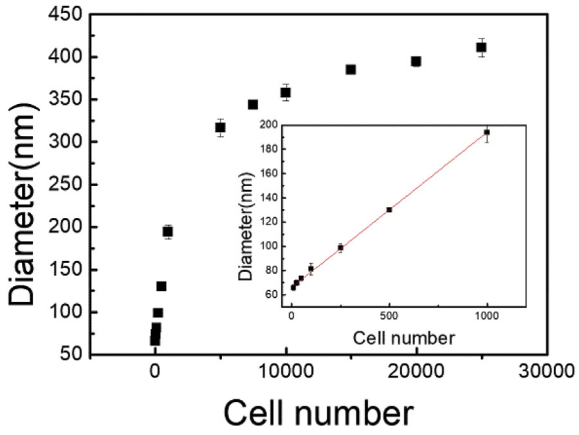


Fig. 3. The relationship between the average diameters of AuNPs and the telomerase extracts from different numbers of MCF-7 cells. Insert: standard curve for cell number ranging from 10 to 1000. Error bars show the standard deviations of three experiments.

with the increasing concentration of telomerase. At higher concentration, telomerase could recognize and elongate more TS primers. This resulted in an increased hybridization of HCR products with the AuNP probes, leading to a higher DLS signal. The inset depicts the linear relationship between the average diameters of AuNPs and the cell numbers ranging from 10 to 1000 with a correlation coefficient (R^2) of 0.9992. The detection limit was estimated to be 4 MCF-7 cells, which was calculated from three times of the signal-to-noise ratio divided the slope of standard curve, it was comparable with or even better than most of the telomerase assays reported previously [49,50].

3.5. Detection of telomerase activity in urine sample

The composition of the urine is complex and it may inhibit or enhance the activity of some enzymes. The complex components present in urine can also interfere with the experiment by affecting the chemical reactions that are involved in the sensor. To overcome the effects of complex composition of urine, we established a

sensor for the detection of telomerase activity based on HCR and DLS, which was enzyme-free and included no chemical reactions. There are many components in urine, and hence, it would be unscientific to investigate the effect of individual component. Therefore, the addition and recovery experiment was carried out in urine sample to investigate the feasibility and reliability of the sensor. As shown in Table 1, when telomerase extracted from 250 to 1000 cells was added to the human urine samples, the recovery varied from 94.8% to 100.8%, and the relative standard deviation values were in the range of 1.2%–3.4%. This indicated that the method had good feasibility and reliability in human urine and that it might be used for the detection of telomerase activity in urine sample.

Telomerase activity can be detected in 85%–95% of tumor cells. When the cancer cells die, the telomerase that is produced by the cells is secreted into the blood. Hence, it is difficult to diagnose cancer based on the telomerase activity in blood. However, it is difficult for telomerase to breakthrough multiple walls of glomerulus and other organs to enter into urine. Telomerase that is produced by brain tumors is blocked by cerebrospinal fluid (CSF), making it more difficult to enter into the urine. Therefore, telomerase from most cancers do not enter into urine. Bladder cancer cells drop into the urine directly, and hence, telomerase activity in urine can be used as an important criterion for diagnosis of bladder cancer.

To explore the application of the proposed method, three cases were measured for each cancer. Urine samples were collected from normal individuals and from patients suffering from bladder cancer, liver cancer, gastric cancer, lung cancer, and prostate cancer. The DLS signal for each case is shown in Fig. 4. In samples from normal human, the DLS signal was closer to that of the control sample. This was similar to that seen in the addition and recovery experiments, indicating that the proposed method can be applied to the complex sample of urine. In samples from patients with bladder cancer, the diameter was higher than normal human dramatically, where as in case of samples from patients with other cancer, the diameter was almost similar to that of the normal human. These results indicated that the telomerase from patients with liver cancer, gastric cancer, lung cancer, and prostate cancer did not enter into the urine, and that patients with bladder cancer might be diagnosed through the level of telomerase activity in their urine. Therefore, the proposed method can be used for non-invasive diagnosis of bladder cancer.

3.6. Compared with other methods for diagnosis of bladder cancer

Cystoscopy was invasive and costly, it was gold standard for the diagnosis of bladder cancer [51]. Conventional cystoscopy intuitively determined the location, number and the size of tumors, and it was easy to operate. It also conducted a biopsy for the lesion site to judge the tumors, and for further clinical treatment guidance. However, it had low sensitivity to the diagnosis of flat tumor, which might lead to misdiagnosis and delay the treatment [52]. In addition, fluorescence cystoscopy was developed for the diagnosis of bladder cancer as well, which was based upon the selective absorb

Table 1
Recoveries of telomerase from the spiked human urine samples. The result is the average values from three measurements.

Urine samples	Add telomerase (cells)	Recovery (%)	Relative standard deviation (%)
1	250	94.8	3.4
2	500	91.2	1.2
3	800	96.3	2.0
4	1000	100.8	3.2

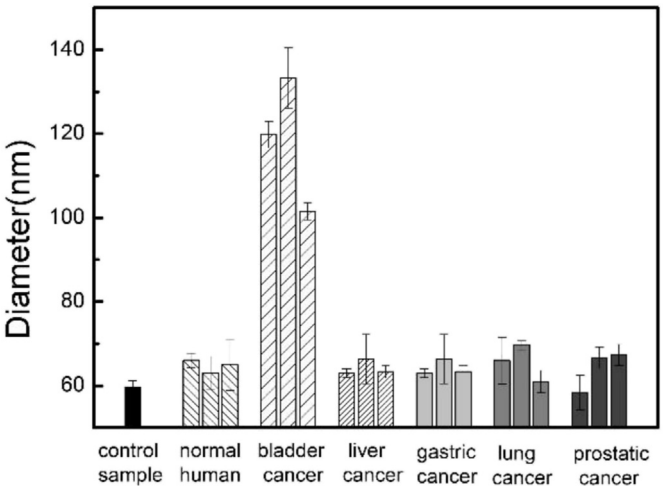


Fig. 4. Detection of telomerase activity by DLS extracted from urine samples.

of photosensitizer by tumor tissue, and demonstrated different color under the appropriate wavelength of light irradiation from normal tissue to bladder cancer [53–55]. However, the concentration of photosensitizer, the intensity of excitation light source and other factors need further investigated.

Cytology was the common noninvasive method for diagnosis, it had high specificity and high value especially in the diagnosis of carcinoma in situ. But their results depended on the level of pathologists, which might exist bias in observation, and the sensitivity was low in the diagnosis of low-grade tumors [56]. Fluorescence in-situ hybridization (FISH) technique was a noninvasive method which had high sensitivity and assisted cytology for the diagnosis of bladder cancer. But it was complex and the efficacy may be affected by some factors, which made it difficult to be popularized and be applied in grass-roots hospitals [57–60]. Microsatellite analysis the urine was another method, fluorescent PCR was used to search DNA microsatellite alterations in urine sediment for diagnosis of bladder cancer, it was rapidly and had high sensitivity, but the result was operator dependent [61]. Compared to above mentioned methods, the proposed method had high sensitivity due to the amplification of HCR and the high sensitivity of DLS technique. Moreover, it was enzyme-free and ease of operation, which avoided the effect of some complex components in urine sample.

4. Conclusion

In summary, we developed a strategy for non-invasive diagnosis of bladder cancer by detecting the telomerase activity in human urine using HCR and DLS. Due to the amplification of HCR and the sensitivity of DLS technique, this method detected telomerase activity of as low as 4 MCF-7 cells. This method is enzyme-free and does not involve any chemical reaction, thus avoiding the effect of complex components in urine. The complex components in urine had no effect on the addition and recovery experiments. Satisfactory recovery was obtained, which proved the feasibility and reliability of the proposed method. We further tested the proposed method in urine samples from normal individuals and in patients with different type of cancer. We found that urine samples from patients with bladder cancer alone produced strong DLS signal. Therefore, this study presents a simple, low cost, and highly sensitive method for the detection of telomerase activity in urine, and to apply it in non-invasive diagnosis of bladder cancer in future.

Declaration of interest statement

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.03.039.

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