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DNA Ligase Mediated Ag^+ Ion Sensor

Razack Abdullah and In Seok Hong*

*Department of Chemistry, Kongju National University, Chungnam 314-701, Korea. *E-mail: ishong@kongju.ac.kr*
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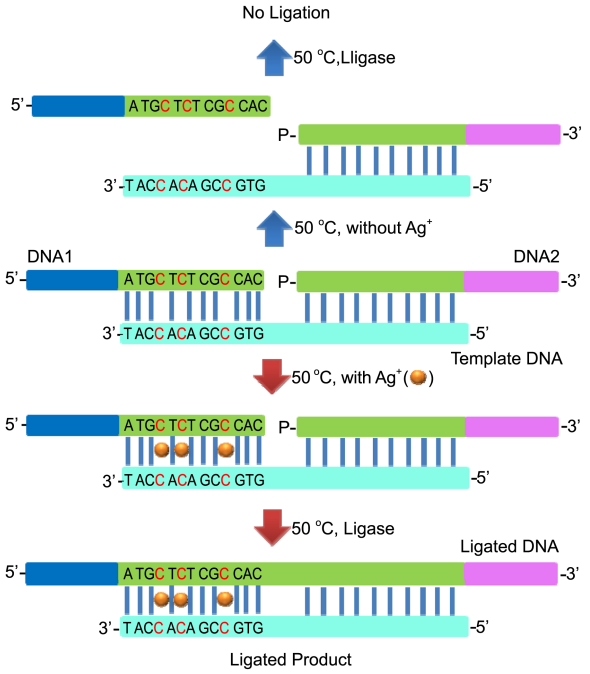
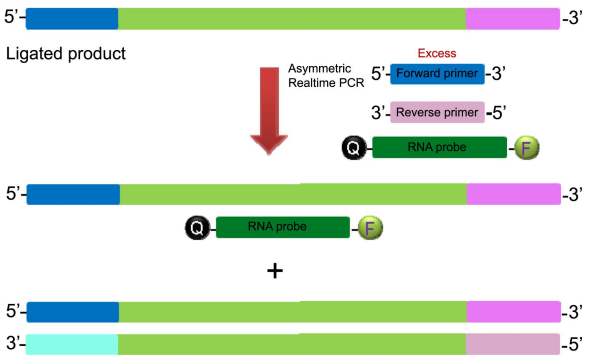
Silver and its alloys are widely used as superconductors and dental amalgam as well as in photography, batteries, medicines, lubricants, mirror, and jewelry.[1] Every year, tons of silver and silver compounds are released into the aqueous environment from emissions and industrial waste.[2] Several compounds of silver are toxic to living organisms.[1] Therefore, it is of high importance to develop highly sensitive and selective methods to detect trace amounts of silver ions in an aqueous environment. A number of methods using organic fluorophores,[3] quantum dots,[4] ion selective electrodes,[5] inductively coupled plasma-mass spectroscopy (ICP-MS),[6] atomic absorption spectroscopy,[7] gold nanoparticles,[8] and carbon based materials[9] have been developed for the sensitive, simple, and rapid detection of silver ions in aqueous solution. Although these methods have their own benefits, they still present some limitations such as low water solubility, poor selectivity, and insufficient sensitivity. Recently, Shionoya *et al.* reported that DNA duplexes possessing metallo-base pairs exchibited higher thermo stability than natural hydrogen-bonded DNA.[10] Furthermore, Ono *et al.* reported that Ag^+ ions are capable of selectively binding to cytosine (C) bases and forming strong and stable C-Ag-C complexes.[11] This discovery led to the development of oligonucleotide-based sensors to detect Ag^+ ions in an aqueous solution.[11,12] This approach provides a highly sensitive detection of Ag^+ ions, but still presents a few limitations including a complex process, low sensitivity, and especially, the requirement of fluorophore labeling. Additionally, many metal ions, including Ag^+ ions, can quench fluorescence signals,[13] thus, possibly affecting the sensitivity of fluorescence measurement.

To overcome these problems, we designed a novel and highly sensitive strategy involving an enzymatic ligation and quantitative PCR (qPCR) to detect Ag^+ ions. As presented in Scheme 1, the first step was DNA ligation, in which silver ions were detected and the second step was signal amplification of the ligated template DNA. The rate of DNA amplification by qPCR can be directly correlated to the amount of silver ions in the ligation reaction. In this system, three oligonucleotides were used for DNA ligation. One is a template DNA for ligation, and the other two oligomers were a full matched sequence DNA and a mismatched DNA oligomer to the template. The mismatched oligomer had three Cs located near the 3′-terminal site and mismatched to the Cs of the template DNA. The full matched DNA strand has a 5′-phosphate group for ligation. All the three oligomers for ligation reaction could be hybridized at a given temperature. The sequence of each oligomer was designed to adjust the melting temperature (T_m) of the template DNA and matched/mismatched oligomers. The calculated T_m value of the template and matched DNA was 59.7 °C under a salt concentration of 25 mM Na^+ and 5 mM Mg^{2+}.



Scheme 1. Ligase-mediated silver ion sensing.

The calculated T_m of the template and mismatched oligomer was designed to be less than 30 °C. Thus, if the ligation



Scheme 2. Amplification of the ligated product by asymmetric qPCR.

reaction is carried out at 50 °C, the ligated product is not formed at this ligation temperature because the mismatched oligomer cannot hybridize to the template DNA. However, in the presence of silver ions, silver ions can bind to the C/C mismatched bases between the mismatched oligomer and the template DNA, which produce stable C-Ag-C complexes. Eventually, the T_m value of the mismatched oligomer to the template was increased up to 50 °C, which is why the two mismatched and matched strands were ligated at 50 °C by thermo-stable DNA ligase.

The ligated products were further amplified by asymmetric qPCR[14] (Scheme 2). Originally, the ligated target sequence was designed to have the sequences corresponding to the 5′- and 3′-primers. Under excess quantities of forward primers, the PNA probe can bind to the forward primer extended single strand DNAs. Thus, the fluorescence intensity increased proportionally to the number of single strand DNAs present in solution, and was recorded in each cycle of qPCR. The Ct values (threshold crossing PCR cycles) can be directly correlated to the amount of ligated products, which are produced by the ligation reaction in the presence of silver ions.

Experimental Section

The oligomers were purchased from Bioneer Inc (Korea). The oligomer sequences were 5′-AAG GCC TGC TGA AAA TGA CTA TGC TCT CGC CAC-3′ (DNA1), 5′-P-TAG CTC CCT TAC CAA TGA CTT CTT TGC ATA TTA CTG GTG CAG GAC C-3′ (DNA2), 5′-GTC ATT GGT AAG GGA GCT AGT GCC GAC ACC AT-3′ (Template DNA), 5′-AAG GCC TGC TGA AAA TGA CT-3′ (Forward primer), and 5′-GGT CCT GCA CCA GTA ATA TGC A-3′ (Reverse primer). PCR premix solution and PNA probe were specially designed from Panagene Corp (Korea). The sequence of the PNA probe was Dab-GGA GCT AGT GGC G-(OEK)-FAM, it contains a dabcyl quencher and a FAM group at the *N* and *C*- terminal sites, respectively. Silver nitrate and co-enzyme (β-NAD) were purchased from Sigma-Aldrich. The thermo-stable DNA ligase (*Tfi* DNA ligase) was purchased from Bioneer Inc. (Korea), and further purified using the desalting method with an Amicon filter (30-kDa cutoff) to remove metal chelating agents such as EDTA and DDT as well as other interfering chloride ions. The desalted ligase was reassembled with freshly prepared reaction buffer (10x; pH 8.3, 100 mM HEPES, 0.5 M $Mg(ClO_4)_2$, and 0.25 M $NaNO_3$).

The enzymatic ligation was carried out as follows. Each oligomer DNA1, DNA2, and template DNA (each 1 μM) were mixed with the reaction buffer. Silver ions were added to each reaction solution and the final volume was adjusted to 20 μL with sterilized water to reach a final 1x reaction buffer condition (pH 8.3, 10 mM HEPES, 50 mM $Mg(ClO_4)_2$, and 25 mM $NaNO_3$). The concentrations of silver ions in each tube were 0, 1.5, 3, 6, 9, and 12 μM respectively. The reaction solutions were kept at 50°C on the preheated heat-block for 30 min, and then the ligase (2 μL, 2 U/μL) and coenzyme (2 μL, 0.5 mM) were added to the solution. The ligation reaction was performed at 50 °C for 2 h, and then quenched by cooling the solution to 0 °C. The reaction mixture was diluted and an aliquot was used for qPCR. qPCR was performed using an asymmetric PCR method on Bio-Rad C1000 thermal cycler. The PCR solution consisted of the ligated template DNA (1 μL), forward primer (0.8 μM), reverse primer (0.06 μM), PNA probe (0.8 μM) and PCR premix (10 μL). qPCR was programmed for 15 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 30 sec at 56 °C and 15 sec at 72 °C.

Results and Discussion

In our system, the T_m value of the mismatched pairs between DNA1 and template DNA is critical to control the ligation reaction. Thus, three Cs mismatched DNA1 was designed to lower sufficiently its T_m value. No ligation was expected to occur in absence of silver ions at high temperature (50 °C). The binding constant of silver ions to a C-C mismatch base is known to be 10^6 M^{-1}.[15] Thus, 1 μM of each DNA was used for stabilization of the enzymatic ligation with increasing concentration of silver ions. The thermo-stable *Tfi* DNA ligase was chosen for the ligation reaction under hot temperature. It is active between 45 °C and 65 °C. As expected in control conditions (absence of silver ions), no amplification of the ligated DNA was observed at 50 °C. After addition of silver ions, the oligomers can specifically form C-Ag-C complexes between the DNA1 and template DNA inducing an increase in the T_m value of the complexes above 50 °C. Therefore, a stable duplex was formed at 50 °C. *Tfi* DNA ligase recognized the nicked duplex strands

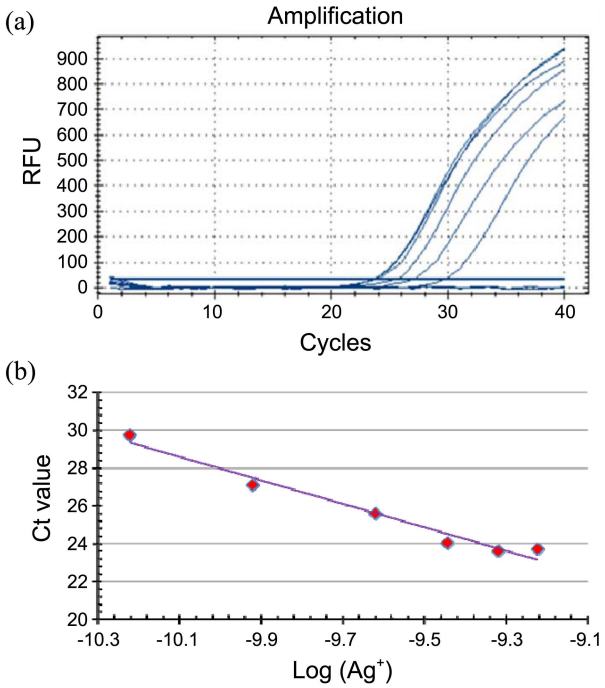


Figure 1. (a) qPCR amplification results, (b) Correlation curve between Ct values and the added molar amounts of silver ions.

and ligated the nicked duplex to produce the ligated DNA target for subsequent qPCR.

As the amount of silver ions in the ligation reaction increased, the quantity of ligated products generated increased. Thus, the amplification signal appeared earlier in qPCR. Figure 1(a) presents the qPCR profile of the ligated targets generated using increasing concentration of silver ions in the ligation reaction. In theory, Ct values are correlated with an initial copy number of DNA target. As shown in Figure 1(b), the obtained Ct values are correlated with the concentrations of silver ions (0.06, 0.12, 0.24, 0.36, 0.48 and 0.60 nM) in qPCR.

If silver ions are sequentially added to the C-C mismatched pair then, theoretically, at least 3 equivalents of silver ions are required to form a stable DNA duplex because of the presence of three Cs mismatches in DNA1. However, when just 1.5 equivalents of silver ions were added, a significant amplification of the ligated target was observed. This demonstrates that silver ions were preferentially added to the already silver-bound C-Ag-C complex strand to make DNA duplexes more stable. In other words, the binding of one silver ion to the C-C mismatch facilitates the other silver ion to bind to the adjacent C-C mismatch on the same strand to stabilize the duplex formation. When over 9 equivalents of silver ions were added to the solution, the Ct values were almost saturated, which reflects that the binding constant of C-Ag-C complex was not strong enough to complete the equimolar binding of the Ag^+ and the C-C mismatches.

The detection limit of silver ions in this system was 1.5 pmol, which corresponds to the 1 μL of the 1.5 μM of silver ion solution used for the ligation reaction. Since the aliquots were diluted, 1000x, the amount of silver ions detectable by qPCR at the femtomolar level. When the ligation reaction was carried out using the full matched sequences of DNA1 in absence of silver ions, a Ct value of 17 was observed indicating that the efficiency of the enzymatic ligation for the C-Ag-C complexes was less than 100%. This may be due to the position of C-Ag-C complex close to the ligation site. To improve the efficiency of the ligation, further studies including adjustment of the sites and the number of C-C mismatches are in progress.

Conclusion

The present work demonstrates a simple two-step strategy for the detection of Ag^+ ions. This is the first report on the use of an enzymatic ligase technique and qPCR for the detection of Ag^+ ions. This strategy makes a way to extend the enzymatic alphabet for the detection of metal ions. The detection limit of silver ions in this method was 1.5 pmol in the ligation reaction.

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