

# Using molecular beacon to monitor activity of *E. coli* DNA ligase

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NAD<sup>+</sup>-dependent DNA ligase has been widely used in gene diagnostics for disease-associated mutation detection and has proved to be necessary for screening bactericidal drugs targeted to DNA ligases. However, further research has been restricted since conventional ligase assay technology is limited to gel electrophoresis, which is discontinuous, time-consuming and laborious. An innovative approach is developed for monitoring the activity of *E. coli* DNA ligase catalyzing nucleic acid ligation in the report. This approach utilizes a molecular beacon hybridized with two single-stranded DNA (ssDNA) segments to be ligated to form a hybrid with a nick, and could therefore be recognized by the enzyme. Ligation of the two ssDNA segments would cause conformation changes of the molecular beacon, leading to significant fluorescence enhancement. Compared to gel electrophoresis, this approach can provide real time information about ligase, is more time efficient, and is easier to use. The effect of quinacrine, a drug for malaria, on the activity of the ligase is detected, thereby certifying the capability of the method for developing novel antibacterial drugs targeted at NAD<sup>+</sup>-dependent ligase. The fidelity of strand joining by the ligase is examined based on this approach. The effects of external factors on activity of the ligase are analyzed, and then an assay of *E. coli* DNA ligase is performed with a broad linear range of  $4.0 \times 10^{-4}$  Weiss Unit mL<sup>-1</sup> to 0.4 Weiss Unit mL<sup>-1</sup> and the detection limit of  $4.0 \times 10^{-4}$  Weiss Unit mL<sup>-1</sup>.

## Introduction

DNA ligase is a key cell protein required for a number of important cellular processes, including the replication and repair of DNA as well as genetic recombination.<sup>1–7</sup> DNA ligases have found widespread use as a tool for *in vitro* DNA manipulation and cloning techniques.<sup>8,9</sup>

Polynucleotide ligases can be divided into two broad classes: those requiring NAD<sup>+</sup> as coenzyme and those requiring ATP. The eukaryotic, viral and archaeobacteria encoded enzymes all require ATP, while NAD<sup>+</sup>-requiring DNA ligases are found exclusively in eubacteria. NAD<sup>+</sup>-dependent DNA ligase was first isolated from *Escherichia coli* in 1967,<sup>10–13</sup> and the *E. coli* enzyme has been regarded as the prototype for mechanistic studies of the bacterial ligase family ever since.<sup>2</sup> It has been reported that while there is some structural similarity between ATP-dependent DNA ligase and NAD<sup>+</sup>-dependent DNA ligase, the nature of some of the residues and the spatial organizations of the two domains of the ligases may differ significantly.<sup>14,15</sup> The differences between the two families of enzymes and the high degree of conservation of the eubacterial DNA ligase<sup>16,17</sup> have led to the suggestion that the NAD<sup>+</sup>-dependent DNA ligases are a potential target for developing novel antibiotics.<sup>15,18</sup> Furthermore, NAD<sup>+</sup>-dependent thermostable bacterial DNA ligases, taking advantage of single nucleotide discrimination, have applications in detecting cancer and disease-associated gene mutations by ligase chain reaction (LCR).<sup>19–22</sup>

Research on DNA ligases has been generally based on polyacrylamide gel electrophoresis and autoradiography since T4 DNA ligase was first purified more than 30 years ago.<sup>2,23–26</sup> Gel electrophoresis is laborious, time-consuming and incapable of providing a real time signal of the activity of the ligase. Some other simple and precise methods need to be developed for further application research on NAD<sup>+</sup>-dependent ligase, such as screening potential drugs targeted to NAD<sup>+</sup>-dependent DNA ligases and checking the fidelity of the ligase for potential use in mutation detection. In this paper a novel method for the study of NAD<sup>+</sup>-dependent DNA ligases based on a molecular beacon was developed, thereby allowing the activity of the ligase to be assayed in real time, which makes it possible to do research on NAD<sup>+</sup>-dependent DNA ligase conveniently.

Previous work from this laboratory has developed a technique based on a molecular beacon for real time monitoring of DNA ligation by T4 DNA ligase, an ATP-dependent ligase.<sup>27,28</sup> NAD<sup>+</sup>-dependent ligase is also of great interest, especially in its potential to be applied in screening antibacterial drugs and gene mutation detection when compared with ATP-dependent ligase.<sup>15,18–22</sup> In this report, the activity of *E. coli* DNA ligase was first assayed in real time for further research on applications associated with the ligase and mechanisms of catalysis by NAD<sup>+</sup>-dependent ligase.

Molecular beacons are a class of novel fluorescence probes, and since their development in 1996 they have been widely used in DNA/RNA and some protein studies.<sup>29–32</sup> Molecular beacons are single-stranded oligonucleotides with a stem-and-loop structure, which can report the presence of a specific

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complementary nucleic acid. The stem is composed of five to seven base pairs that are complementary. A fluorophore and a quencher are linked to the 5'- and 3'-ends of the stem, respectively. The stem keeps the two moieties in close proximity, causing the fluorescence of the fluorophore to be quenched due to the transfer of energy. When the probe meets a target nucleic acid molecule that is complementary to the loop, the stem is forced apart, leading to the restoration of fluorescence.

## Experimental

### Experimental principle

The two ssDNA fragments to be ligated are attached to MB's 5'-half and 3'-half loops, respectively. First, hybridization occurs between the two ssDNA fragments and a molecular beacon to form a DNA substrate containing a nick with 5'-phosphate and 3'-hydroxyl, which can be recognized by *E. coli* DNA ligase. The molecular beacon's stem remains closed in this situation. When  $\text{NAD}^+$  exists together with the ligase, an attack on the  $\alpha$ -phosphate of  $\text{NAD}^+$  by the *E. coli* DNA ligase results in the displacement of pyrophosphate and the formation of a covalent ligase-adenylate intermediate in which AMP is linked to the  $\epsilon$ -amino group of a lysine. Adenylated *E. coli* DNA ligase then recognizes the nick and activates the 5'-phosphate of the nick by transferring AMP to the 5'-monophosphate terminus, which consists of an inverted (5')-(5') pyrophosphate bridge structure. A nucleophilic substitution reaction then occurs between activated 5'-phosphate and 3'-hydroxyl. The two fragments are then ligated, resulting in the opening of the molecular beacon's stem together with significant fluorescence enhancement. Real-time monitoring of ligase activity thus can be realized based upon this phenomenon. A schematic of the principle is shown in Fig. 1. It is clear that the molecular beacon in this design

acts not only as the probe that reports on the process of the ligation reaction by means of fluorescence changes, but also as the template for the formation of the substrate that is recognized by *E. coli* DNA ligase.

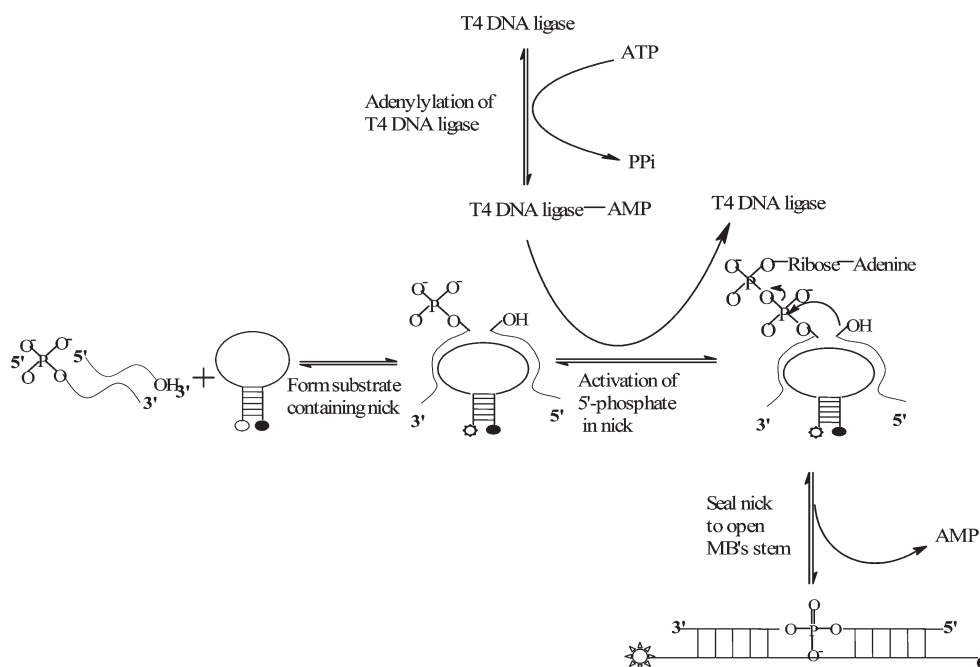
### Molecular beacon and DNA substrates

The molecular beacon (MB) was synthesized by TriLink Biotechnologies Inc. (San Diego, USA), with tetramethylrhodamine (TAMRA) labeled at its 5'-terminus as the fluorophore and 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) coupled to 3' terminus as the quencher. The MB is composed of 32 nucleotides, with a 20-nt loop, and a 6-bp stem constituted by two 6-nt complementary arm sequences. The other oligonucleotides (ODNs) were synthesized by SANGO Biological Engineering Technology and Service Co. Ltd. (Shanghai, China). Sequences of MB and ODNs are listed in Table 1. ODN1 is an 18-nt oligonucleotide complementary

**Table 1** Synthesized oligonucleotides

Code	Sequence (5'-end to 3'-end)
MB	(TAMRA) - <u>CGTTGA</u> TGG TTC CAC TTC TCG TGC GT <u>TCAACG</u> -(DABCYL)
ODN1	CGC ACG AGA AGT GGA ACC
ODN2	CGC ACG AGA
ODN3	AGT GGA ACC
ODN4	CGC ACG AGG
ODN5	CGC ACG AGC
ODN6	CGC ACG AGT
ODN7	GGT GGA ACC
ODN8	CGT GGA ACC
ODN9	TGT GGA ACC

<sup>a</sup> The underlined sequences in MB sequence represent stem of MB. ODN4, ODN5 and ODN6 are similar to ODN2, but one base mismatch at 3' end. ODN7, ODN8 and ODN9 are similar to ODN3, but one base mismatch at 5' end.



**Fig. 1** Principle scheme of monitoring activity of *E. coli* DNA ligase catalyzing DNA ligation based on molecular beacon.

to the middle 18 nucleotides of the MB's loop. ODN2 and ODN3 were designed to complement the 3'- and 5'- half part of the MB's loop, respectively. From ODN4 to ODN9 are single base mismatch oligonucleotides with similar sequences to ODN2 and ODN3. *E. coli* DNA ligase was purchased from Takara Biotechnology Co. Ltd. (Dalian, China).

### Reagents

Acrylamide, *N,N,N',N'*-tetramethyl-1,2-diaminomethane (TEMED) and ammonium sulfate were purchased from Sigma Chemical Co. (St Louis, MO). *N,N*-Methylene-bisacrylamide and urea were purchased from Bebcos (Kansas, AZ). NAD was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). MgCl<sub>2</sub> and EDTA were purchased from Amresco (Solon, OH), Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). All other chemicals were products of research or analytical purity and used without further purification. Deionized water that was obtained through a Nanopure Infinity™ ultrapure water system (Barnstead/ThermoLynx Corp., Dubuque, IA) and had an electrical resistance larger than 18.3 MΩ.

### Phosphorylation of ODNs

Typical phosphorylation reactions contain 50 nM oligonucleotide, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 5 mM DTT and 1 mM ATP in the 20 μL solution. After keeping the reactions at 37 °C for 2 h, they are stopped and enzyme-killed by heating them to 70 °C for 5 min. ODN3, ODN7, ODN8 and ODN9 are phosphorylated for subsequent ligation reactions and p-ODN3, p-ODN7, p-ODN8 and p-ODN9 are used to represent their products of phosphorylation at the 5'-terminus, respectively.

### Hybridization between MB and oligonucleotides

The buffer used for hybridization has the same composition as the ligation reaction buffer for the following preparation of electrophoresis samples, as well as for the real time monitoring of ligation and assay of the ligase. The ligation reaction buffer contained 30 mM Tris-HCl (pH 8.0), 2.5 mM MnCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 40 mM LiCl, 1.2 mM EDTA, 0.1 mM NAD<sup>+</sup> and 0.05% BSA. MB and ssDNA segments are added to a 120 μL buffer and their concentrations are 400 nM and 600 nM, respectively. The reaction temperature is kept at 37.0 °C. Fluorescence changes in the solution as well as the elapsed time are monitored on a Jobin-Yvon Fluolog 3 fluorescence spectrophotometer (France). After the fluorescence of the solution kept unchanged for 200 s, fluorescence intensities were noted.

### Denaturing polyacrylamide gel electrophoresis

Eight samples are prepared for electrophoresis. The ligation reaction mixtures contained MB (400 nM) and oligonucleotides (600 nM) in a 10 μL ligation reaction buffer. The nine samples are composed of (1) MB; (2) MB; (3) MB, ODN2 and ODN3; (4) MB, ODN2 and ODN3; (5) MB, ODN2 and

*p*-ODN3; (6) MB, ODN2 and *p*-ODN3; (7) MB and ODN1; (8) MB and ODN1, respectively. *E. coli* DNA ligase (0.48 U) is added to (2), (4), (6) and (8) before the reactions are incubated at 16 °C overnight. The reactions are stopped by the addition of 50% final volume formamide and 5 mM EDTA. The samples are heated at 95 °C for 5 min and cooled quickly in a mixture of ice and water. Then the ligated and unligated oligonucleotides are analyzed by electrophoresis on 20% denaturing polyacrylamide gels (20% acrylamide, 19 : 1 acrylamide : bisacrylamide, 7 M urea, 2 mM EDTA, 89 mM Tris-borate, 25% formamide). Electrophoresis is carried out in 1 × TBE (pH 8.3) at 80 V constant voltage for about 3 h. After fixing and silver staining, gels are scanned using an ImageMaster VDS-CL (Amersham Biosciences).

### Real-time monitoring of ligation process

MB, ODN2 and *p*-ODN3 are added into the 120 μL ligation reaction buffer and their concentrations are 400 nM, 600 nM and 600 nM, respectively. The temperature of the reaction solution is kept at 37.0 °C. Fluorescence changes in the solution as well as elapsed time is monitored on a fluorescence spectrophotometer. *E. coli* DNA ligase could not be added to the system until the fluorescence keeps unchanged for 200 s; at that time hybridization of the two ODNs to MB is completed. The quantity of the added ligase is 0.48 Weiss Unit.

The effects of external factors and quinacrine-2HCl are inspected by comparing initial ligation velocities calculated from monitoring the curve of ligation at different concentrations of the factors with other conditions unchanged. Mismatch ligations by the ligase are carried out using substrates containing a one-base mismatch at the 3'-side or 5'-side of the nick in the same conditions as with ligation monitoring.

### Ligase assay

The assay of the ligase is achieved by measuring the initial rates of the ligation reaction catalyzed by ligase that is concentrated in series (ranging from 4.0 × 10<sup>-4</sup> Weiss Unit mL<sup>-1</sup> to 0.4 Weiss Unit mL<sup>-1</sup>), and discerning the function between initial ligation velocity and quantity of ligase. Concentrations of MB and ssDNA segments are respectively fixed at 400 nM and 600 nM in all the reactions. The initial rate of ligation product formation is represented by the initial fluorescence enhancement rate, which can be calculated from a real-time monitoring curve of the ligation process.

### Fluorescence detection methods

The fluorescence group labeled at the 5'-terminus of MB is TAMRA, of which the maximal emission wavelength is 578 nm and the maximal excitation wavelength 521 nm. The fluorescence intensity of the solution containing MB is obtained through setting the excitation and emission wavelengths at 521 nm and 578 nm, respectively. Real-time monitoring curves are achieved by scanning fluorescence changes of the sample over a period of time, with the excitation and emission wavelengths being set at 521 nm and 578 nm, respectively. All fluorescence detection experiments are carried out on a

Jobin–Yvon Fluolog 3 fluorescence spectrophotometer (France) with a Thermo Neslab temperature bath (USA) to keep the temperature of the samples at 37.0 °C.

## Results and discussion


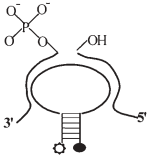
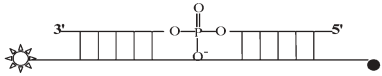
### Responses of molecular beacon to oligonucleotides

Earlier study indicates the MB can significantly distinguish the ligation product from the two ssDNA fragments to be ligated, and thus can be used to monitor the nucleic acids ligation process catalysed by T4 DNA ligase.<sup>27,28</sup> Because the reaction condition for *E. coli* DNA ligase is slightly different from that for T4 DNA ligase, hybridization experiments were carried out again under the specific condition to test the disparity between the MB's responses to the oligonucleotides before and after ligation. ODN1 is used as a substitute for the product. As shown in Table 2, the fluorescence intensity of MB with ODN1 is 9.45 times that of MB, and 7.33 times of that of MB with ODN2 and *p*-ODN3. The fluorescence of MB is enhanced by 29% after it is hybridized with ODN2 and *p*-ODN3, indicating that the stem of MB is only slightly loosened. When MB encounters the ligation product (ODN1), a remarkable fluorescence enhancement suggests that the stem has been taken apart. The results show that MB is also capable of responding to a ligation reaction catalysed by *E. coli* DNA ligase with high sensitivity.

### Polyacrylamide gel electrophoresis and real-time monitoring of DNA ligation

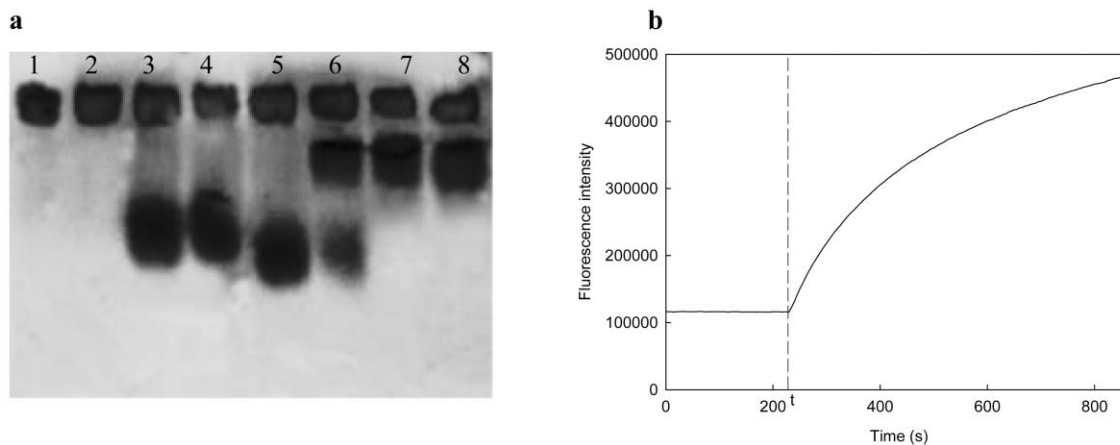
Electrophoresis experiments were carried out to make sure that the ligation product could be obtained only while a DNA substrate containing a nick that could be recognized by ligase was formed in the ligation mixture. The results are shown in Fig. 2a. Line 1 to line 8, respectively, represent sample 1 to sample 8. Oligonucleotides in sample 1 to sample 8 were: (1) MB; (2) MB; (3) MB, ODN2 and ODN3; (4) MB, ODN2 and ODN3; (5) MB, ODN2 and *p*-ODN3; (6) MB, ODN2 and *p*-ODN3; (7) MB and ODN1; (8) MB and ODN1, respectively. Samples 2, 4, 6 and 8 have similar compositions as samples 1,

**Table 2** Fluorescence intensities of MBs with different oligonucleotides

Substances	Structure	Normalized fluorescence intensity
MB		1.00
MB+ODN2+ <i>p</i> -ODN3		1.29
MB+ODN1		9.45

3, 5 and 7, respectively, except that samples 2, 4, 6 and 8 contained *E. coli* DNA ligase. Comparing lines 2, 4, 6 and 8 with lines 1, 3, 5 and 7, it can be ascertained that no new oligonucleotide is produced in samples 2, 4 and 8 after the addition of ligase. However, a new strip exists in line 6 compared with line 5. The position of the new strip suggests it is an oligonucleotide with the same length of ODN1, which indicates that a ligation product is obtained. It can also be found that *p*-ODN3 in lines 5 and 6 runs faster than ODN3 in lines 3 and 4. That is perhaps because *p*-ODN3 has a phosphate at the 5'-terminus, which causes it to contain an extra negative charge compared with ODN3.

A sample containing the same substances as sample 6 is monitored on the fluorescence spectrophotometer, and the real time curve is shown in Fig. 2b. The curve rapidly rises after the addition of 0.48 U of *E. coli* DNA ligase, implying the



**Fig. 2** Correlation of gel electrophoresis assay and fluorescence assay for DNA ligation. (a) Polyacrylamide gel assay for ligation reaction. (b) Real time monitoring curve of DNA ligation. The ligase added into sample at time *t*.

beginning of the ligation reaction. The ligation process and the activity of the ligase can thus be monitored in real time.

### Effect of quinacrine·2HCl

Quinacrine·2HCl, a drug commonly used for malaria, is reported to be a specific inhibitor of *E. coli* DNA ligase.<sup>18</sup> The effect of this drug on the activity of *E. coli* DNA ligase is examined using the innovative technology provided in this report. The results are shown in Fig. 3. The activity of the ligase is represented by initial ligation velocities calculated from monitoring the curve of ligation. The data have been normalized.

It can be concluded from Fig. 3 that the activity of the *E. coli* DNA ligase is inhibited by 50% when the concentration of quinacrine·2HCl is around 8  $\mu\text{M}$ , and by over 90% when the concentration is higher than 17  $\mu\text{M}$ . The result is consistent with previous report about the effect of the drug on *E. coli* DNA ligase.<sup>18</sup> This is an example demonstrating the capability of the approach to be used for the identification of potential and specific inhibitors of  $\text{NAD}^+$ -dependent DNA ligases, which will be helpful for the development of eubactericidal drugs.

### Specificity and fidelity of strand joining by *E. coli* DNA ligase

The fidelity of strand joining refers to the extent to which an enzyme can ligate substrates containing mismatched bases on either side of the nick. Previous studies suggest that the bacterially derived DNA ligases are more sensitive to the nature of their oligonucleotide targets than viral or eukaryotic DNA ligases. DNA ligases are widely used with oligonucleotides in processes such as oligonucleotide ligation assays (OLA),<sup>8,23,34,35</sup> the ligase chain reaction (LCR)<sup>19–22</sup> and to provide primers for DNA sequencing.<sup>36</sup> The success of these methods is built on the fidelity of ligation catalysed by the ligases.

The approach provided here can be used to conveniently analyze the specificity and fidelity of nucleic acids ligases. Experiments are carried out to examine the effect of base mismatches at the reactive 5'-phosphate and 3'-hydroxyl positions on the ligation reactions operated by *E. coli* DNA ligase (data not shown).

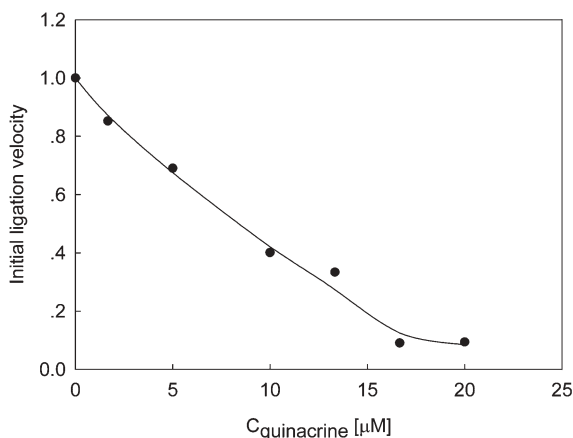


Fig. 3 Effect of quinacrine·2HCl on activity of *E. coli* DNA ligase.

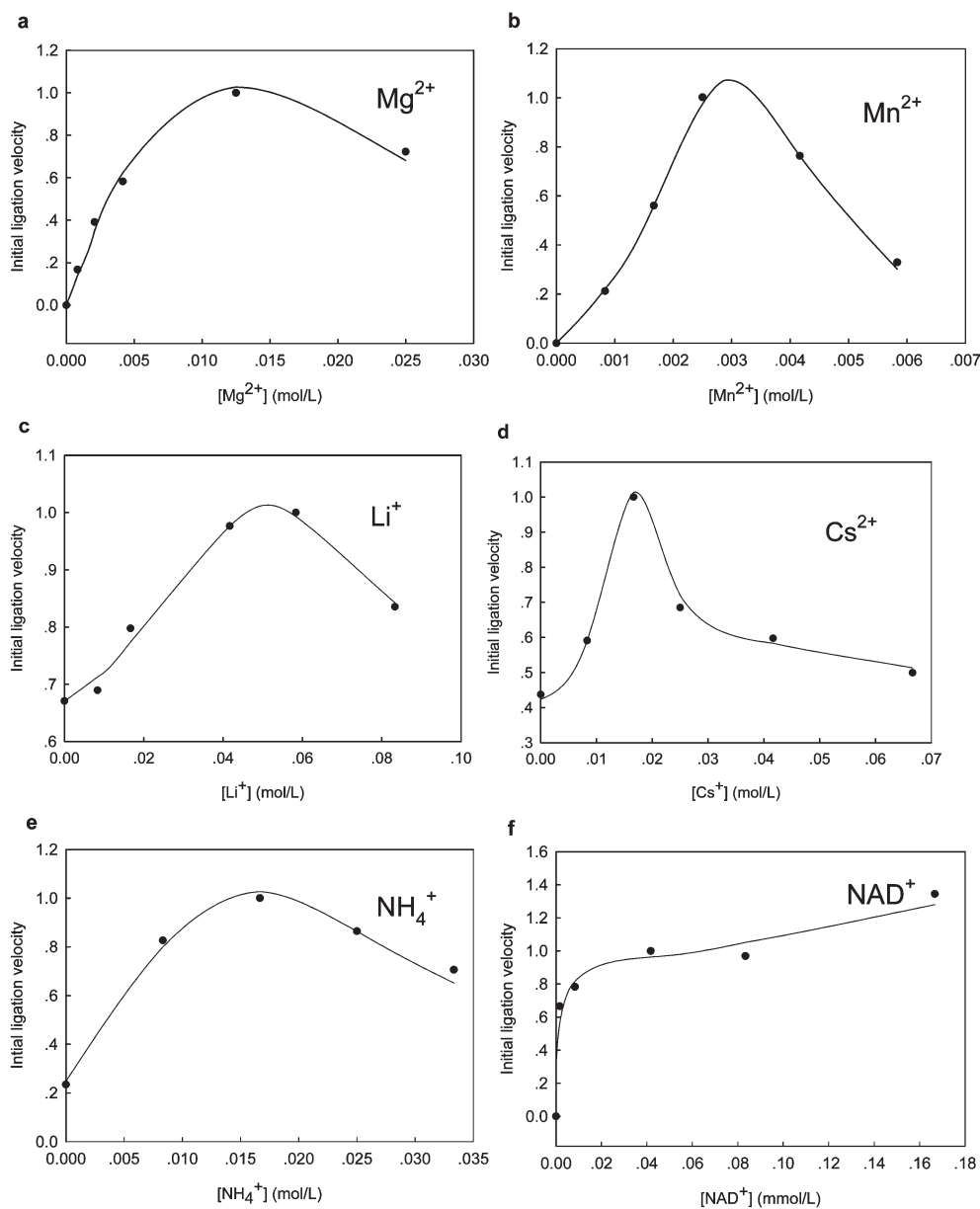
MB serves as a template of the ligation reaction as well as a probe for real-time monitoring. According to the base sequence of MB, the six types of base mismatches at the nick should be 5'-C:T, 5'-G:T, 5'-T:T and 3'-C:T, 3'-G:T, 3'-T:T, respectively. The mismatch effects on ligation are gauged by the initial velocity of the formation of 18-mer ligation products as a function of enzyme concentration. The initial velocity of the formation of ligation products is represented by an initial increasing velocity of the MB's fluorescence intensity. It turns out that *E. coli* DNA ligase is fairly critical for the base match at the nick, and its fidelity is better than the T4 DNA ligase studied before, which is more tolerant to base pair mismatch on both termini of the nick for successful ligation.<sup>27,28</sup> For *E. coli* DNA ligase, ligation almost cannot happen when a base mismatch exists at the nick. The experiments show that no ligation occurs for 5'-C:T, 3'-C:T, and 3'-T:T mismatches at the nick. Additionally, only less than 1% of the initial ligation velocity of the perfect base match could be obtained as the mismatches were 5'-G:T, 5'-T:T and 3'-G:T, respectively. This indicates that *E. coli* DNA ligase is more sensitive to mismatched base pairs on the 3'-side of the nick and more tolerant on the 5'-side, which is consistent with previous research results about the fidelity of  $\text{NAD}^+$ -dependent and ATP-dependent ligases.

The G:T base pair proves to cause a minimum of structural disturbance within the duplex of DNA. This mismatch is also found to be the most amenable to the *E. coli* DNA ligase reaction, which agrees with the previous study on T4 DNA ligase.<sup>27,28</sup> The geometry of the 5'-phosphate and 3'-hydroxyl group at the nick may have an effect on the mismatch ligation by DNA ligase. Mismatched base pairs close to the ligation point may disrupt the geometry of the nick on substrate DNA and change the relative position between the 3'-hydroxyl and 5'-phosphate, causing them to leave the AMP group, and thereby leading to alteration of the ligation rate.<sup>37</sup>

### Effects of external factors

Some monovalent cations and divalent cations among certain concentration ranges act as activators stimulating intermolecular ligation with *E. coli* DNA ligase, while high concentration  $\text{K}^+$  and  $\text{Na}^+$  serve as inhibitors to T4 DNA ligase.<sup>27,28</sup> By determining the effects of those cations on the ligase activity, useful information is obtained for the mechanism study, in general terms, of the metal-dependent enzymatic catalysis of the phosphoryl transfer reactions. Furthermore, an optimal reaction condition for the ligase can be achieved through the analysis of external factors, which will hopefully improve the sensitivity of the ligase assay.

As shown in Fig. 4, DNA ligases, like all enzymes, have cofactors concentration optima: a plot of initial ligation velocity against ion concentration produces a bell-shaped curve. However, the substrate for ligases and the complex of ligation products hybridized with MB, which are all duplex DNA, are also ion-sensitive. Experiments are carried out to examine the effects of  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Cs}^+$  and  $\text{NH}_4^+$  on those DNA hybrids formed by MB and ODNs. These experiments detect the fluorescence changes of the complexes in a series of solutions with different ions of various



**Fig. 4** Effects of metal-cofactors, monovalent ions and coenzyme on activity of *E. coli* DNA ligase. (a) Effect of Mg<sup>2+</sup>; (b) effect of Mn<sup>2+</sup>; (c) effect of Li<sup>+</sup>; (d) effect of Cs<sup>2+</sup>; (e) effect of NH<sub>4</sub><sup>+</sup>; (f) effect of NAD<sup>+</sup>.

concentrations. The results suggest that the fluorescence intensities of the complexes change very little in the ion concentration ranges while the ions act as activators for the ligase (data not shown). It indicates that these activators do not affect hybridization of the substrates, but may work in the following two ways: they combine with the enzyme, causing structure and conformation changes in the enzyme, and consequently affect the affinity of the enzyme for the DNA substrate or catalysis function of the enzyme.

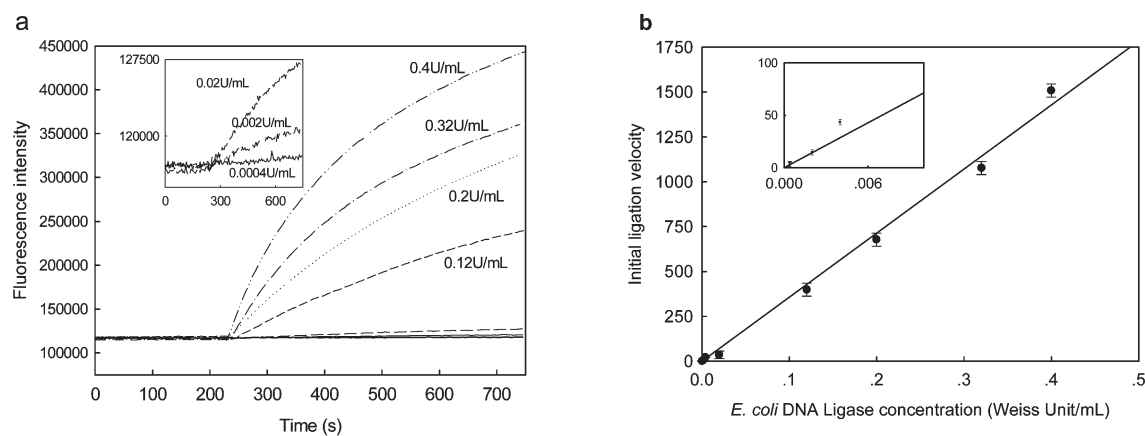
The ligase is active with either Mg<sup>2+</sup> or Mn<sup>2+</sup> as the metal cofactor. It also appears to be more active with Mn<sup>2+</sup> than with Mg<sup>2+</sup> at all metal concentrations. According to the experimental data, the higher initial ligation velocity and yield using Mn<sup>2+</sup> as the metal factor is probably due to a higher nick closure rate, resulting in lower accumulation of AppDNA.<sup>26</sup>

The ligase requires NAD<sup>+</sup> as a coenzyme, and the effect that the quantity of NAD<sup>+</sup> has on the catalysis efficiency of the ligase is also detected. It can be inferred from Fig. 4(f) that increased NAD<sup>+</sup> concentration greatly stimulates the catalysis of the ligase when the concentration is lower than 0.01 mM and cannot enhance the activity of the ligase much when the concentration is higher than 0.01 mM. The optimal initial ligation velocity has normalized all data.

#### Assay of the ligase

The ligation reaction in this experiment can be simply expressed as the following eqn. 1:





**Fig. 5** a, Real time monitoring curve of ligation catalyzed *E. coli* DNA ligase of different concentrations. b, Assay of *E. coli* DNA ligase: linear curve of initial ligation velocity to concentration of *E. coli* DNA ligase.

where E represents *E. coli* DNA ligase, S is the substrate formed by MB and the DNA fragments to be ligated, P is the product of ligation and  $K_1$  is the reaction constant of nucleic acids ligation reaction.

According to the Michaelis–Menten equation, the initial reaction velocity is determined by the concentration of enzyme and substances (as eqn. 2):<sup>33</sup>

$$V_0 = \frac{K_1[E][S]}{K_m + [S]} = \frac{K_1}{\frac{K_m}{[S]} + 1} [E] \quad (2)$$

where  $V_0$  is the initial reaction velocity,  $K_m$  is the Michaelis–Menten constant and  $[E]$  and  $[S]$  are the concentrations of total enzyme and substance, respectively. If  $[S]$  is kept as a settled value, the initial velocity would be directly proportional to  $[E]$  in a certain range. A fast assay method for *E. coli* DNA ligase has been developed based on the above proportional relationship, and the results are shown in Fig. 5.

If fluorescence intensity is directly proportional to the quantity of ligation product, then the initial velocity of DNA ligation can be represented by the initial enhancement rate of fluorescence intensity, and the relationship between  $V_0$  and  $[E]$  is illustrated in Fig. 5b. As we expected, in the range from  $4 \times 10^{-4}$  Weiss Unit  $\text{mL}^{-1}$  to 0.4 Weiss Unit  $\text{mL}^{-1}$ ,  $V_0$  is directly proportional to  $[E]$ , and this linear relationship could be elucidated as the following eqn (3):

$$V_0 = 3.57 \times 10^6 \times C_{\text{ligase}} \quad (3)$$

$V_0$  is the initial velocity;  $C_{\text{ligase}}$  represents the concentration of *E. coli* DNA ligase in  $\text{U mL}^{-1}$ . 8 min after the addition of  $4 \times 10^{-4}$   $\text{U mL}^{-1}$  ligase, the enhanced fluorescence signal is 3 times that of background noise. So, the detection limit of this assay method within 10 min is  $4 \times 10^{-4}$   $\text{U mL}^{-1}$ , which is at the same scale as the assay for T4 DNA ligase.<sup>27,28</sup>

## Conclusion

The activity of *E. coli* DNA ligase has been monitored in real time for the first time based on a developed innovative technique using a molecular beacon. Compared

with traditional methods for ligase study, the technique has four major advantages: (1) the novel approach has the potential to take the place of gel electrophoresis in drug screening for discovering novel bactericidal drugs targeting to  $\text{NAD}^+$ -dependent eubacterial DNA ligase because of its simplicity, veracity, and time-saving property; (2) it can be used for real time monitoring of ligases' activity, which would hopefully make it beneficial for research fields relevant to ligases, such as gene damage and repair, and detecting disease-associated mutations by LCR and OLA; (3) it could provide real time process information about nucleic acids ligation, which is of special significance for the study of kinetics as well as the mechanism of the nucleic acids' ligation process, and it blazes a brand-new trail leading to research on interactions between nucleic acids and proteins (enzymes); (4) moreover, it can be used for a sensitive and quick assay of ligases with many advantages compared with conventional methods. This novel approach provides a convenient, fast, and precise way for many investigations involving *E. coli* DNA ligase, such as DNA damage and repair, drug research, etc.

## Acknowledgements

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