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A Novel Label-Free Fluorescence Strategy Based on Dumbbell Probe for Sensitive Detection of DNA Ligase

Wenping Zhu*, Xinlu Wang, Liyan Dai, Weijie Yang, Yanxia Li, Ruilan Liu

College of Chemistry and Chemical Engineering, Zhoukou Normal University, Zhoukou, China
Email: *wenping315@163.com

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Abstract

Based on the principle that DNA ligation reaction mediates hydrolysis of the dumbbell probe (DP), a novel label-free strategy for sensitive detection of DNA ligase was developed. DNA ligase ligated the nick on DP to generate a fully closed dumbbell DNA structure, which could prevent the hydrolysis of exonuclease, after combining with SYBR Green I (SG I), a strong fluorescent signal was obtained. In the absence of DNA ligase, DP was hydrolyzed into single nucleotides by exonuclease, resulting in a rather weak signal. The linear range of this method for detecting T4 DNA ligase is 0.00004 - 0.004 U/μL, and the detection limit is 0.00003 U/μL. The proposed strategy is sensitive, inexpensive and easy to operate, which may offer an effective tool for further applications in new drug screening.

Subject Areas

Analytical Chemistry

Keywords

Dumbbell Probe, DNA Ligase, SYBR Green I, DNA Ligation Reaction, Exonuclease

1. Introduction

Deoxyribonucleic acid (DNA) ligase is a necessary and ubiquitous enzyme involved in the processes of DNA replication, repair and recombination [1], it is the first ATP-dependent ligase derived from bacteriophage, which catalyzes the formation of phosphodiester bonds between the juxtaposed 5' phosphate end and 3' hydroxyl end in double-stranded DNA with the help of ATP. T4 DNA li-

gase is not only widely used in biological genetic engineering, but the abnormal expression and defect of this nuclease are closely related to the pathogenesis of cancer and neurodegeneration [2]. Therefore, DNA ligase is a powerful target for the synthesis of broad-spectrum antibacterial agents and cancer-related inhibitors. In addition, DNA ligase has become an indispensable tool enzyme in *in vitro* DNA manipulation techniques, such as the detection of specific nucleic acid sequences or protein analysis, DNA nanotechnology and DNA computing [3]. The determination of DNA ligase is of great significance for basic biochemical research, medical diagnosis, and drug development [4]. Therefore, the development of simple, sensitive, economical methods for DNA ligase detection is still in progress.

The traditional detection methods of DNA ligase mainly include polyacrylamide gel electrophoresis and autoradiography, which are complex, discontinuous, or not sensitive. Researchers have developed several new alternative methods, such as molecular beacon-based methods [5], hairpin-based fluorescence methods [6], and surface plasmon resonance-based approach [7] and electrochemical methods [8]. Most of these methods rely on DNA chemical modification and cumbersome operation process, which significantly increases the cost and complexity of the assay. Therefore, it is of great value to develop simple, label-free methods for sensitive detection of DNA ligase.

Label-free fluorescence methods often provide rapid and economical biomolecular detection by combining fluorescent dyes, which have received great attention. SYBR Green I (SG I) is an asymmetric cyanine dye with green excitation wavelength that often used as the reporter group in label-free methods [9]. SG I has high sensitivity, good temperature stability and good photophysical properties, it has been widely used in the determination of biomolecules (DNA, protein) and the selective detection of metal ions [10] [11]. In order to develop novel, simple and sensitive methods for further application research on DNA ligase, taking SG I as a fluorescent reporter group to construct biosensors is obviously a good choice. Therefore, in this paper, a label-free strategy for sensitive detection of DNA ligase was developed based on dumbbell probe and DNA ligation reaction.

2. Experimental Section

2.1. Chemicals and Reagents

DNA oligonucleotide was synthesized and purified by Shanghai Sangon Biotechnological Co., Ltd. (Shanghai, China). T4 DNA ligase, Klenow Fragment (KF exo-), T4 Polynucleotide Kinase (PNK) and SG I (10,000× concentrated stock in H_2O) were purchased from Shanghai Sangon Biotechnological Co., Ltd. (Shanghai, China). Exonuclease I (Exo III) and Exonuclease III (Exo I) were purchased from New England Biolabs (Beijing, China). Other reagents and chemicals were of analytical grade and used as received. The reaction buffer solutions employed in this work were DNA ligase buffer (40 mM Tris-HCl, 10 mM

$MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 8.0) and MOPS buffer (10 mM MOPS, 150 mM $NaNO_3$, pH 7.0). DNA sequence for dumbbell probe (DP) is as follows:

(5'-P-*GCGTGG*GAAAT*CCACGC*CCCAACCCTAGGGTAGGGCGGGTTGGG-3').

The italicized part and the underlined sequences are complementary, respectively.

2.2. Experimental Condition

DNA oligonucleotide was firstly dissolved in MOPS buffer, then pre-annealed by heating to 95˚C for 5 min and allowed to cool slowly to room temperature to form DP structure. For a typical detection experiment for DNA ligase, 5 μL of 10× DNA ligase buffer, 3 μL of 3 μM DP, 5 μL of T4 DNA ligase diluent, and 37 μL of pure water were mixed, kept at room temperature for 1 h. Then, 2 μL of 5 U/μL Exo III and 2 μL of 2.5 U/μL Exo I were added into the above mixture and incubated at 37˚C for 30 min. Subsequently, 240 μL of MOPS buffer, 6 μL of SG I (100×) were added into the above mixture and incubated at room temperature for 10 min.

2.3. Fluorescence Measurements

Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Agilent, USA) with an excitation wavelength (λ_{ex}) of 495 nm, and the fluorescence emission spectra were collected from 510 to 600 nm at room temperature. The fluorescence spectrum at λ_{em} = 527 nm was recorded, the PMT detector voltage was fixed at 800 V, and each measurement was carried out in a final volume of 300 μL.

3. Results and Discussion

3.1. Working Principle of the DP-Based Fluorescence Strategy

A novel label-free fluorescence strategy for sensitive detection of DNA ligase was developed based on DP and DNA ligation reaction, which is presented schematically in **Figure 1**. As shown in **Figure 1**, the designed DNA strand can self-hybridize to form a dumbbell probe (DP) with a nick on its stem and a phosphate (P) at the 5' end. In the absence of DNA ligase, DP was hydrolyzed by exonuclease into single nucleotides from the nick, resulting in a weak background signal. However, the nick on DP was ligated to form a fully closed dumbbell DNA structure in the presence of DNA ligase, which prevented the hydrolysis of exonuclease. Finally, a strong fluorescence signal was observed after combining with fluorescent dye SG I. The proposed fluorescence strategy is sensitive, inexpensive and easy to operate.

3.2. Verification the Feasibility of DP-Based Strategy

Figure 2 depicts the typical fluorescence spectral responses of the DP-based strategy for detection of DNA ligase. Verification the feasibility of DP-based

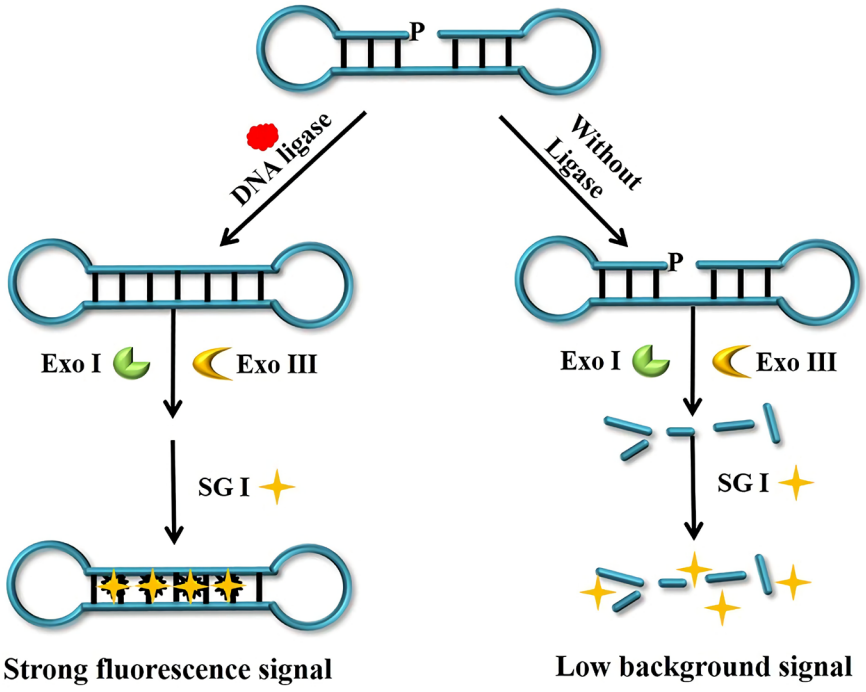

Figure 1. Schematic illustration of the fluorescence strategy for DNA ligase detection based on dumbbell probe.

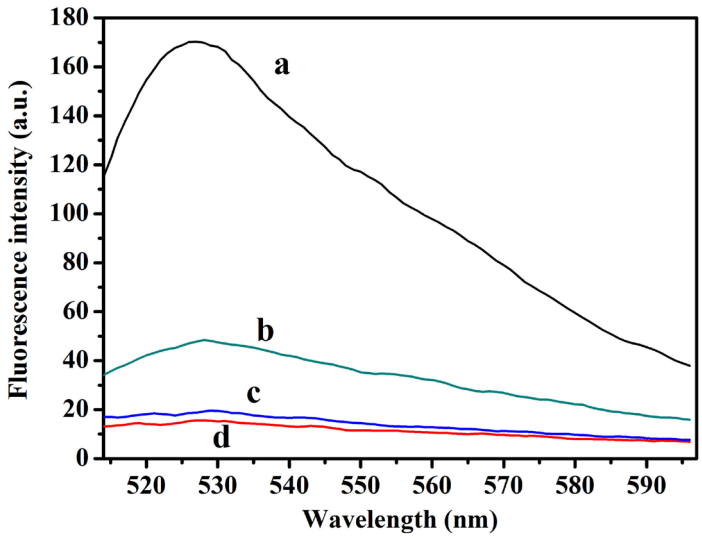

Figure 2. Typical fluorescence spectra of DNA ligase detection. Each sample contains 30 nM DP (final volume of 300 μL) and the fluorescence measurements were performed at the optimal experimental condition. (a) DP + 0.01 U/μL DNA ligase; (b) DP + 0.001 U/μL DNA ligase; (c) DP + denatured DNA ligase; (d) DP.

strategy based on the difference in fluorescence spectra under varying conditions. As shown in Figure 2, a significant strong fluorescence signal (curve a) was observed in the both presence of DP and DNA ligase (0.01 U/μL). As expected, a lower fluorescence signal was obtained when less DNA ligase (0.001 U/μL) was present in the reaction mixture (curve b). Moreover, it was obvious

that there was a low background signal in the absence of DNA ligase (curve d). Furthermore, one could get a weak signal similar to curve d when the denatured DNA ligase and DP were added in the reaction mixture. The results indicated that the proposed DP-based strategy was correct and feasible.

3.3. Optimization of Experimental Conditions

In this experiment, the concentrations of SG I, Exo I and Exo III have great impact on the performance of the fluorescence strategy. Those experimental conditions were investigated and optimized one by one, and the results are as follows.

Figure 3 depicts the relationship between the fluorescence signal ratio and the concentration of SG I. As shown in Figure 3, fluorescence signal ratio increased with SG I concentration up to its maximum peak then decreased gradually, the signal peak was obtained at the concentration of SG I (2×). Therefore, SG I (2×) was used as the optimal concentration of SG I and used for further experiments.

Figure 4 depicts the relationship between the fluorescence signal ratio and the concentrations of exonuclease. It can be seen from Figure 4, fluorescence signal ratio showed a trend of first increasing and then decreasing with the increase of exonuclease concentration, which indicated that excessive or too small concentration of exonuclease is detrimental to assay performance. Therefore, the optimal concentration of Exo I was chosen to be 0.1 U/μL, and the optimal concentration of Exo III was 0.2 U/μL.

3.4. Sensitivity of DNA Ligase Assay

The fluorescence spectra responses to DNA ligase of varying concentration were performed under the optimal experimental conditions. As shown in Figure 5, it

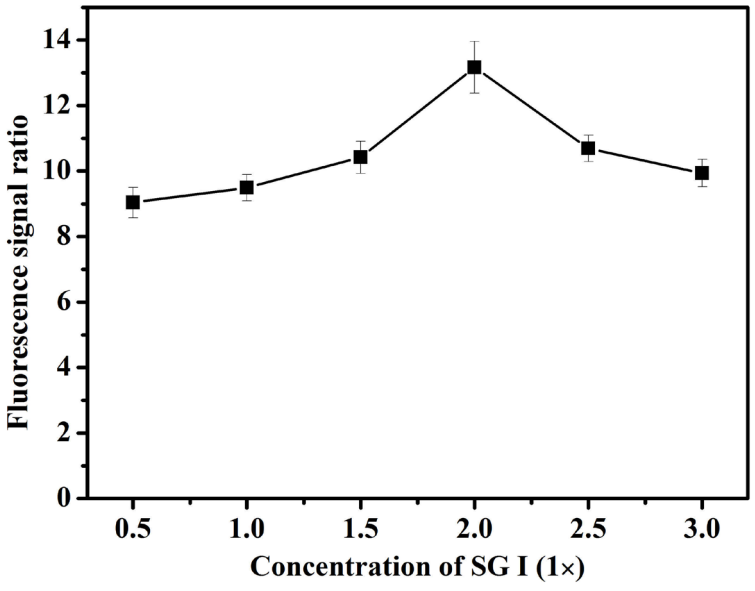

Figure 3. Dependence of fluorescence signal ratio on concentration of SG I. Fluorescence signal ratio is defined as the ratio of fluorescence peak intensity at 527 nm from DNA ligase to that from the blank. Error bars show the standard deviations of three experiments.

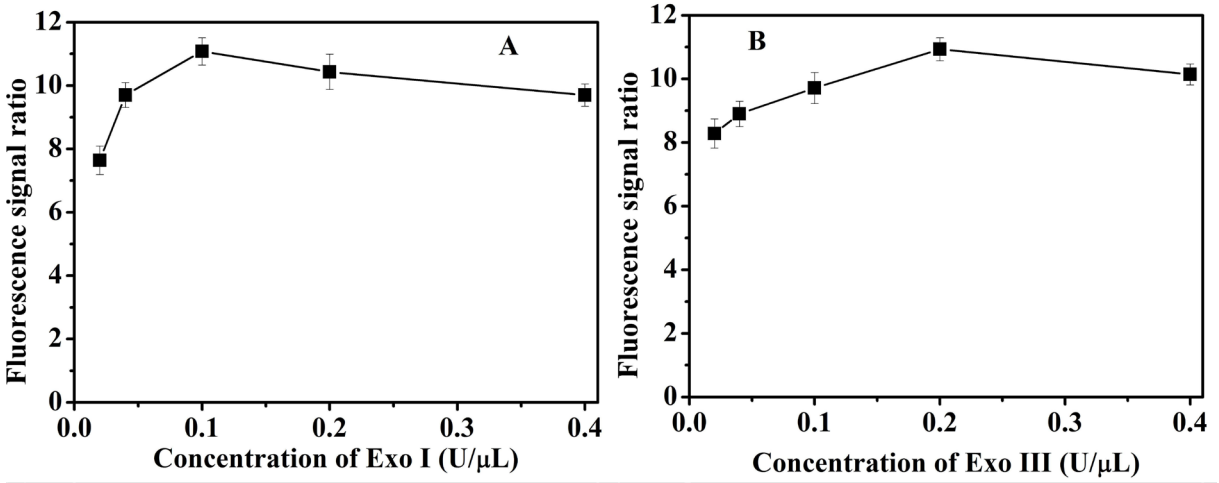

Figure 4. Dependence of fluorescence signal ratio on concentrations of Exo I and Exo III. Fluorescence signal ratio is defined as the ratio of fluorescence peak intensity at 527 nm from DNA ligase to that from the blank. Error bars show the standard deviations of three experiments.

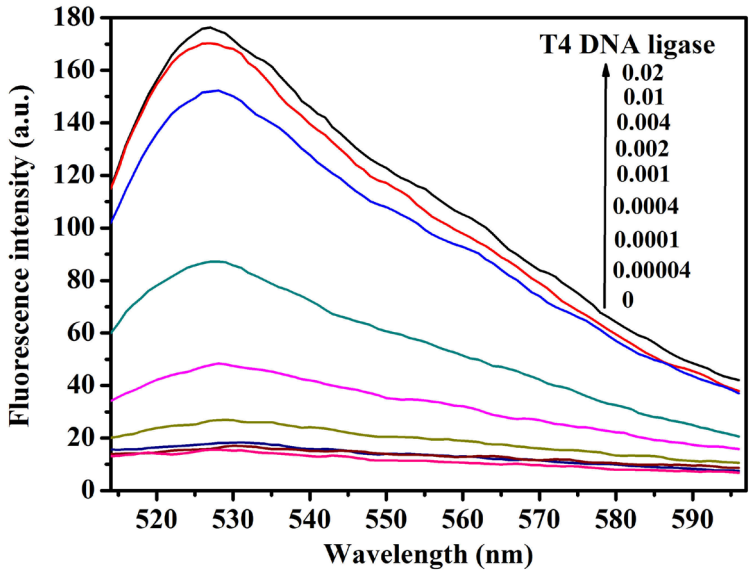

Figure 5. Fluorescence spectra in response to different concentrations of T4 DNA ligase.

is observed that the fluorescence signals gradually increased as the concentrations of T4 DNA ligase varied from 0.00004 to 0.02 U/μL, which indicated the generation of more fully closed DP after ligation reaction.

Figure 6 illustrates the relationship between fluorescence intensity and ligase concentration. Notably, the fluorescence intensity of spectra response at 527 nm significantly increased with the increasing of DNA ligase concentration, but reached a plateau above the concentration of 0.01 U/μL. Fluorescence intensity showed a good linear correlation with the concentration of T4 DNA ligase in the range from 0.00004 - 0.004 U/μL. The correlation equation is $F = 34901C + 13.24$ ($R^2 = 0.9969$), where F represents the fluorescence intensity and C represents the concentration of T4 DNA ligase. The detection limit is estimated to be 0.00003

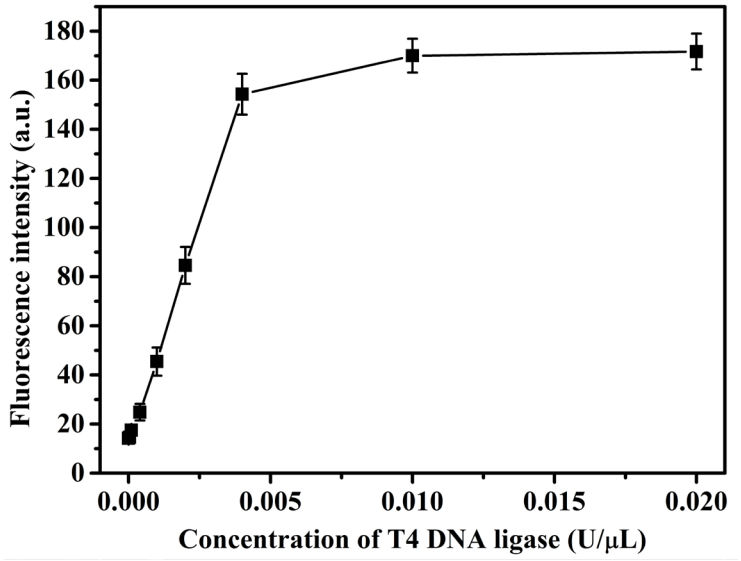

Figure 6. Linear relationship between the fluorescence intensity at 527 nm and the concentration of T4 DNA ligase. Error bars show the standard deviations of three experiments.

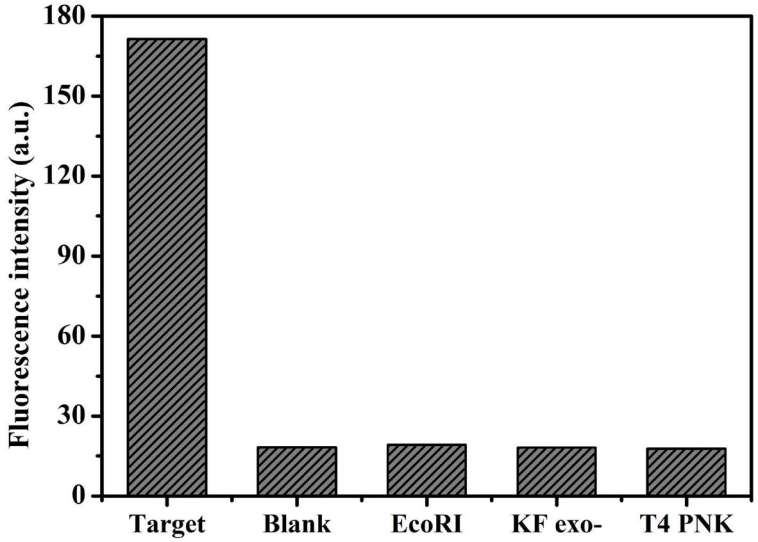

Figure 7. Measurement of fluorescence intensity in response to the reaction buffer (Blank), 0.05 U/μL EcoRI, KF exo-, T4 PNK, respectively.

U/μL based on the principle of 3 times the standard deviation over the signal of the negative control. The proposed method is easy to operate and inexpensive, it may be helpful for novel drug screening.

3.5. Specificity of the Strategy

Several irrelevant DNA modification enzymes were used as the negative control samples to evaluate the detection specificity. As shown in **Figure 7**, a remarkable fluorescence signal was observed in the presence of target DNA ligase (Target column), while no significant fluorescence signal was detected in response to the

reaction buffer (Blank column). Under the identical conditions, similar weak fluorescence signals were obtained in response to other three enzymes. These results confirm the excellent specificity of the proposed strategy toward DNA ligase.

4. Conclusion

In summary, a label-free fluorescence strategy for sensitive detection of DNA ligase was developed based on dumbbell probe and DNA ligation reaction. This approach is based on the principle that the DNA ligation reaction mediates the exonuclease hydrolysis of dumbbell probe. Taking SG I as the fluorescent reporter group, the proposed strategy can realize sensitive and inexpensive detection of DNA ligase, which may offer an effective tool for further applications in new drug screening.

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Conflicts of Interest

The authors declare no conflicts of interest.

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