Hairpin DNA probe based surface plasmon resonance biosensor used for the activity assay of *E. coli* DNA ligase

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Received 30th September 2009, Accepted 3rd December 2009
First published as an Advance Article on the web 22nd December 2009
DOI: 10.1039/b920228e

Using hairpin DNA probe self-structure change during DNA ligation process, a sensitive, label-free and simple method of *E. coli* DNA ligase assay *via* a home-built high-resolution surface plasmon resonance (SPR) instrument was developed. The DNA ligation process was monitored in real-time and the effects of single-base mutation on the DNA ligation process were investigated. Then an assay of *E. coli* DNA ligase was completed with a lower detection limit (0.6 nM), wider concentration range and better reproducibility. Moreover, the influence of Quinacrine on the activity of *E. coli* DNA ligase was also studied, which demonstrated that our method was useful for drug screening.

Introduction

DNA replication, repair and recombination are essential life processes in which many biomolecules such as proteins, enzyme and ribozymes participate. Among them, DNA ligases play an indispensable role in catalyzing the formation of phosphodiester bonds between adjacent 3′-hydroxyl and 5′-phosphate termini of duplex DNA.[1–4] DNA ligase assays are very important for researchers to expound the secrets of life, develop new functionalized medicine surmounting many difficult diseases and promote the development of information science.[3,5]

Traditionally, DNA ligase assays are usually limited to polyacrylamide gel electrophoresis and autoradiography, which are complex, discontinuous, or not sensitive.[6–8] Recently, there have been efforts to develop electrochemical and fluorescent detection methods.[9–13] Among them, one interesting research of electrochemical and fluorescent detection is that based on hairpin DNAs.[11,13,14] Hairpin DNAs are single-stranded DNAs with a stem-loop structure, which have been found to exhibit good stability, high sensitivity and excellent specificity. Due to these advantages, hairpin DNAs can be used for many applications including DNA/DNA, DNA/RNA studies and DNA-protein interaction assays.[15–18] Using hairpin DNAs, Zauner Gerhild and his coworkers realized the electrochemical assay of DNA ligation;[13] Wang's group explored the fluorescent detection of T4 DNA ligase and *E. coli* DNA ligase.[11,14] However, the fluorescent labeling technique and the purification process are time consuming, labor intensive and have a high cost, while some of the electrochemical measurements need to be labeled or feature low sensitivity, which have limited their practical applications. Therefore, some other simple and sensitive methods based on hairpin DNAs need to be developed for further application research on DNA ligase.

Surface plasmon resonance (SPR) has been demonstrated as a viable analytical method for rapid, sensitive and label-free analysis. Recently, in order to boost up the detection sensitivity and satisfy the aim of trace analysis, some home-made high-resolution SPRs have been developed. Combined with flow injection, a home-built high resolution SPR (FI-SPR) was used for DNA sensitive detection and with the FI-SPR, attomole quantities of target DNA could be detected.[19,20] Moreover, the home-built high resolution SPR was also combined with electrochemical detection (ESPR) and successfully used for the study of conformational changes of molecules like cytochrome c, peptides and $FcC_{11}SH$ self-assembled monolayers (SAMs).[21–23] All of these results indicated that the home-built high-resolution SPR instrument could achieve simple and high sensitive detection of molecular conformational changes.

Therefore, in this paper, combining our home-built high-resolution SPR with hairpin DNA probe, we explored a sensitive, label-free and simple *E. coli* DNA ligase biosensor. The principle (Fig. l) is as follows: After being immobilized on the Au film, the hairpin DNA probe hybridized with two single-stranded target DNA segments to form a hybrid with a nick. When encountered with *E. coli* DNA ligase, the nick was ligated, which would cause a conformation change of the hairpin DNA probe from the stem-loop structure to a rigid, linear double helix. This conformation conversion finally resulted in a great change in the SPR dip shift that could be recorded in situ. Using this method, we monitored the DNA ligation process in real-time and investigated the effects of single-base mutation on the DNA ligation process. Then an assay of *E. coli* DNA ligase was performed and a detection limit of 0.6 nM was obtained. Moreover, an effect of quinacrine on the activity of the ligase was detected, which certified that our method is helpful for drug screening.

Experimental

Materials

E. coli DNA ligase was purchased from Takara Biotechnology Co. Ltd. (Dalian, China). The DNA sequences for both probe and targets are as follows: Probe (5′-SH-$(CH_2)_6$-*CCTCTC* GTG

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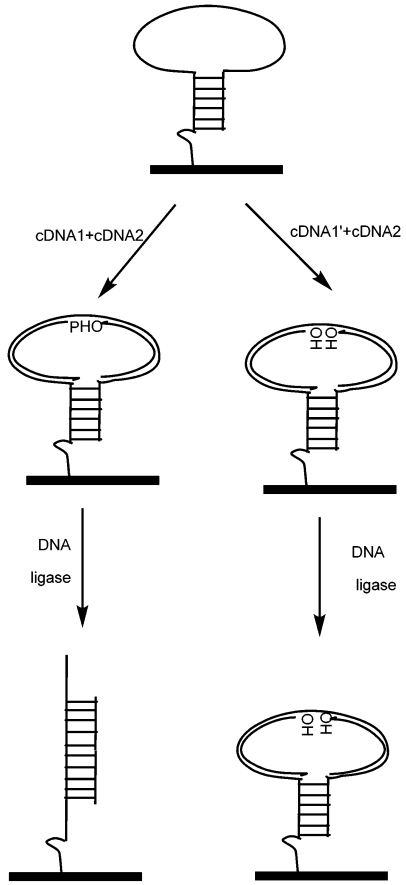

Fig. 1 Principle scheme of monitoring activity of *E. coli* DNA ligase based on hairpin DNA probe. P indicates 5′-phosphate; OH indicates 3′-hydroxyl. For clarity, all of the molecules were not drawn to scale.

TCT TG ATT CTC CCG TCA *GAGAGG*-3′); cDNA1 (5′-p-ATC AAG ACA -3′), cDNA1′ (5′-ATC AAG ACA -3′), cDNA2 (5′-GAC GGG AGA-3′), mDNA1 (5′-GAC GGG AG*C*-3′), mDNA2 (5′-GAC GGG AG*G*-3′), mDNA3 (5′-GAC G*T*G AGA-3′). cDNA and mDNA are the abbreviated forms of Complementary target DNA and Single-base mismatch target, respectively. The sequences in italics in the probe represent the stem of the hairpin DNA; the bases in italics in the mDNAs represent the mismatched ones. cDNA1′ has the same base sequence as cDNA1 but there is a hydroxyl at the 5′ end of cDNA1′ and a phosphate (P) at the 5′ end of cDNA1; mDNA1, mDNA2 and mDNA3, the single-base mismatched sequences of cDNA2 have a different mismatched base or mutation position. All of the sequences were purchased from Shanghai Shenggong Biotechnology Co. (Shanghai, China), and used without further purification. The probe DNA stock solution was prepared with 50 mM phosphate buffer solution (PBS, pH 7.0) containing 50 mM NaCl; the target stock solutions and *E. coli* DNA ligase diluents were prepared with 30 mM Tris-HCl buffer solution containing 4 mM $MgCl_2$ (pH 8.0), 0.1 mM NADH Na_2 and 0.005% BSA. 6-mercapto-1-hexanol (MCH) was acquired from Sigma (USA). Other reagents used were all obtained from Beijing Chemical Reagents Co. (Beijing, China). Millipore Milli Q (18MΩ cm) water was used in all experiments.

Instruments

Our home-built FI-SPR instrument, equipped with a bi-cell detector for high-resolution measurements, was used in this investigation. Briefly, the SPR instrument recorded reflected light falling onto the two cells at two (A and B) photodetectors, then both the subtraction (A − B) and the sum (A + B) signals were measured by a PCI-1731 interface card (Advantech, Taiwan) controlled by a Labview program. The ratio of the subtraction to sum signals, which is linearly proportional to the SPR angular shift, was obtained numerically by dividing (A − B) by (A + B).[20,21] To convert the ratio of (A − B)/(A + B) to the SPR dip shift, an instrumental calibration experiment was carried out using a method similar to that reported by Kolomenskii and his coworkers.[24] The incorporation of a bi-cell photodiode detector to measure the different signals resulted in a much-improved angular resolution (10^{-4}–10^{-5} degrees).[20,25] All of the *E. coli* DNA ligase solutions were introduced into the carrier solution by a 50-μl sample loop on a six-port valve and subsequently were delivered to the flow cell at a constant speed of 1.0 mL/h by a syringe pump (KDS100, KD Scientific Inc, Holliston, MA, USA).

Preparation of the SPR sensor surface

BK7 glass slides (Fisher) were treated in a hot piranha solution (30% H_2O_2 and 70% concentrated H_2SO_4 at 80 °C) for 30 min (Note: This is a dangerous cleaning solution, and care must be taken when handling the solution). On cooling to room temperature, the glass slides were rinsed thoroughly with deionized water. Before preparation of the gold substrate, the slides were dried by N_2 steam. Then, Au films of a 50-nm thickness and a 2-nm Cr underlayer were deposited onto the dried glass slides using a sputtering coater (Model 108, Kert J. Lester Inc., Clairton, PA).

For each experiment, 20 μl of 1 μM hairpin DNA probe solution was dropped onto the pretreated film and incubated overnight at 4 °C. After rinsing with PBS and drying with N_2, the modified film was blocked by 1.45×10^{-5} M MCH for 3 min. Then a 200 nM mixed solution containing two different single-stranded target DNA segments, both of which were matched with the loop of hairpin DNA probe, was dropped onto the modified film and allowed to react for 3 h. Finally, the modified films were rinsed with water thoroughly and dried with N_2.

Results and discussion

Selectivity and specificity of strand joining by *E. coli* DNA ligase

E. coli DNA ligase, a typical NAD^+-dependent DNA ligase, has been reported to catalyze the formation of phosphodiester bonds between adjacent 3′-hydroxyl and 5′-phosphate termini of duplex DNA.[26] In order to prove the feasibility of our method and demonstrate the DNA ligation process clearly, two groups of experiments were carried out. In one group, two target DNA segments (cDNA1 and cDNA2) respectively contained 5′-phosphate and 3′-hydroxyl; in the other group, both of the two target DNA segments (cDNA1′ and cDNA2) contained hydroxyl. Then, *E. coli* DNA ligase was introduced into the flow cell. The SPR dip shifts were recorded and the data are shown in Fig. 2. It

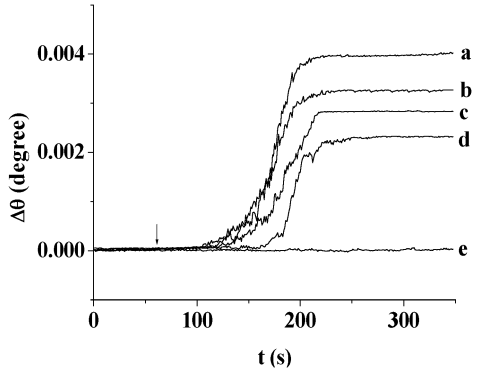

Fig. 2 SPR responses of hairpin DNA probe with different target modified Au films to the injections of 60 μM *E. coli* DNA ligase: (a) cDNA1 and cDNA2, (b) cDNA1 and mDNA3, (c) cDNA1 and mDNA2, (d) cDNA1 and mDNA1, and (e) cDNA1′ and cDNA2. The arrow indicates the time when the samples were injected to the flow cell.

is obvious that there is no SPR signal change for cDNA1′ and cDNA2 (curve e) while a much larger SPR dip shift occurs for cDNA1 and cDNA2 (curve a, 0.0038°). This experimental result verifies that *E. coli* DNA ligase doesn′t react on 3′-hydroxyl and 5′-hydroxyl but can catalyze the formation of phosphodiester bonds between adjacent 3′-hydroxyl and 5′-phosphate termini of duplex DNA. The ligation causes a hairpin DNA probe self-structure change from the stem-loop structure to a rigid and linear double helix, which induces a large refractive index change and finally causes a great SPR dip shift change. This result validates that this simple and label-free method is amenable to monitor DNA ligation processes in real-time.

To further understand the DNA ligation process, the effects of base mutation on DNA ligation process were also studied. Three different targets were used: cDNA1 was complementary with one side of hairpin DNA probe while both mDNA1 and mDNA2 were incompletely matched with the other side of hairpin DNA probe. mDNA1 contained a single mismatched base C (C:T) while mDNA2 had a single mismatched base G (G:T) at the 3′ end of their own segment, and both of the mutation positions were just at the nick. As shown in Fig. 2, the net SPR dip shift for cDNA1 and mDNA1 (curve d) is smaller than that for cDNA1 and mDNA2 (curve c) and both of these SPR dip shifts are smaller than that for cDNA1 and cDNA2 (curve a), which demonstrates that DNA ligation efficiency is affected by base mutation and our method can be used to conveniently analyze the specificity of DNA ligation. These differences of DNA ligation efficiency may be due to two reasons. On the one hand, mismatched base pairs close to the ligation point may disrupt the geometry of the nick on the substrate DNA and change the relative position between the 3′-hydroxyl and the 5′-phosphate, causing them to leave the AMP group leading to a decrease in the efficiency of DNA ligation.[11,27] On the other hand, even at the same mutation position, the influence of different bases on hybridization efficiency is different. Perhaps more mDNA2 hybridizes with the hairpin DNA probe and forms more hybrids with a nick. When encountered with *E. coli* DNA ligase, more nicks were ligated making larger conformation changes occur and causing a greater change in SPR dip shift.

In order to confirm which is the main reason, another group of target DNAs, cDNA1 and mDNA3, was investigated. mDNA3 (C:T) had the same mismatched base as mDNA1 (C:T), however, different from mDNA1, its mutation position was in the center of the fragment. According to the reference, mDNA3 would have a lower hybridization efficiency than mDNA1.[28,29] If the second reason mentioned above is the main one, there will be a smaller SPR dip shift for cDNA1 and mDNA3 than that for cDNA1 and mDNA1. However, the experimental result is the opposite: The SPR dip shift for cDNA1 and mDNA3 (curve b in Fig. 2) is larger than that for cDNA1 and mDNA1, and even greater than that for cDNA1 and mDNA2.

From the results, it can be seen that the geometry of the 3′-hydroxyl and the 5′-phosphate group at the nick has a mainly effect on the mutation ligation by DNA ligase. From this point, the increasing specificity of DNA ligation in this present system should owe to the conformational constrains of the loop structure of hairpin DNA probe, that is, the special loop structure of hairpin DNA probe makes the geometry conditions of ligation much harsher. In addition, at both 3′ ends, the effects of different base mutation were different. Compared with 3′-C:T, the 3′-G:T base pair causes a smaller structural disturbance within the duplex of DNA, so it causes a higher ligation efficiency and leads to a greater change in SPR dip shift. This study is helpful for us to further understand the DNA ligation mechanism.

Assay of *E. coli* DNA ligase

Then, using this simple hairpin DNA probe system, the effect of the *E. coli* DNA ligase concentration on the ligation efficiency was investigated. As shown in Fig. 3, the SPR dip shifts increase with the increasing concentrations of *E. coli* DNA ligase, which suggests that the method can be used for quantitative measurement of DNA ligase. In order to display this trend more clearly, a linear relationship between the SPR response and the logarithm of the target concentration from 0.6 nM to 60 μM is also given ($r = 0.997$) (Fig. 4). It is obvious that the SPR dip shifts proportionally increase with the ligase concentration in a wide concentration range (5 orders of magnitude), which suggests this method can certainly be used to assess DNA ligase activity in a wide concentration range.

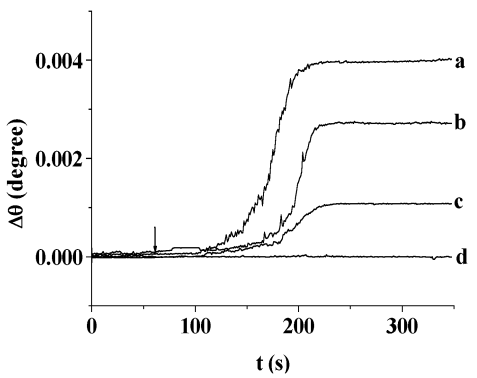

Fig. 3 SPR responses of hairpin DNA probe with cDNA1 and cDNA2 modified Au films to the injections of (a) 60, (b) 6×10^{-1}, (c) 6×10^{-4} and (d) 0 μM *E. coli* DNA ligase. The arrow indicates the time when the samples were injected to the flow cell.

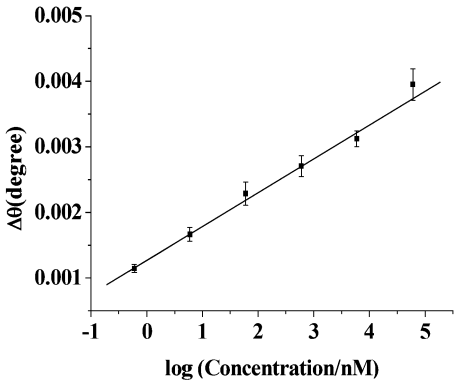

Fig. 4 SPR responses plotted as a function of *E. coli* DNA ligase. The six DNA ligase concentrations are 0.6 nM, 6 nM, 60 nM, 600 nM, 6 μM, 60 μM, respectively. The absolute uncertainties are computed from at least three replicate measurements and shown as the error bars.

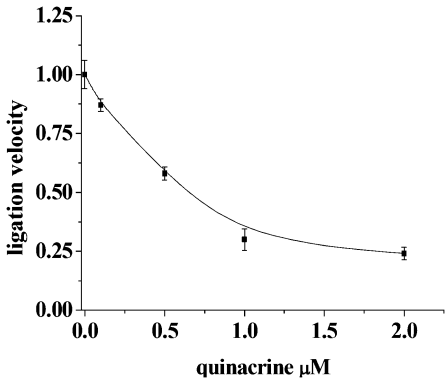

Fig. 5 Effect of quinacrine·2HCl on the activity of *E. coli* DNA ligase (60 μM). The five quinacrine·2HCl concentrations aere 0, 0.1, 0.5, 1, 2 μM, respectively. The absolute uncertainties are computed from at least three replicate measurements and shown as the error bars.

In addition, from Fig. 3, it can be seen that the net dip shift in curve c is approximately 0.0011° greater than that shown in curve d, indicating that 0.6 nM *E. coli* DNA ligase can be detected by our high-resolution SPR easily. Such a detection value is far lower than that of measurements based on electrochemistry,[12] compares favourably with those of fluorescence measurements based on hairpin DNA,[11,14] and offers a detection limit that is also comparable to those of fluorescence resonance energy transfer.[9,10] Moreover, the home-built SPR instrument has been used for DNA ultrasensitive detection[19,20] and successfully used for the study of conformational changes of molecules like cytochrome c, peptides and $FcC_{11}SH$ SAMs,[21–23] which demonstrated that this instrument has advantages on sensitive detection. In this study, for 0.6 nM *E. coli* DNA ligase, the SPR response is about 0.0011°, which is higher than the detection limit of this instrument (10^{-4}–10^{-5} degrees),[20,25] so the data is believable. Importantly, compared with fluorescence measurements, our present system without any labeling can not only greatly simplify the experimental process but also reduce the cost of the experiment. We attribute the improvement of detection limit to the high sensitivity of our home-built SPR instrument and the conformation change of hairpin DNA probe. During the DNA ligation process, the hairpin DNA probe change from the stem-loop structure to a rigid and linear double helix, this conformation conversion results in a larger refractive index change and finally causes a greater change in the SPR dip shift.

Besides the wide range of measurements and high sensitivity, good reproducibility is also one of the features of this method. Just because of the simpleness of the hairpin DNA probe system, the relative standard deviations (RSD, all <8%) computed from at least three replicate measurements, shown as the error bars in the figure, are relatively small and acceptable among the typical analytical techniques,[30] suggesting that the method is quite reproducible. Then with a label-free and simple system, a DNA ligase assay with a wider range of measurement, lower detection limit and better reproducibility is developed.

Effect of Quinacrine·2HCl

Quinacrine·2HCl, a drug commonly used for malaria, is reported to be a specific inhibitor of *E. coli* DNA ligase and useful for the study of the interaction of the inhibitor with ligases.[5] Here, we extended our method to the study of *E. coli* DNA ligase reactions in the presence of different concentrations of Quinacrine·2HCl, ranging from 0 to 2 μM. The results are shown in Fig. 5. The activity of the ligase is represented by the ligation velocity calculated from the net SPR dip shift of a mixture of 60 μM ligase and appropriate concentrations Quinacrine·2HCl divided by that of the ligase (60 μM). As shown in Fig. 5, Quinacrine·2HCl caused significant inhibition of *E. coli* DNA ligase activity in a dose-dependent manner. Moreover, it is estimated that the activity of the *E. coli* DNA ligase is inhibited by 50% when the concentration of Quinacrine·2HCl is around 0.58 μM. This study demonstrates that our method can be used for the identification of specific and potential inhibitors of NAD^+-dependent DNA ligases, which is helpful for novel drug screening.

Conclusion

In summary, making use of hairpin DNA probe self-structure change during the DNA ligation process we have developed a sensitive, label-free and simple bioassay for real-time monitoring of *E. coli* DNA ligase. This method could provide real time process information about DNA ligation and could be used to investigate the specificity of strand joining, which is especially significant for the study of the mechanism of the DNA ligation process. Using this method we obtained a detection limit of 0.6 nM for *E. coli* DNA ligase, which was lower than those of fluorescence measurements of DNA ligase based on hairpin DNA and even comparable to those of fluorescence resonance energy transfer. Moreover, the effect of Quinacrine on the activity of *E. coli* DNA ligase was also studied, which demonstrated that our method is useful for novel drug screening.

Acknowledgements

This work was supported by grants from the Ministry of Science and Technology of China (973 program No. 2006CB705601), State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, KF2008-15.

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