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Introduction

Endonucleases are one kind of nucleases and they are easy to cut the phosphodiester linkages of the nucleic acid backbone. Endonucleases play an important role in a variety of fields, such as genetic recombination, molecular cloning, DNA replication and repaired.\textsuperscript{1,4} Besides, they are also used for drug development in medicinal chemistry like antiviral drugs screening.\textsuperscript{5,6} So it is necessary to develop the methods for detecting endonuclease activity and inhibition screening in the fields of biotechnology and pharmaceutical studies. The early methods included gel electrophoresis, chromatography, P\textsuperscript{32}-radioactive labeling, enzymelinked immunosorbent assay (ELISA).\textsuperscript{7-11} However, most of those methods have some innate drawbacks, such as time-consuming, insensitive, heavy workload, isotope labeling and so on.\textsuperscript{12} In order to overcome these shortcomings, more and more alternative ways have been developed for detecting endonucleases. The fluorescent method was the common analytic method that had aroused great interests among researchers, because the fluorescent method has the advantages like high sensitivity, rapid analysis, and economy.\textsuperscript{13} Various fluorescent assays have been developed to detect endonucleases later, for example, Huang group used quantum dots (QDs) as fluorophore and made used of different kinds of quenchers to realize multiplexed detection of endonuclease based on fluorescence resonance energy transfer (FRET),\textsuperscript{12} they also utilized gold nanoparticle as quencher and volume enhancement factor for detecting endonucleases.\textsuperscript{14,15} The Dong group constructed a method based on G-quadruplex DNAzyme as signal probe for endonuclease detection.\textsuperscript{16} Noble metal nanoclusters (NCs) have attracted great research interests around the field of bio-analytic chemistry, because they exhibit excellent properties, such as facile synthesis, tunable fluorescence emission (ranging from visible to near-IR), ultratine size, low toxicity, good photostability and biocompatibility.\textsuperscript{17,20-27} When the Dickson group first reported the water-soluble gold and silver nanoclusters with highly fluorescence,\textsuperscript{20-22,28} more and more analytic chemists would like to utilize noble metal nanoclusters as biological fluorescent probe to develop the label-free biosensors. The noble metal nanoclusters can be synthesized by using thiolated compounds or polymeric compounds (thiolated compounds, DNAs, proteins and dendrimers) as scaffold.\textsuperscript{29-33} Among of those templates, the water-soluble DNA-scaffolded fluorescent silver nanoclusters (DNA-AgNCs) are widely used by analysts.\textsuperscript{31,34} They made use of DNA-AgNCs as label-free fluorescent probe to detect many types of materials, for examples, the Qian group took advantage of DNA-AgNCs as fluorescent probe for detection of endonuclease activity and inhibition;\textsuperscript{17} the Peng group also determine micrococcal nucleaseions by DNA-AgNCs;\textsuperscript{31} The Ye group used DNA-AgNCs as probe to monitor specific DNA;\textsuperscript{35} the Zhang group utilized DNA-AgNCs as the signal response of fluorescent molecule to...
detect cocaine, the Willner group realized multiplexed analysis of genes by using DNA-AgNCs. All the described methods above have been demonstrated that DNA-AgNCs was an useful fluorescent probe and could be used in a wide range of areas of bio-analysis.

Recently the Nie group reported that long-chain cytosine (C)-rich DNA could serve as an excellent template to synthesize DNA–AgNCs with strong fluorescence. They took the advantage of terminal deoxynucleotidyl transferase (TdT) to polymerize C-rich sequence and the strong fluorescence of DNA-AgNCs was able to be synthesized. At the same time, the Zhang group utilized nicking endonuclease to cleave the DNA which used as template to synthesize DNA–AgNCs for the detecting of cocaine. Combined with their results of experiments, we deduced that the long C-rich DNA which was used as template to synthesize DNA–AgNCs could be changed to become short DNA, the fluorescent signal would become weak. EcoRI pertains to type II restriction endonuclease, it can recognize and cleave a defined the DNA sequence of GAATTC to protect living cells, so it is important to analysis of EcoRI activity and inhibition. Many label-free methods for detecting endonuclease, such as G-quadruplex DNAzyme, DNA-AgNCs, DNA-hosted Hoechst dyes for fluorescence detection. There also label-free method on the basis of light scattering (LS) of carbon nanotubes to detect nuclease.

Based on this theory, we developed a label-free platform to detect endonucleases named EcoRI and its inhibitions.

**Experimental**

**Materials and reagents**

All the DNA oligonucleotides used in this work were synthesized and purified by Sangon Biotech Co; Ltd. (Shanghai, China), they were C-rich DNA (5’-CCCCCGCGGATTTCCCGGGGTTTCCCTTCC-3’) and G-rich DNA (5’-GGGGGGGAATTCGGGGGGGAATTCGGGG-3’). EcoRI, EcoRV, BamHI, PvuII were all purchased from New England Biolabs Co; Ltd. (Beijing, China). Silver nitrate (AgNO3), Sodium borohydride (NaBH4) and trihydroxymethyl aminomethane (Tris) were purchased from Sinopharm Chemical Reagent Co; Ltd. (Shanghai, China). Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Xilig Chemical Co. Ltd. (Guangdong, China). Sodium acetate (NaAc), magnesium acetate (Mg(Ac)2) were obtained from HWRK Chemical Co. Ltd (Beijing, China). 5-fluorouracil and Pyrophosphate bought from Solarbio Co. Ltd (Beijing, China). All other reagents were of analytical reagent grade and used without more purification and modification. Ultrapure fresh water (resistivity>18.2Ω) was used throughout the present study.

**Apparatus**

Photoluminescence (PL) measurements were obtained through a LS-55 luminescence spectrometer (Perkin Elmer, USA). UV–vis absorption spectra were obtained through a TU-1901 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd.).

**Endonuclease activity and inhibition assay**

The assay were performed in the reaction buffer (50 mM Tris-Ac, 50 mM NaAc, 10 mM Mg(AC)2, pH 7.5@25℃). Firstly, 1 μM C-rich DNA and G-rich DNA were mixed equally in the reaction buffer in a volume of 180 μL, the solution was heated at 90℃ for 10 min and gradually cooled down to ambient temperature to form double-strand DNA (dsDNA). Then different concentrations of EcoRI endonuclease were added and incubated at 25℃ for 80 min.

For the inhibition assay, the inhibitors of EcoRI were pyrophosphate (PP) and 5-fluorouracil, varying concentrations of inhibitors were incubated with 1 μM dsDNA at 25℃ for 30 min, then the EcoRI endonuclease was added and incubated at 25℃ for 80 min.

**The synthesis and determination of fluorescent DNA-templated silver nanoclusters**

The DNA–AgNCs were synthesized based on the reported method with slight modification. Briefly, 6 μM AgNO3 solution was added to the solution described above, after vigorous stirring with vortex for 2 min and incubating in the dark at 4℃ for 20 min, 6 μM NaBH4 solution was added and vigorous stirred with vortex for 1 min, the final solution was incubated at 4℃ more than 3 h in the dark. Fluorescence intensity of the solution of DNA-AgNCs was recorded by using a LS-55 luminescence spectrometer with an excitation wavelength of 560 nm and an emission wavelength of 614 nm.

**Agarose gel electrophoresis**

The 4% agarose gel electrophoresis analysis of the DNA was carried on in 1×TBE (90 mM Tris, 90 mM boric acid, 2 mM ethylenediaminetetraacetic acid (EDTA)) at room temperature, the constant voltage was 80 V and the time was set for 1 h, then stained for 30 min in ethidium bromide solution and scanned using an Omega 16 Gel Document System (ULTRALUM, USA).

**Detection of EcoRI in human serum samples**

Normal human serum sample was provided by the Fifth People Hospital of Guilin (Guilin, China). The serum was added into a 10 kDa molecular weight filter. Then, it was centrifuged at 8000 rpm for 20 min. Subsequently, the treated serum samples were kept at 4℃ before used. For the standard addition assays, six kinds of concentrations of EcoRI were spiked into diluted human serum and the identical assay procedure as in the reaction buffer described above.

**Results and discussion**

**Principle of detecting EcoRI**
Scheme 1 Schematic illustration of using DNA-AgNCs as fluorescence probe for detection EcoRI

Scheme 1 had showed the principle of the label-free assay based on DNA-AgNCs for detection EcoRI, two kinds of especial DNA sequences were designed, a C-rich DNA that was modified with two sequences of EcoRI recognition site GAATTTC and three sequences of AgNC nucleic acid sequences (each nucleic acid sequence of AgNC was six cytosine bases sequence) between the EcoRI recognition site, a G-rich DNA was designed to complete complement with the C-rich DNA. After mixing with a certain concentration of AgNO$_3$ and NaBH$_4$, the integrated C-rich DNA contained twenty cytosine bases sequence could used as the scaffold to synthesize DNA-AgNCs with strong fluorescence intensity. When the C-rich DNA was mixed with G-rich DNA, they hybridized and formed dsDNA, which was the substrate of EcoRI, so the EcoRI endonuclease could cut the recognition site of the dsDNA and the integrated C-rich DNA was cleaved to be three kinds of nucleic acid sequences, they contained six or seven cytosine bases sequence only, the fluorescence intensity of the solution of DNA-AgNC became weak.$^{36,38}$ Opposite, DNA-AgNCs was synthesized by the long chain C-rich DNA exhibited strong fluorescence intensity in the absence of EcoRI. Therefore, the EcoRI endonuclease would be quantified and qualitative throughout monitoring the change of fluorescence intensity of the DNA-AgNCs. This proposed assay also would detect and screen the inhibitors of EcoRI in a label-free way.

Feasibility study

In order to verify the feasibility of our assay, we carried on a series of experiments. First of all, we obtained the UV-vis absorption spectra of the solutions of DNA-AgNCs, the characteristic absorption peak of DNA-AgNCs appeared around 560 nm in the UV-vis absorption spectra of dsDNA-AgNCs (Fig. 1A, curve a), one small absorption peak around 400 nm attributed to the absorption of Ag nanoparticles (AgNPs).$^{43,44}$ In the present of EcoRI, the characteristic absorption peak appeared around 560 nm was disappeared (Fig. 1A, curve b). Two photographs contained the solution of DNA-AgNCs under room-light and UV irradiation from the inset of Fig. 1A had been showed. Under the room light, the color of the solution of DNA-AgNCs was pale yellow showed in Fig. 1A ① and a pink color was observed under UV lamp excitation (Fig. 1A, ②). It had also indicated that the DNA-AgNCs had been formed.

Then the fluorescence emission spectra of the DNA-AgNCs were obtained under different conditions. As shown in Fig. 1B, when the C-rich DNA used as the template alone to form DNA-AgNCs, the fluorescent intensity was the most strongest (Fig. 1B, curve a), and the DNA-AgNCs which was synthesized by G-rich DNA only, its fluorescent intensity was the weakest (Fig. 1B, curve e). Furthermore, C-rich DNA hybridized with G-rich DNA to form dsDNA, a slight lessening and red shift of the fluorescent intensity of dsDNA-AgNCs would be seen (Fig. 1B, curve c), when the EcoRI was reacted with dsDNA, the value of fluorescence signal response of the dsDNA-AgNCs became very weak(Fig. 1B, curve d). Moreover, once the C-rich DNA-AgNCs was mixed with EcoRI, the fluorescent intensity was not weaken too much, that was to say that it was not the EcoRI to
quench the fluorescence of DNA-AgNCs (Fig. 1B, curve b).

The feasibility of the experimental principle was also demonstrated by agarose gel electrophoresis analysis (Fig. 2). There were no band appeared in lanes 1 and 2 when the C-rich DNA and G-rich DNA were alone, respectively. After C-rich DNA and G-rich DNA were mixed and form dsDNA, a clear band appeared (lane 3). Once the EcoRI was added with dsDNA, the dsDNA was cut and just a little had been remained, so the band became very weak and blurry (lane 4). It can be suggested that EcoRI could cut the dsDNA.

All those had demonstrated that our method could potentially be applied to detection EcoRI without label anything.

**Optimization of assay condition**

For getting the optimal experimental parameters to the highly sensitive detection of EcoRI, we carried it out in several optimal conditions for the assay. The concentration of dsDNA was the first factor to consider. As shown in Fig. S1, with the concentration of dsDNA increased tardily, the relative fluorescence intensity of the DNA-AgNCs decreased rapidly. 0.5 μM of dsDNA should be used in the further experiments theoretically, but the fluorescent intensity of DNA-AgNCs which was synthesized by the 0.5 μM of dsDNA was very weak actually. So the 1μM of dsDNA was used in the further assays.

Then the effect of reaction temperature of EcoRI also was optimized in the experiment (Fig. S2). There was no too much difference at the fluorescence intensity of the dsDNA-AgNCs with and without EcoRI under 25°C and 30°C in Fig. S2, and 25°C was chose as the optimizing condition to the further experiments. At last, the effect of reaction time of EcoRI was investigated for the assay. As illustrated in Fig. S3, when the reaction time reached to 80 min, the fluorescence intensity of the dsDNA-AgNCs changed little and the 80 min was used as the optimal conditions for the further assay.

![Fig. 2 Gel electrophoresis of the biosensing system under different conditions: lane M: DNA marker; lane 1: C-rich DNA; lane 2: G-rich DNA; lane 3: C-rich DNA+ G-rich DNA; lane 4: C-rich DNA+ G-rich DNA+EcoRI. Conditions of reaction: 5 μM G-rich DNA, 5 μM C-rich probe and 5.45 × 10⁻⁴ U/μL EcoRI.](image)

![Fig. 3 (A) Fluorescence emission spectra of the experiment system in the presence of EcoRI, the concentration of EcoRI from top to bottom were 0 U/μL, 4.34 × 10⁻⁴ U/μL, 6.45 × 10⁻⁴ U/μL, 8.70 × 10⁻⁴ U/μL, 1.09 × 10⁻³ U/μL, 1.30 × 10⁻³ U/μL, 1.52 × 10⁻³ U/μL, 1.74 × 10⁻³ U/μL, 1.96 × 10⁻³ U/μL, 2.17 × 10⁻³ U/μL, 4.35 × 10⁻³ U/μL, 6.52 × 10⁻³ U/μL, 8.70 × 10⁻³ U/μL, 1.09 × 10⁻² U/μL, 1.30 × 10⁻² U/μL. (B) The calibration curve for detection EcoRI ranging from 4.34 × 10⁻⁴ U/μL to 1.74 × 10⁻³ U/μL and 4.35 × 10⁻³ U/μL to 1.09 × 10⁻² U/μL. F_r represented for the fluorescence intensity of blank sample, F represented for the fluorescence intensity with different concentrations of EcoRI. Conditions of reaction: 1 μM G-rich DNA and 1 μM C-rich probe. Error bars were calculated from three replicate measurements.](image)

**Quantification of EcoRI endonucleases**

To research the dynamic response range of EcoRI in the proposed assay, a series of concentrations of EcoRI (ranging from 0 to 1.30 × 10⁻³ U/μL) under the optimal conditions were detected. As illustrated in Fig. 3A, with the increasing of EcoRI concentrations, the fluorescence intensity of DNA-AgNCs gradually decreased, there were two linear relationships in the range from 4.34 × 10⁻⁴ U/μL to 1.74 × 10⁻³ U/μL and 1.74 × 10⁻³ U/μL to 1.09 × 10⁻² U/μL (Fig. 3B and 3C). The regression equations were ΔF = 0.08781C - 0.02168 (R²=0.999) (Fig. 3B) and ΔF = 0.04444C + 0.3148 (R²=0.996) (Fig. 3C), respectively, where ΔF = (F_r - F)/F_r (F_r represented for the fluorescence intensity of blank sample, F represented for the fluorescence intensity with different concentrations of EcoRI), C meant the concentration of EcoRI. The limit of detection of EcoRI was evaluated to be 2.1 × 10⁻⁷ U/μL (S/N=3). All these clearly demonstrated that the method could be used to sensitively detect EcoRI, it would comparable to the reported methods.²⁵,46

**Selectivity of this assay**

We investigated the specificity of the method based on DNA-AgNCs for monitoring EcoRI, three kinds of non-targeted endonucleases were introduced to identify the selectivity of...
Conclusions

In summary, take the advantage of the properties that the fluorescence DNA-AgNCs synthesized by long-chain C-rich DNA was stronger than the shorter one; a label-free method had been developed based on DNA-AgNCs as fluorescence probe to selective determine EcoRI endonuclease. As a proof-of-concept, we clearly testify that our assay could monitor the activity and screen the inhibitors of EcoRI endonuclease. What’s more important was that our method used DNA-AgNCs as fluorescence probe without label any other things, and the synthetic method of DNA-AgNCs was easy, facile and cost-effective, so this assay is convenient and cheap. Besides, The method enable to sensitive detect EcoRI with the limit of detection $2.1 \times 10^{-4} \text{U/µL}$. Moreover, this method was able to distinguish the endonuclease from others which also belongs to type II restriction endonuclease well, the even when the concentration of the other endonucleases were 50-fold higher than EcoRI. At the same time, this strategy is able to research the inhibitors of EcoRI and detect the target endonuclease in real human serum samples. At last, it was also low toxicity and highly fluorescent and could be used it in biological imaging and other more areas like.

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Notes and references
