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A simple and sensitive fluorescence method for detection of telomerase activity using fusion protein bouquets

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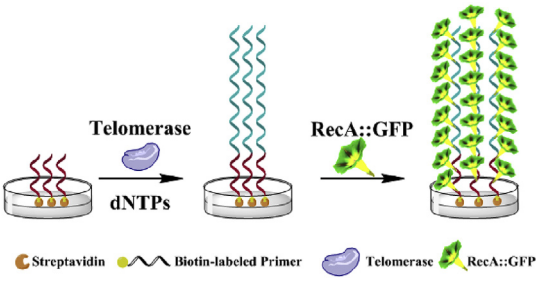
HIGHLIGHTS

- PCR-free, label-free, simple fluorescence method was developed for the telomerase activity detection.
- The RecA-GFP fusion protein was used as the active biological element in the realization of a telomerase biosensor.
- The proposed method was cost, effective, sensitive and applicable in biological samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Telomerase is considered as a widely accepted cancer biomarker for early cancer diagnostics. Herein, we develop a simple, ultrahigh sensitivity method for detection of telomerase activity, which relied on that RecA-GFP fusion proteins wrapped around telomeric DNA to form fluorescence bouquets. RecA-GFP fusion protein was synthesized through fusion protein technology. In the presence of telomerase, telomerase elongation products are wrapped around by RecA-GFP fusion protein to form big fluorescent bouquets, which resulted in strong fluorescence. This method has the linear range from 50 to 1000 HeLa cells and the detection limit is 8 HeLa cells, based on a signal-to-noise ratio (S/N) of 3. Compared with conventional methods, this method has the advantages of low toxicity, outstanding sensitivity, and excellent selectivity. Hence, it provides a promising approach for the detection of telomerase activity and diagnosis of cancer.

1. Introduction

Telomeres are unique nucleic acids which composed of constant repeat sequences $(TTAGGG)_n$ at the end of eukaryotic chromosomes which protect the regions of chromosome end from fusion or degradation using their DNA-protein complex [1,2]. However the telomeres in cancer cells are elongated and achieve unlimited replicative potential in the presence of telomerase [3]. It is well known that telomerase is over-expressed in the majority of cancer cells, whereas it is absent or repressed in most somatic cells. Therefore, telomerase has been considered as a cancer biomarker for early cancer diagnosis and therapeutic target [4]. So far, several methods have been developed for telomerase activity detection. Among them, polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) has been the most extensively

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used method [5]. Although it exhibits extremely high sensitivity and wide detection range, TRAP suffers from severe limitations such as laborious and time consuming. Meantime, the method is susceptible to the inhibition of the PCR process by the cell extract. To solve these problems, several kinds of PCR-free methods have been developed recently, including fluorescence [6–8], colorimetric [9,10], electrochemistry [11,12], surface enhanced Raman scattering (SERS) [13], and so on. For example, Xiao et al. used single molecule approach for amplification-free telomerase activity detection [14]. With the method, telomerase extracted from cancer cells was accurately detected with sensitivity down to 10 cells within 10 min. Although single molecule or single particle counting methods are well-equipped to provide excellent sensitivity and high resolution [14–17], the potential of practical application is limited due to sophisticated instruments and complicated manipulation. Thus, the development of a simple, reliable method for the telomerase assay still remains a great challenge.

Since 1962, green fluorescent protein (GFP) has been found in the jellyfish Aquaria Victoria [18]. P-hydroxybenzylidene-2,3-dimethylimidazolinone (HBDI) of GFP is light-emitting molecular unit, and the fluorescence quantum yield of the chromophore is close to unity [19,20]. Although GFP is not a good choice due to the overlap of emission wavelength with that from the biological fluids. GFP has the advantages of good biocompatibility, water-soluble, low toxicity and easy modification [21]. So it was widely used as fluorescent markers in cell biology and molecular biology. As fluorescent markers, GFP need be labeled. The traditional chemical modification methods often destroy proteins. They are usually accompanied by harsh reaction conditions such as strong acid or alkali. Herein, fusion protein technique is introduced to modify proteins. The fusion protein technique is aimed to synthesize fusion proteins through the target genes fusion and expression of protein [22]. The basic method of constructing fusion protein is to encode multiple specific functions coding to form a polypeptide sequence which lead to realize the common expression of two genes. In the fusion protein technique, the fusion proteins are encoded by the whole sequence of proteins and generally retain the activity of the original proteins [23]. Because the fusion protein technology has enormous advantages of genetic stability, repeatable, and simple purification, the fusion proteins have been widely used in genes expression and multifunctional enzymes and so on [24,25]. However, to the best of our knowledge, the application of fusion protein technique in analytical chemistry has not been reported.

RecA, a DNA binding protein, plays a central role in homologous recombination and repair of broken DNA molecules [26]. It is wild used in antigenic variation, induction of toxin biosynthesis, swarming motility, and antibiotic resistance [27–29]. The RecA protein strongly binds with ssDNA to form a long clusters helical nucleoprotein filament [30]. Thus, combined fluorescent dye or nanoparticle with RecA, the fluorescence method will be built to detect ssDNA. Although fluorescent dye or nanoparticle has high fluorescence quantum yield, they were poison. Moreover their conjugation methods often destroy proteins. They are usually accompanied by harsh reaction conditions. In our work, RecA-GFP fusion protein was synthesized through fusion protein technology. RecA-GFP fusion protein didn't only have the fluorescence of GFP, but also the interaction of RecA and ssDNA. In the presence of telomerase, telomerase elongation products are wrapped around by RecA-GFP fusion protein to form big fluorescent bouquets, which resulted in strong fluorescence. Therefore, the activity of telomerase in urine samples can be sensitively detected by measuring fluorescence of bouquets. RecA-GFP fusion proteins bind three nucleotides, the telomere DNA could enrich more fluorescence signal of fusion proteins [30]. So without signal application, this design can reach such low detection limit. Furthermore, owing to the biocompatibility of RecA-GFP fusion proteins, the method has low toxicity. Hence, the proposed fluorescence strategy holds great potential for the early diagnosis of cancers.

2. Experimental

2.1. Reagents and apparatus

The biotin-labeled telomerase primer DNA: 5′-biotin-AAA AAA AAA TCC GTC GAG CAG AGT T-3′ was synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonic acid (CHAPS), β-mercaptoethanol, glycerol, phenylmethyl¬sulfonyl fluoride (PMSF), ethyleneglycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Tween 20 and tris-(hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The plasmid pMD18, *Hin*d III enzyme, and Taq DNA polymerase were purchased from Takara Biomedical Technology Co. Ltd. (Kusatsu, Shiga, Japan). The dNTPs were from Sangon Biotechnology Co. Ltd. (Shanghai, China). The gel-electrophoresis loading buffer, ladder DNA were purchased from Thermo Fisher Scientific Co. Ltd. (MA, USA). All cells were purchased from Boster Bio-Engineering Limited Company (Wuhan, China). All other reagents were of analytical reagent grade and were purchased from Tianjin chemical reagent Co. Ltd. (Tianjin, China). Millipore Milli-Q water (18 MΩ cm^{-1}) was used in all experiments.

The fluorescence intensity was measured by Infinite M − 200 automatic multifunction microplate reader (Tecan, Switzerland) and Hitachi F-7000 fluorescence spectrophotometer (Kyoto, Japan). The gel-electrophoresis image was measured by Gel Doc XR + molecular imager (Bio-Rad, USA). The EMSA image was measured by FLA-7000 bioimager (GE Healthcare Japan, Japan). Polymerase Chain Reaction (PCR) process was performed on Applied Biosystems 2720 thermal cycler (Thermo Fisher Scientific, USA).

2.2. Cells culture

Human cervical cancer cells (HeLa), human lung cancer cells (A549), human breast cancer cells (MCF-7), and normal human liver cells (HL-7702) were cultured with 10% fetal bovine serum in Dulbecco's modified Eagle's medium. The cells were maintained at 37 °C in a humidified atmosphere (95% air and 5% CO_2). The cells number was determined by a Petroff-Hausser cell counter (USA).

2.3. Telomerase extension reactions

Cells extracts were serially diluted in lysis buffer (pH 7.5, 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.5% CHAPS and 10% glycerol). Then the extracts (1 μL) and dNTPs (1 μL, 10 mM) were added into 20 μL of telomerase substrate primer (TSP) solution (pH 8.3, 20 mM Tris-HCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 63 mM KCl, 0.05% Tween 20, 0.1 mg mL^{-1} BSA). The solution was then incubated at 37 °C for 1 h. For thermal inactivation control experiment, telomerase extracts were incubated at 95 °C for 10 min.

2.4. Preparation of RecA-GFP fusion protein

The pMD18 plasmid (30 μL, 10 mM) and *Hin*d III enzyme (2 μL, 15 U/μL) were added into 8 μL reaction buffer (pH 7.5, 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM Dithiothreitol, and 500 mM NaCl). The solution was incubated at 37 °C for 1 h. Then, the green fluorescent protein gene (30 μL, 10 mM) and *Taq* enzyme (2 μL, 10 U/μL) were added. The mixture was incubated through a RCR process. The recombinant plasmids were inoculated into Escherichia coli in LB

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