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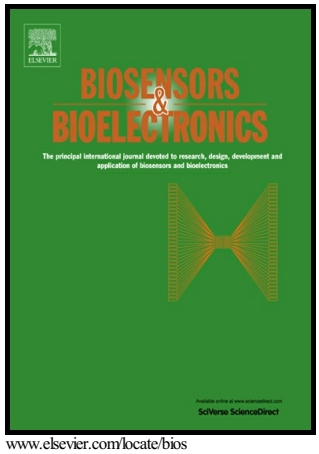

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Sensitive detection of telomerase activity in cancer cells using portable pH meter as readout

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Abstract

This study developed a portable and sensitive biosensor for the detection of telomerase activity extracted from HeLa cells by monitoring urease regulated pH change. In the presence of telomerase, the telomerase substrate (TS) primer was elongated by adding telomeric repeats $(TTAGGG)_n$ firstly. The TS primer was then immobilized onto streptavidin magnesphere paramagnetic particles (PMPs). The elongated repeat unit can hybridize with biotinylated complementary DNA (cDNA) to specifically capture biotinylated urease onto PMPs. After magnetic separation, the PMPs complex was mixed with urea solution. As urease can catalyze the hydrolysis of urea into ammonia and induce pH increasing of the solution, the activity of telomerase can be detected by pH-sensitive dyes or by a portable pH meter easily. The changed pH has a linear relationship with the logarithm of the number of HeLa cells from 50 to 10000 and with a detection limit of ~20 HeLa cells, which indicated the proposed method have potential application in telomerase-related cancer diagnosis.

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Key words: Telomerase ; HeLa cell; pH meter; bromocresol purple; Urease.

1. Introduction

Telomerase is a ribonucleoprotein that can catalyze the addition of telomeric repeats $(TTAGGG)_n$ onto the end of telomeres (Cesare and Reddel, 2010; Nakamura et al., 1997; Wang et al., 2017a), and it plays an important role in telomere maintenance and protection (Hayflick, 1965; Lemieux et al., 2016; Shay and Bacchetti, 1997). In most normal human cells, telomerase activity either is repressed or absent. In contrast, in cancer cells, such as bladder, uterine, stomach, esophageal, breast, thyroid, colorectal, laryngeal squamous cell, the activity of telomerase is prominent (Ivancich et al., 2017; Patel et al., 2004). About 85-90% of human cancer cells are found with overexpression of telomerase (Eitsuka et al., 2018; Li et al., 2010; Morin, 1989). Therefore, telomerase is considered as a common biomarker for human malignant tumors. Thus, the sensitive detection of telomerase is important for cancer diagnosis and therapy.

Many methods have been developed for telomerase activity detection. The most commonly used method is polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP). The method is sensitive but has the drawbacks such as time-consuming and susceptible to amplification related errors (Herbert et al., 2006; Kim and Wu, 1997; Weizmann et al., 2004; Zheng et al., 2005). So PCR-free methods, including fluorescence (Gao et al., 2016; Wang et al., 2015; Wang et al., 2016; Zhang et al., 2016b; Zhang et al., 2014b), electrochemistry (Liu et al., 2013; Yi et al., 2014), colorimetric assay (Zhang et al., 2016a), electrochemiluminescence (Zhang et al., 2014a), surface plasmon resonance (Qian et al., 2017) and so on, have been developed for telomerase detection. Some point-of-care testing (POCT) sensors have also been developed for telomerase activity detection. For example, Lu's group reported a portable personal glucometer-based sensing platform for quantitative monitoring of telomerase activity (Wang et al., 2014). Zhu's group constructed a portable cellulose paper microzone plate for the detection of telomerase based on colorimetry and

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up-conversion fluorescence dual-mode methods (Fu et al., 2018). Yang's group reported a POCT strategy for detection of telomerase activity using portable pressure meter as readout (Wang et al., 2017b). All these reports indicated that POCT for telomerase activity are reliable and simple.

With the characteristics of low price, high accuracy and simple operation, pH meters are widely used to measure the pH of solutions. The pH signal of the system can be semi-quantified by pH-sensitive dye or pH paper strips with the naked eye and quantified by a hand-held pH meter. Hence, the pH tests have the advantages of portable size, cost effectiveness, easy operation and reliable quantitative results. Recently, pH meters are used as readout to construct electrochemical biosensors, most of which are based on labels such as glucose oxidase (GO_x) (Zhang et al., 2016c), alkaline phosphatase(Mousty et al., 2008) or urease (Tram et al., 2014). These conjugates catalyze their corresponding substrates to generate a pH change in solution. And diverse targets, such as mycotoxin (Zhao et al., 2018), proteins (Zhang et al., 2016c), DNA sequence (Xie et al., 2014), and bacterial pathogen (Ye et al., 2016) were detected.

In this study, we first constructed a portable biosensor for telomerase activity detection using pH meter as readout. The proposed sensor combines the advantages of self-amplification of telomerase and magnetic separation. More importantly, unlike optical detection devices which need light sources, optical filters and photon detectors, pH based measurement requires only a handheld pH meter or cheap and widely available pH indicator. With the advantages of low-cost, easy-to-use, and high sensitivity, the pH-based strategy will be a very promising strategy and will be widely applied in POCT. The proposed biosensor was eventually employed to detect telomerase activity in a complex sample.

2. Experimental Section

2.1. Chemicals and Reagents

Streptavidin magnesphere paramagnetic particles (PMPs) were purchased from

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Promega Corporation (Madison, USA), Tween-20, mercaptoethanol, glycerol, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), bovine serum albumin(BSA), human serum albumin (HSA), bromocresol purple (BCP), streptavidin (SA), tris-(hydroxymethyl) aminomethane (Tris), tris-buffered saline Tween (TBST, referring to 10 mM Tris buffer, 0.15 M sodium chloride, 1% Tween-20, pH 7.5) were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Biotinylated urease, ethylene glycolbis (aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), deoxynucleotide solution mixture (dNTPs), Human cervical carcinoma cells (HeLa), and all DNA oligonucleotides used in this work were synthesized and HPLC-purified by Sangon Inc. (Shanghai, China). Human normal liver cell HL-7702, human cancer cell MCF-7 and human cancer cell SW480 were purchased from Shanghai Cell Bank, CAS (Shanghai, China). The Healthy human serum specimens were obtained from Fujian Provincial Hospital (Fujian, China). The DNA oligonucleotides sequences were listed as follows:

Telomerase substrate (TS) primer: 5'- biotin-TTTTTTAATCCGTCGAGCAGAGTT -3'

Complementary DNA (cDNA): 5'-biotin-TTTCTAACCCTA-3' (cDNA contained the complementary sequence to the extended TS telomeric repeat strand)

All other chemicals were of analytical reagent grade. All solutions were prepared with deionized water (Milli-Q, Millipore, resistance 18.2 MΩ•cm).

2.2. Extraction of telomerase

1.0×10^6 cells were dispersed into a 1.5 mL EP tube, washed twice with ice-cold PBS (0.1 M, pH 7.4). Subsequently, the cells were resuspended into 200 μL ice cold CHAPS lysis buffer (10 mM Tris-HCl, 1 mM EGTA, 0.1 mM PMSF, 1 mM $MgCl_2$, 5 mM mercaptoethanol, 0.5% CHAPS, 10% glycerol, pH 7.5), incubated on ice for 30 min, and centrifuged at 12,000 rpm for 20 min at 4 ℃, The supernatant was used immediately for telomerase assay or stored at -20 °C for further use.

For detecting the telomerase activity from directly captured cells, a certain number of HeLa cells were collected. Then, these cells were mixed with ice-cold CHAPS lysis buffer to extract the telomerase. The crude cell lysate was used immediately as

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the source of telomerase to detect the telomerase activity.

2.3. pH biosensor fabrication and telomerase activity detection

Telomerase extracts were diluted in lysis buffer with respective number of cells, and telomerase extracts (2.5 μL) were incubated with 5'-biotinylated TS primer (75 nM) in extension buffer (20 mM Tris-HCl, 1 mM EGTA, 63 mM KCl, 1.5 mM $MgCl_2$, 0.005% Tween-20, 0.1 mg/mL BSA, 1 mM dNTPs, pH 8.3) at 37 °C for 3 h to proceed the extension reaction by telomerase.

Reaction products of telomerase were directly added to PMPs (10 μL, 1 mg/mL) which was washed two times with TBST buffer (0.1 M), and this was incubated by rolling the vial for 60 min at 37 °C. The reaction mixture was separated by using magnetic racks and washed twice with TBST buffer. Then, the mixture were resuspended in the reaction buffer (0.75 μM biotinylated cDNA, 50 mM Tris-HCl, 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, pH 7.4) for 1.5 h at 37 °C, the mixture was shaken to realize the hybridization between the cDNA and elongated telomeric repeats. After magnetic separation and washing, the mixture was added to SA (1 μg/mL) diluted in Tris-HCl buffer for incubation (45 min, 37 °C). Then, the mixture was washed three times with TBST buffer to remove unbound SA, and biotinylated urease (1 μg/mL) diluted in Tris-HCl buffer was added to the mixture for another 45 min at 37 °C. Afterwards, the mixture was washed six times with TBST buffer and then further washed twice with pure water to reduce the nonspecific binding.

Finally, 100 μL of the urea solution (0.1 M urea, 0.128 M NaCl, pH 5.95) was added to each tube. After reaction 10 min at 37 °C, urea solution was carefully transferred into a new centrifuge tube, the pH signal of the system can be semi-quantified by pH-sensitive dye or quantified by a hand-held pH meter.

2.4. Telomerase activity Detection in human serum

Telomerase extracts, dNTPs and 5'-biotinylated TS primer were added into human serum. The mixture was incubated at 37 °C for 3 hours to proceed the extension reaction by telomerase. The following process of telomerase activity detection was same as described above.

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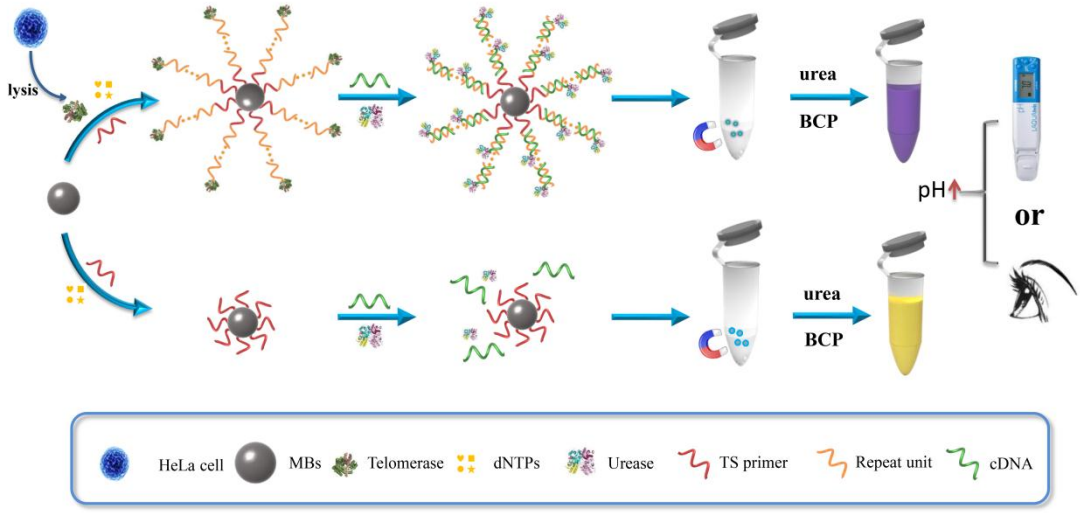
3. Results and Discussion

3.1. Principle of the portable pH biosensor for telomerase activity assay

The principle of the portable pH biosensor is illustrated in Scheme 1. In the presence of telomerase and dNTPs, the biotinylated TS primer could be elongated by adding numerous telomeric repeats TTAGGG at the 3' end by telomerase extracted from cancer cells. Subsequently, PMPs were added to the solution. Because of the specific recognize between biotin and streptavidin, the telomerase reaction products could bound with PMPs tightly. Then, elongated telomeric repeatedly hybridized with cDNA, because the 3' end of cDNA was modified with biotin, the biotinylated urease could conjugate with cDNA with the help of SA through biotin-avidin-biotin linkage and could be captured specifically onto the PMPs. After magnetic separation and washing, the unconnected urease was removed. And then the PMPs complex was mixed with urea solution. Urease can catalyze hydrolysis urea into ammonia, resulting in an obvious increased pH signal of the system. In the absence of telomerase, TS primer could not be extended. So the pH value of system had no significant change because nearly no urease was linked on the PMPs. Thus, no obvious pH change would



Scheme 1 The principle of the portable pH biosensor based on the self-amplification of telomerase and magnetic separation for telomerase activity assay in HeLa cells.

be detected. The change of the pH value can be detected by pH indicator or pH meter easily. On the basis of this principle, the activity of telomerase can be detected by pH-sensitive dye with the naked eye or by a portable pH meter. As the ΔpH has a relationship with the activity of telomerase, a portable biosensor for telomerase activity analysis can be developed.

A simple control assay was performed to test the feasibility of the proposed mechanism. BCP is a suitable pH indicator for the detection of urease activity (Chandler et al., 1982; Schussel and Atwater, 1995). When pH ranges from 5.8 to 7.5, BCP can show a vivid colour change from yellow to purple (Lvov et al., 2001), so BCP was chosen to check the pH changing of the system in this study. ed. As shown in Fig. 1A, when all components existed (column a), the pH value of the system enhanced to 6.6 in 10 min and the color of the reaction mixture turned into purple after the addition of BCP, which can be distinguished by human eyes easily. For comparison, we monitored the pH signals of the detection mode in the absence of telomerase (column b), TS primer (column c), cDNA (column d), streptavidin (column e) and urease (column f). As shown from column b to f, the pH value of the system was almost unchanged (about 5.9), and the colour of the solution was yellow if BCP was added. These results indicat that all components including TS primer, cDNA streptavidin and urease are specifically capturing onto PMPs virtually and the influence of the nonspecific adsorption can be ignored. To further verify the feasibility of the proposed system, gel electrophoresis was applied to analyze the reaction products. As shown in Fig. 1B, no bands were observed when the TS primer was mixed with CHAPS lysis buffer (Lane 1). However, an obvious smear appeared when the TS primer was incubated with HeLa cells extracts (Lane 2). The reason lies in that the molecular weight of products is different but not an exact numerical value. These results manifest that various lengths of DNA strands are generated through the TRAP, which could be used to detect the activity of telomerase. These results also confirmed the feasibility of the proposed biosensor.

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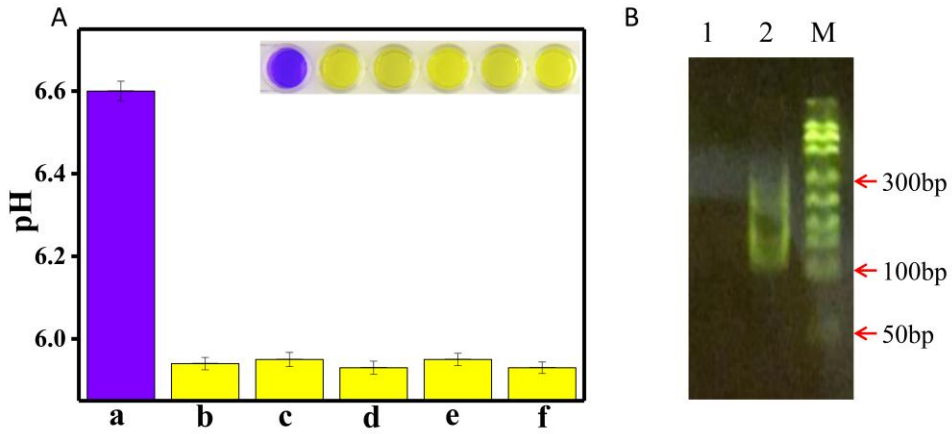

Fig. 1 (A) pH responses in the presence of (a) telomerase + TS primer + cDNA + streptavidin + urease; (b) TS primer + cDNA + streptavidin + urease; (c) telomerase + cDNA + streptavidin + urease; (d) telomerase + TS primer + streptavidin + urease; (e) telomerase + TS primer + cDNA + urease; (f) telomerase + TS primer + cDNA + streptavidin. **(B)** Agarose gel electrophoresis images in the absence (lane 1) and presence (lane 2) of HeLa cells extracts. Lane M: DNA size marker. The number of HeLa cells was 1000.

3.2. Optimization of the reaction conditions

To achieve the best analytical performance, various experimental conditions were optimized. Firstly, the concentration of TS primer was optimized. As shown in Fig. 2A (red curve), the pH value increased with the increasing of the TS concentration and reached the maximum at 75 nM, and then the pH value decreased as the concentration of the TS primer further increased, which is probably because the increased concentration of TS primer could hinder the DNA hybridization due to steric hindrance. And the pH value of background remained unchanged (black curve), thus, 75 nM of TS primer was used in our system. The incubation time of extension reaction and the concentration of dNTPs affect the final length of elongated DNA directly. As shown in Fig. 2B, the pH signal increased significantly with the extension

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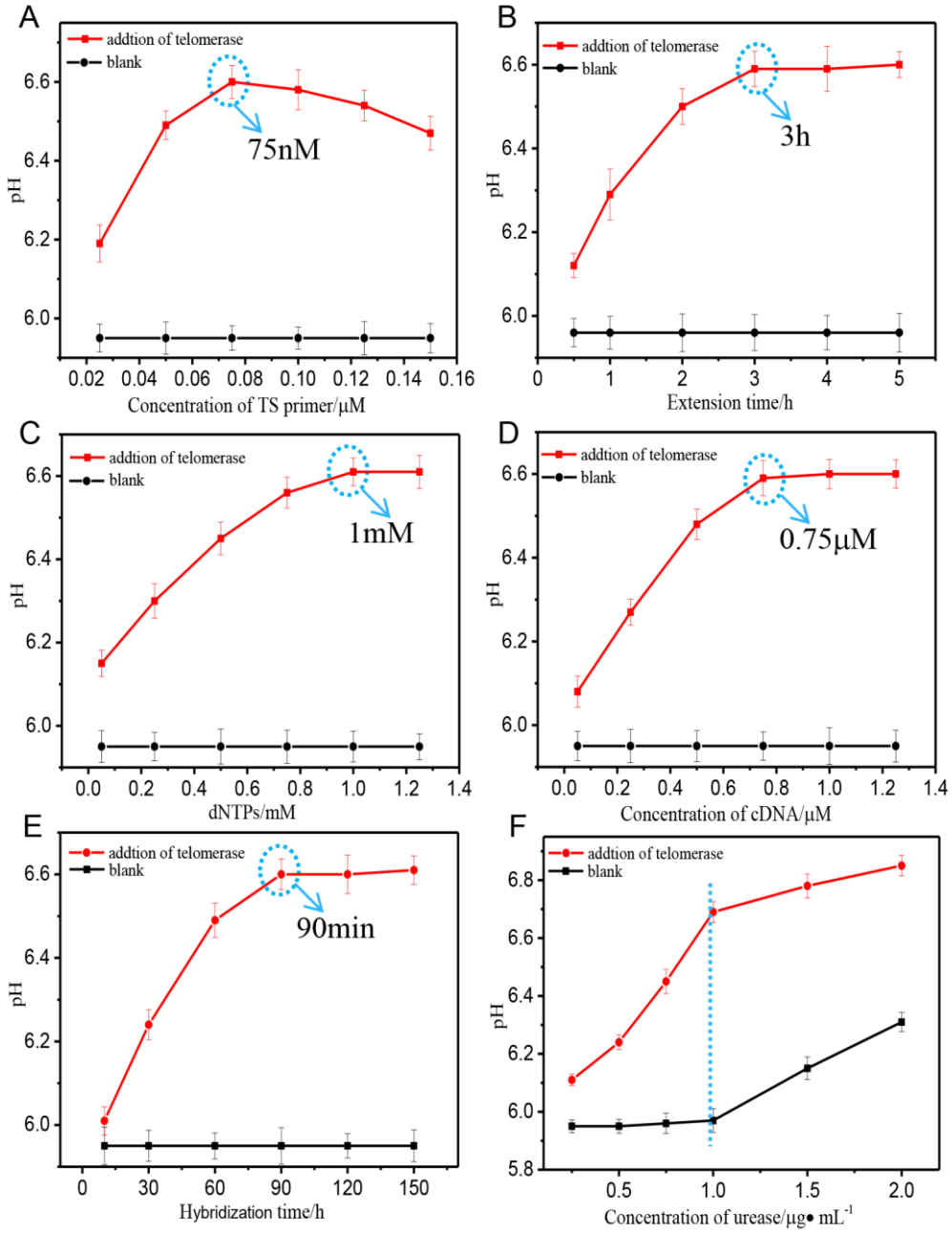

Fig. 2 Optimization of the detection conditions: **(A)** Effect of the concentration of TS primer on the pH signal. **(B)** Effect of the extension time on the pH signal. **(C)** Effect of the concentration of dNTPs on the pH signal. **(D)** Effect of the concentration of cDNA on the pH signal. **(E)** Effect of the hybridization time on the pH signal. **(F)** Effect of the concentration of urease on the pH signal.

of incubation time from 0.5 to 3h and then reached a plateau after 3 h but the background signal had no change. This result indicates that TS primer gradually elongates in the first 3 h and remains unchanged later. Thus, the optimal incubation

time of extension reaction was 3 h.

The effect of dNTPs concentration was also studied. As shown in Fig. 2C, the pH signal increased with the increasing of the dNTPs concentration and reached the maximum at 1 mM, suggesting that 1 mM of dNTPs was enough for the extension reaction; furthermore, the background signal did not affected by the concentration of dNTPs, so, 1 mM of dNTPs was chosen as the best condition in the following study.

To obtain effective hybridization, the concentration of cDNA and the hybridization time were also optimized. As presented in Fig. 2D, the pH signal increased with the increasing of the cDNA concentration and reached the maximum at 0.75 μM, and the pH signal of background nearly had no change. Therefore, 0.75 μM of cDNA was used for the hybridization of elongated TS primers with cDNA. Fig. 2E shows that the pH signal increased gradually and then reached a plateau after 90 min, indicating that 90 min was necessary to ensure a sufficient hybridization and the background signal did not affected by the hybridization time. Thus, 90 min was chosen as the best hybridization time for the following study. The concentration of the urease affects the sensitivity of the biosensor greatly. As presented in Fig. 2F (red curve), with the increasing of the concentration of biotinylated urease, the pH value of the system increased greatly firstly and then increased slowly after 1 μg/mL, this is probably because the increased concentration of urease could connect more urease to the PMPs and therefore consumes more hydrogen ions of the solution; but too high concentration of urease can generate false positive signals, as shown in Fig. 2F (black curve). The largest pH change detected in the presence and absence of telomerase was obtained when the concentration of urease was 1 μg/mL. Therefore, 1 μg/mL of urease was chosen in our further study.

3.3.Calibration curve of telomerase activity detection

The proposed pH biosensor was then applied to detect telomerase activity extracted from different numbers of HeLa cells under the optimized conditions. As shown in Fig. 3A, the ΔpH (the changing of the pH value after and before the addition of

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telomerase) increased gradually as the number of HeLa cells increased in the range of 20-10000. The inset figure of Fig. 3A shows the photographs of corresponding colour of urea/BCP solution when the number of HeLa cells ranged from 0 to 10000, a sharp colour variation (from yellow to grayish yellow, grayish purple and purple) was easily observed by the the naked eye, which indicates that the pH value increased gradually. ΔpH had a linear relationship with the logarithm of the number of HeLa cells from 50 to 10000. The regression equation was:

$$\Delta pH = 0.28lgN - 0.24,\ R^2 = 0.9911$$

where *N* is the number of HeLa cells and *R* is the regression coefficient. The detection limit is 20 HeLa cells at the signal-to-noise ratio of 3 (S/N = 3). In spite of telomerase of 20 HeLa cells can be easily differentiated from the blank control, it only reflected the mean telomerase activity of HeLa cells. So, we also analyzed the telomerase activity in the cell lysates of a certain number of

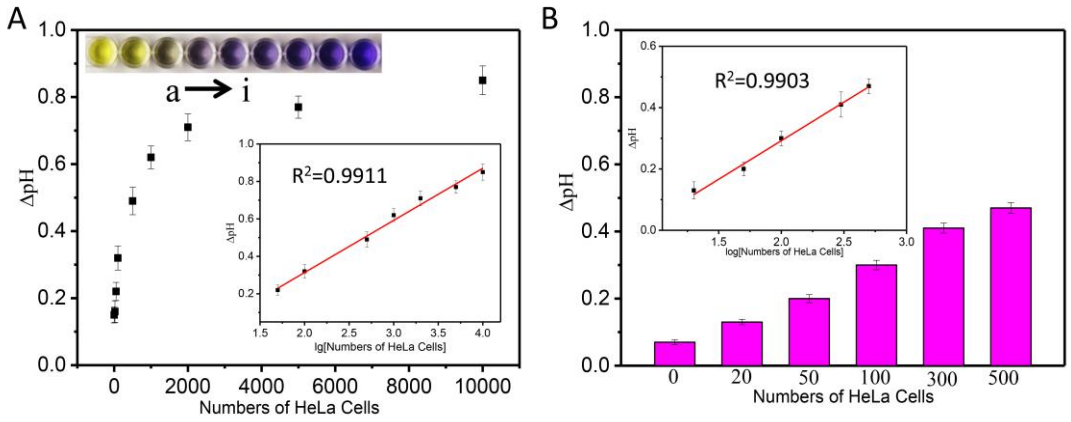

Fig. 3 (A) pH response to diluted telomerase extracts from different numbers of HeLa cells: (a) 0, (b) 20, (c) 50, (d) 100, (e) 500, (f) 1000, (g) 2000, (h) 5000, (i) 10,000. The inset figures are the corresponding photos after the addition of BCP (HeLa cells from 0 to 10000) and the calibration curve between the logarithm of the number of HeLa cells from 50 to 10000 and pH value. **(B)** Detection of telomerase activity from cell lysate of HeLa cells via portable pH. The numbers of cells were 0, 20, 50, 100, 300 and 500. The inset figures are the calibration curve between the logarithm of the number of HeLa cells from 20 to 500 and pH value.

HeLa cells. The experimental procedures is the same as the detection of the diluted extracts. As shown in Fig. 3B, the ΔpH increased gradually when the number of HeLa cells increased from 0 to 500. ΔpH had a linear relationship with the logarithm of the number of HeLa cells from 20 to 500. And the telomerase activity in 20 HeLa cells can also be well detected with portable pH meter, indicating that the proposed pH biosensor could realize accurate and sensitive detection of telomerase activity.

Table 1 compared the performance of the proposed pH biosensor with those of other POCT methods. Firstly, it is clear that the sensitivity of this work is comparable to that of the reported POCT analysis technique. Visual inspection can provide rapid answers, which can help doctor to make an immediate decision in clinical analysis. Digital analysis with a portable pH meter can help to improve the accuracy and sensitivity of early diagnosis. Second, the pH of system is not affected greatly by the surrounding environment, such as humidity, light sources and elevation, as that of pressure (Lin et al., 2018; Wang et al., 2017b) or fluorescence (Fu et al., 2018). Third, nearly all basic laboratories have pH meter, so it is unnecessary to purchase additional equipment. So the proposed pH biosensor can meet the testing at various locations and arbitrary times.

Table 1 Comparison of the analytical performance of different POCT for detecting telomerase activity.

Device type	Detection limit	Detection time	Dual readout	References
Personal glucometer	80 cells/mL	80 min	No	Wang et al., 2014
Paper microzone plate	5 cells/μL	60 min	Yes	Fu et al., 2018
Portable pressure meter	1 cell	90 min	No	Wang et al., 2017b
Portable pH meter	20 cell	10 min	Yes	This work

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3.4. The selectivity and reproducibility study of the proposed biosensor

The selectivity and reproducibility of the proposed biosensor were also studied, the telomerase activities in eight different kinds of samples including lysis buffer, BSA (0.1 mg/ mL), HSA (0.1 mg/ mL), the telomerase extracts from 1000 cells of HeLa, MCF-7, SW480, HL-7702 and heated HeLa was studied. As shown in Fig. 4, lysis buffer, BSA, HSA, heated extracts of HeLa cells and the normal human cell HL-7702 all caused no effect to the pH assay. On the contrary, although the telomerase activity from different cancer cell was different, all these cancer cells could induce a significant pH signal changing, which means cancer cells have much higher telomerase activity and is consistent with previous reports. These results indicate that the proposed biosensor can be applied to telomerase activity analysis. The reproducibility of the proposed method was also investigated for practical application. Five parallel replicated experiments were performed with telomerase extracted from 1000 HeLa cells, the relative standard deviation (RSD) was 5.9%, indicating that the proposed strategy had good precision and reproducibility. These results indicate that the proposed pH biosensor could become a powerful tool for early cancer detection and diagnosis.

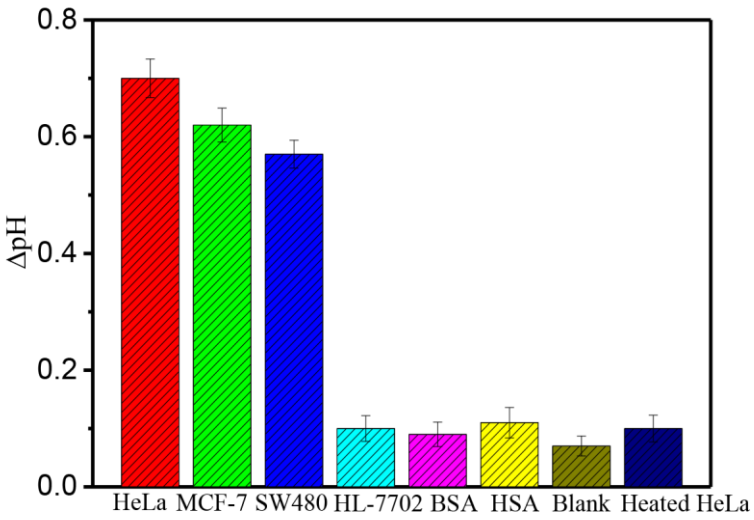

Fig. 4 Selectivity of the proposed pH biosensor for telomerase activity analysis. The assays were carried out in the presence of telomerase extracts from 1000 cells.

3.5. Detection of telomerase activity in serum samples

In order to confirm the practical application, the proposed method was applied to detect telomerase activity in human serum. We tested mimetic samples which contained telomerase extracts from different numbers of HeLa cells in normal human serum. As shown in Table 2, four samples were investigated, the results revealed that the proposed method has a good accuracy and precision. The standard addition recovery rates were in the range of 81.0-90.0%, and the RSD were in the range of 2.45-6.03%. These results illustrate that the proposed portable biosensor can be applied to detect telomerase activity in a complex matrix.

Table 2 Recoveries and RSD of the proposed sensor for the detection of telomerase activity in human serum.

Sample numder	1	2	3	4
Added (numbers of HeLa cells)	50	100	200	500
Detected (numbers of HeLa cells)	43	81	180	421
Recovery rate (%)	86.67	81.00	90.00	84.17
RSD (%)	3.89	6.03	4.74	2.45
Colour (adding BCP)				
ΔpH (using portable pH meter)	0.23	0.31	0.40	0.50

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4. Conclusion

In summary, a portable and sensitive biosensor for the detection of telomerase activity is successfully developed using pH meter or naked eye as the readout.The detection of telomerase activity is translated into the detection of pH changing of the system with the introduction of a high-efficiency of catalytic action of urease, which

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can be quantitative detected by a portable pH meter, while semi-quantitative assay with the naked eye is achieved by the addition of a pH-sensitive dye. The detection limit of the proposed method is ~20 HeLa cells. The proposed method combines the advantages of self-amplification of telomerase and enrichment by magnetic separation. It is worth to note that the pH of a system is not affected significantly by the surrounding environmental conditions such as temperature, humidity, light sources and altitude. Moreover, the pH-based assay is low-cost and easy-to-use, and can avoid cumbersome equipment and professional operators. Thus, our proposed strategy could have potential applications for the detection of telomerase activity in a broad area with good stability and reproducibility, which can provide a convenient approach for telomerase-related cancer screening or diagnosis.

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Highlights

- The detection of telomerase activity is translated into the detection of pH with the introduction of a high-efficiency urease.
- A portable pH meter is used for quantitative detection of telomerase with high sensitivity, while semi-quantitative assay with the naked eye can be achieved easily in case of the addition of a pH-sensitive dye.
- The proposed method is demonstrated for the detection of telomerase activity in HeLa cells with a LOD of 20 HeLa cells.

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