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Label-free ultrasensitive determination of *Eco*RI activity based on terminal deoxynucleotidyl transferase generated G-quadruplexes

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ABSTRACT

In this study, a label-free ultrasensitive fluorescent biosensor for monitoring of *Eco*RI activity has been developed based on terminal deoxynucleotidyl transferase (TdT) directed G-quadruplexes formation. Double-stranded DNA is specifically cleaved by *Eco*RI into single-stranded fragments with the newly generated free 3′-OH terminus. In the condition of the polymerization pool containing 10% dTTP, 40% dATP and 50% dGTP, TdT can elongate the cleaved fragments at their 3′-OH ends to produce G-rich DNA sequences which could subsequently form into G-quadruplexes motif in the aid of K^+. The obtained G-quadruplexes structure can be recognized by the typical G-quadruplex-selective probe *N*-methylporphyrin dipropionate IX (NMM). Thus, *Eco*RI activity could be easily and sensitively determined by using this detection platform. The detection limit for *Eco*RI is as low as 0.07 U/mL according to the linear concentration range of 0.1–30 U/mL. Moreover, the proposed assay showed high potential in real sample detection. This detection platform is label-free, highly sensitive, simple to operate and cost effective, possessing high application potential as a useful tool for endonuclease detection. Moreover, we also demonstrated the capability of this strategy to detect *Eco*RI in serum sample, showing high application potential as a useful tool for endonuclease detection.

1. Introduction

Endonucleases, as one of the most important nucleases, play important roles in many biological processes, such as DNA replication, recombination, repair, genotyping, mapping, molecular cloning, polymerase chain reaction and so on, through cleaving the internal phosphodiester bonds of nucleic acids [1–4]. In addition, endonucleases are also considered as promising pharmacological targets for drug development, especially for antimicrobial and antiviral drug [5]. Thus, to simply assay the activity and inhibition of enzymes is highly crucial in modern molecular biology [6]. Among nucleases, *Eco*RI belongs to one of the type II restriction endonucleases [7]. It is considered to be a part of defense system to protect living cells from foreign DNA by recognizing and cleaving the defined DNA sequence –GAATTC–. Therefore, its activity assay has attracted wide interest of the researchers in various fields.

Conventional techniques for the determination of endonuclease activity include high performance liquid chromatography (HPLC), gel electrophoresis, radiolabelling, and enzyme-linked immunosorbent assay (ELISA) [8–11]. However, these methods are usually high cost, time consuming, low sensitive and involved unstable antibodies [12–14]. Recently, some new technologies have been developed to improve the assay of endonuclease activity [15], including fluorescence analysis [16–18], colorimetric detection [19–21] and electrochemical methods [22–24]. As one of the mostly employed analytical method, fluorescence strategies draw dramatic attentions due to their high sensitivity, simple operation and high throughput capacity [25–27]. Various fluorescent strategies have been developed for the detection of endonucleases [28–31]. For instance, Li and Tan used molecular beacons to determine restriction endonucleases with superior sensitivity through intramolecular fluorescent quenching techniques [32]. Liang and co-workers designed fluorescent polarization detection of endonuclease activity and inhibition, and this fluorescent biosensor possesses up to 100-fold sensitive than conventional gel electrophoresis and chromatographic analysis. Zhang et al. constructed a highly sensitive and selective fluorescence AND logic gate platform based on the hairpin DNA probe with *Eco*RI and K^+ as the input elements [33]. Generally, all of these fluorescent methods possess the advantages of high sensitivity, easy operation and time-saving.

G-quadruplex structure is a non-canonical DNA secondary configuration consisting of four guanines (G) stabilized planar stacks, and has drawn increasing interest in label-free analytical applications [34,35]. *N*-methyl porphyrin dipropionate IX (NMM), as a G-quadruplexes selective probe [36–38], exhibits a significantly enhanced fluorescence

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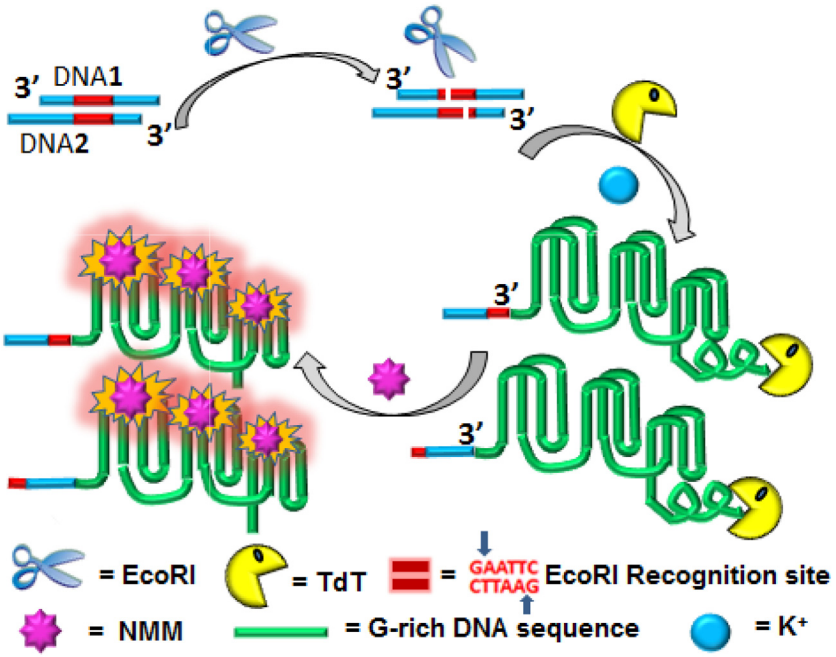

Scheme 1. Schematic illustration of the label-free fluorescent detection of *Eco*RI activity.

upon combining with G-quadruplex and thus plays important roles in the development of G-quadruplex-based fluorescence sensors [39–44]. Meanwhile, in order to sensitively assay the activity of *Eco*RI endonuclease, a unique DNA polymerase, terminal deoxynucleotidyl transferase (TdT), was used in the study [45]. Most DNA polymerases require a DNA template during the replication of genomic DNA [46], whereas TdT can continuously add dNTPs at the free 3′-OH group of the oligonucleotide primers to produce random sequences in the absence of a template [47]. As reported, a random array of G-rich sequences can be achieved when the dNTP reaction pool contains dTTP, dATP, and dGTP with the mole ratio of 1:4:5 [48–50], which could form G-quadruplex structures in the aid of K^+ [51,52]. In addition, Dong et al. verified the need for K^+ to form stable G-quadruplexes [53]. The newly formed strands of G-quadruplex by TdT can be used as a signal transducer and amplifier in G-quadruplex-based detection.

Inspired by these concepts, herein, an ultrasensitive label-free fluorescent biosensor based on G-quadruplex has been developed for the assay of *Eco*RI activity (Scheme 1). *Eco*RI could specifically cleave the double-stranded DNA1/DNA2 hybrid into single-stranded fragments. These newly generated DNA fragments contained free 3′-OH terminals, which could be elongated by TdT. Then, the TdT-produced G-rich products can be recognized quickly by NMM and resulting in an increase in fluorescence. The biosensor just consists of one double-stranded DNA probe and one kind of G-quadruplex generating enzyme, which is simple, easy to operate, cost effective and ultrasensitive.

2. Materials and methods

2.1. Chemicals and materials

All oligonucleotides were synthesized by Shanghai Sangon Biotech Co. (Shanghai, China) and were purified by high performance liquid chromatography (HPLC). In DNA strands, the recognition site of *Eco*RI endonuclease is underlined. DNA1 and DNA2 bases are partially complementary. The sequences of DNA are listed as follows:

DNA**1**: 5′-AATATATGAATTCTATA-3′

DNA**2**: 5′-TTATATAGAATTCATAT-3′

*Eco*RI endonucleases, terminal deoxynucleotidyl transferase (TdT), 2′-Deoxyadenosine-5′-triphosphate (dATP), 2′-Deoxythymidine-5′-triphosphate (dTTP), 2′-Deoxyguanosine-5′-triphosphate (dGTP), pyrophosphate (PP) and serum were purchased from Shanghai Sangon Biotech Co. (Shanghai, China). NMM was bought from Frontier Scientific, Inc. (Logan, Utah, USA). Insulin was obtained from sigma-Aldrich (Beijing, China). NF-κB p50 was bought from Cayman Chemical Co. (Shanghai, China). Rsal was purchased from Beyotime Biotech Co. (Shanghai, China). BSA was obtained from solarbio (Beijing, China). Other chemicals were analytical grade and used without further purification. All oligonucleotide stock solutions were prepared with Tris-HCl buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH = 7.4). Millipore Milli Q (18 MΩ cm) water was employed in all of the experiments.

2.2. Apparatus

All fluorescence measurements were performed on Hitachi F-7000 fluorescence spectrophotometer (Kyoto, Japan). All fluorescence spectra were recorded at λ_{ex} 399 nm and λ_{em} 611 nm. When the samples were excited at 399 nm, the emission was scanned from 500 to 700 nm in steps of 0.2 nm. A slit width is 10 nm. The vertical electrophoresis system was purchased from Bio-Red Laboratories, Inc.

2.3. EcoRI endonuclease cleavage reaction

DNA**1** (400 nM) and DNA**2** (400 nM) were mixed and heated at 90 °C for 5 min to make them fully hybridize, and then was gradually cooled to room temperature. The prepared double-stranded DNA**1**/DNA**2** is mixed with certain amount of *Eco*RI endonuclease in reaction buffer (100 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, pH = 7.4) and incubated at 37 °C for 30 min. The total volume of reaction solution was 25 μL.

2.4. Polymerization of G-quadruplexes by TdT

5 μL of TdT 5 × reaction buffer (1 M potassium cacodylate, 0.125 M Tris, 0.05% Triton X-100, 5 mM $CoCl_2$, pH = 7.2), 0.1 mM dTTP, 0.4 mM dATP, 0.5 mM dGTP, and certain amount of TdT were added into the as-prepared samples, respectively. The solution was filled with Milli Q water and kept at 37 °C for 2 h, and the total volume of the reaction solution was 50 μL.