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The ODN probes conjugating the Cu(II) complex enhance the luminol chemiluminescence by assembling on the DNA template

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ABSTRACT

Potent peroxidase-like activity of the β-ketoenamine (**1**)–dicopper (II) complex (**2**) for the chemiluminescence (CL) of luminol either in the presence or absence of H_2O_2 has been previously demonstrated by our group. In this study, the β-ketoenamine (**1**) as the ligand unit for copper(II) was incorporated into the oligonucleotide (ODN) probes. It has been shown that the catalytic activity of the ODN probes conjugating the ligand–Cu(II) complex is activated by hybridization with the target DNA with the complementary sequence. Thus, this study has successfully demonstrated the basic concept for the sensitive detection of nucleic acids by CL based on the template-inductive activation of the catalytic unit for CL.

1. Introduction

The sensitive detection of nucleic acids plays an essential role in gene diagnosis and biological research for both extra- and intracellular events.[1] A high selectivity is required to recognize not only the sequence, but also any minute difference in a nucleoside such as single nucleotide polymorphism (SNP) that is closely related to the onset of diseases and response to drugs.[2] Conventionally, DNA is analyzed after PCR amplification. On the other hand, it has become an attractive theme to develop an efficient method for the detection of intact nucleic acids without any amplification procedure. In the molecular beacon strategy, the fluorescence intensity of the probe is enhanced by forming a complex with the target nucleic acids.[3–5] The alternative approach utilizes the target nucleic acid as the template to catalyze the chemical reactions[6–9] in order to amplify the signal of the complex formation. Several chemiluminescence (CL) detection systems have been developed based on the peroxidase-like activity that is induced when the hemin–quadruplex assembly is formed on the target sequence.[10] We have previously developed a new catalytic system for the CL of luminol, in which the dicopper(II) complexes catalyze the reduction of dissolved molecular oxygen and subsequent oxidation of luminol to induce CL.[11] This system is characteristic in that the corresponding monocopper is inactive and gains catalytic activity upon assembling the dicopper complex. In this paper, we describe in detail the synthesis and the application of the oligonucleotides probes incorporating the copper(II) complex for detection of nucleic acids by CL.

2. Results and discussion

The β-ketoenamine (**1**) as the ligand part for copper(II) was designed to be conjugated with the oligonucleotide (ODN) probe at the 5′ end (probe**1**) or at the 3′ end (probe**2**) through an appropriate linker (Fig. 1B). It was expected that the two probes would be assembled on the target nucleic acid to form a dicopper complex, thus inducting the catalytic activity for the CL of luminol (Fig. 1A). In our previous study, the β-ketoenamine ligand (**1**) was mixed with $Cu(OAc)_2$ to form the dicopper complexes, which exhibit a high catalytic activity for the CL of luminol either in the presence or in the absence of H_2O_2 (Fig. 2). To determine the site of the ligand for conjugation with the ODN probe, the complex structure formed with the ligand **1** and $Cu(OAc)_2$ was investigated by an X-ray crystal analysis. It has been revealed that the complex is multinuclear with a cubane substructure (Fig. 2A).[12] The dicopper complex part (**2**) and the tetranuclear complex part (**3**) are assembled to form a cubane structure as shown by the dotted line in Figure 2B. Although it was previously anticipated that the primary hydroxyl groups participated in the coordination with the copper ion,[11,13] the complex structure shown in Figure 2 has clearly indicated that some primary hydroxyl groups remain un-coordinated. Crystals suitable for an X-ray analysis were only obtained from a solution in AcOEt as the complexes with AcOEt

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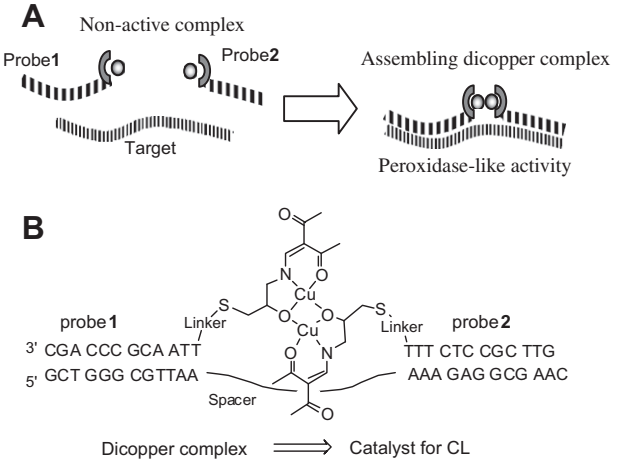


Figure 1. (A) Strategy for assembling dicopper complex on the target sequence. (B) Schematic structure of the ODN–ligand conjugates for the dicopper complex as the catalysis for CL.

molecules. The ESI-MS of the complexes obtained in MeOH indicates only 2+2 complexes. Thus, the primary hydroxyl group was chosen as the site for attachment to the ODN probe at the 3′-terminal or at the 5′-terminal.

The synthesis of the ODN–ligand conjugated probe is shown in Scheme 1. The primary alcohol group of **1** was converted to the tosylate. The 5′-thiol-modified ODNs and 3′-thiol-modified ODNs having different alkyl linkers ($n = 1$ or 2) were reacted with the tosylate (**4**) in a solution of sodium bicarbonate buffer and DMF (9:1) at 50 °C, and the reaction was followed by HPLC. An example of the HPLC charts is shown in Figure 3. After 6 h, a peak around 12 min of the 5′-thiol-modified ODN disappeared, and a new peak corresponding to the conjugated product (Probe**2** ($n = 2$)) appeared around 13 min. After purification by HPLC, the conjugated structure was confirmed by MALDI-TOF MS measurements.

Before investigation of the catalysis of the ODN–ligand conjugates for the CL of luminol, we checked the appropriate pH for the formation of the dicopper complex by UV–vis spectroscopy using the monomeric ligand (**1**) in a buffer solution. The absorption bands at 650 nm for the copper acetate at pH 9 was shifted to 620 nm at pH 12, indicating the formation of the dicopper complexes (Fig. 4).[12] These results suggested that the CL experiments with the ODN–ligand conjugates should be carried out at pH 12.

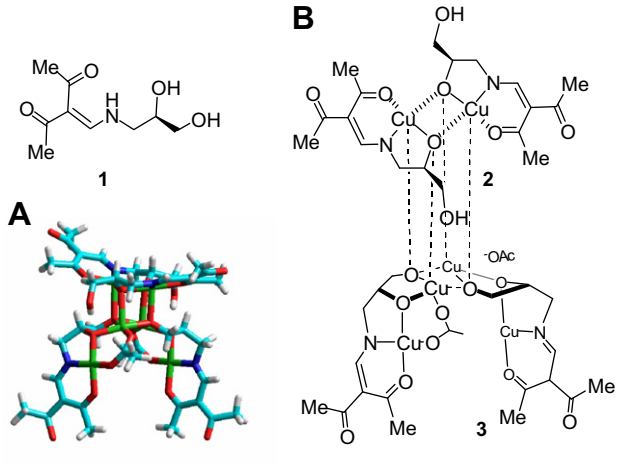
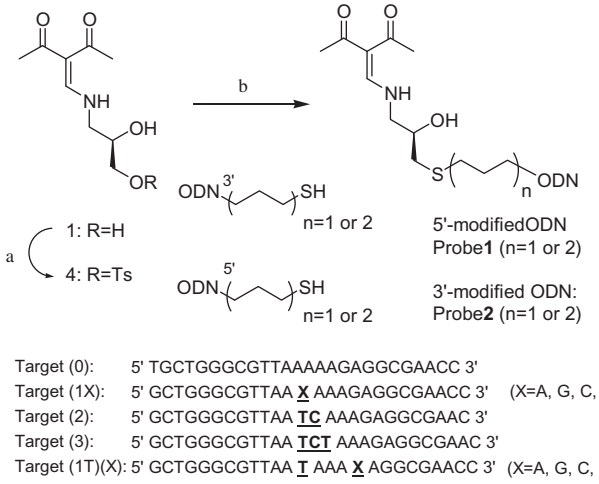


Figure 2. The crystal structures of the multinuclear copper complex (A) and its schematic drawing (B). Solvents (AcOEt) are not shown for clarity. AcO^- represents an acetate bridging two Cu(II).



Scheme 1. The synthesis of the ligand conjugated ODN probes (**1** and **2**) and the target sequence. Reagents and conditions: (a) *p*-TsCl, pyridine, 0 °C, 81%; (b) thiol-modified ODNs, $NaHCO_3$ aq/DMF (9:1), 50 °C, 6 h.

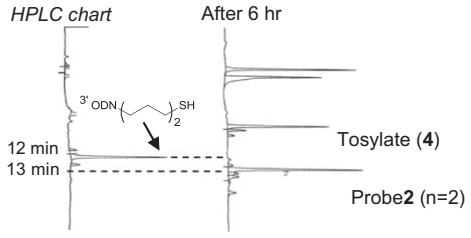


Figure 3. The HPLC chart of the conjugation reactions. HPLC conditions: column; shiseido CAPCELL PAK C18, 4.6 × 250 mm, flow rate; 1.0 mL/min, buffer; A 0.1 M TEAA, B CH_3CN, B concn 10–30%/20 min linear gradient, column oven; 35 °C, UV-detector; 254 nm.

To optimize the alkyl linker length of the ODN probes ($n = 1$ or 2) and the spacer part of the target sequences with the different nucleotide numbers between the probe binding sites, we tested the luminol CL properties using all combinations of the ODN–ligand probes and the target sequences (Scheme 1). The number in the parenthesis of the Target represents the number of nucleotides between the probe binding sites, for instance, Target (**2**) contains the C–T spacer and Target (**3**) has the T–C–T spacer. The different nucleotide is inserted in Target (**1**), for example, Target (**1T**) contains the T nucleotide as the spacer between the probe binding sites.

The CL reactions were started by the addition of luminol sodium and ascorbic acid into the mixture of the probes and the target in the presence of $Cu(OAc)_2$, and the CL intensities were periodically recorded. The results are summarized in Figure 5. Among all the combinations, Probe**1** ($n = 2$) and Probe**2** ($n = 1$) showed a good response to the Target (**1T**). Although a detailed structure of the complex between the probes and target was not clear, it was clearly shown that the catalytic activity of the probes for the CL reaction significantly depended on the distance of the linker of the probes and spacer of the target DNA. Interestingly, although the combination of Probe**1** ($n = 1$) and Probe**2** ($n = 2$) would have an equivalent alkyl linker length with that of Probe**1** ($n = 2$) and Probe**2** ($n = 1$), they showed a low activity for the CL reaction. In the case of the combination of Probe**1** ($n = 2$) and Probe**2** ($n = 2$), the CL intensity is low in comparison to the other combinations. Based on these results, it turned out that the combined use of Probe**1** ($n = 2$) bearing

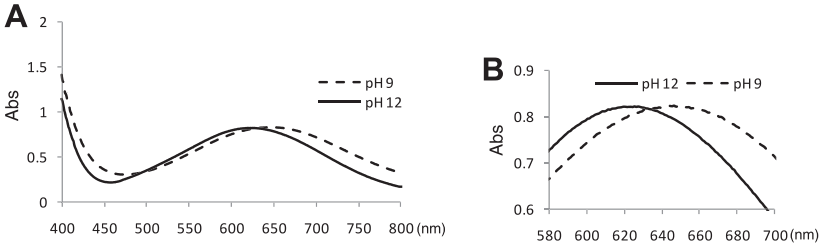


Figure 4. (A) UV-spectra of 10 mM ligand (**1**) in a borate buffer (H_3BO_3–NaOH) contains the 10 mM $Cu(OAc)_2$ at pH 9 or pH 12. (B) Expanded UV-spectra.

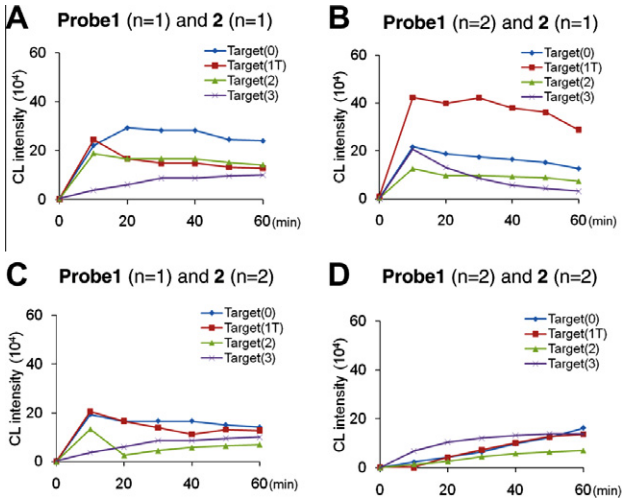


Figure 5. The CL intensity of the combination use of probes**1**, **2**, and Target. The reaction was performed using 1 μM of each oligonucleotides, 25 μM luminol sodium salt, 25 μM ascorbic acid, and 5 μM $Cu(OAc)_2$ in a borate buffer (50 mM H_3BO_3–NaOH, pH 12). Reaction was started by the addition of luminol sodium salt and ascorbic acid.

the C6 alkyl linker and Probe**2** ($n = 1$) bearing the C3 alkyl linker is suitable for detecting the target sequence (1) with one nucleotide spacer between the probe binding sites. The UV melting temperature (T_m) of the combination of Probe**1** ($n = 2$), Probe**2** ($n = 1$), and Target (**1T**) were measured in the presence or absence of $Cu(OAc)_2$.[14] The T_m value in the presence of $Cu(OAc)_2$ was 3.9 °C higher than in the absence of $Cu(OAc)_2$. In contrast, the T_m value of the combination of Target (**1T**) and the corresponding non-conjugated probes decreased by 1.3 °C in the presence of $Cu(OAc)_2$. These results also supported the fact that the hybridized complexes formed between the ODN probes and the target DNA are stabilized by forming complexes with $Cu(OAc)_2$.

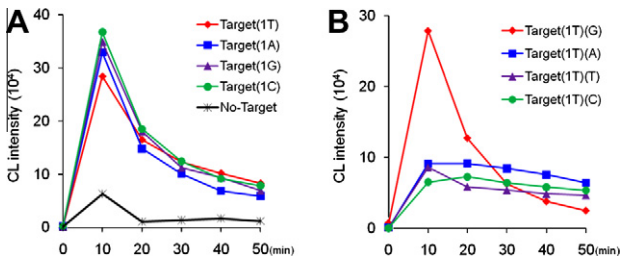


Figure 6. The selectivity of the Probe**1** ($n = 2$) and Probe**2** ($n = 1$) to the target sequence. (A) The comparison of CL with Target (**1T**), (**1A**), (**1G**), and (**1C**). (B) The discrimination of the single nucleotide difference in Target (**1T**)(**G**), (**1T**)(**A**), (**1T**)(**T**) or (**1T**)(**C**). These reactions were performed using 1 μM of each oligonucleotide, 25 μM luminol sodium salt, 25 μM ascorbic acid, and 5 μM $Cu(OAc)_2$ in a borate buffer (50 mM H_3BO_3–NaOH, pH 12). Reaction was started at adding of $Cu(OAc)_2$.

Using the probe combination of Probe**1** ($n = 2$) and Probe**2** ($n = 1$), we next investigated the selectivity toward the target sequence. This combination produced a high CL activity in the presence of Target (**1T**) compared to that in the absence of the target (Fig. 6A). These CL reactions were started by the addition of $Cu(OAc)_2$, because it was found in the preliminary investigation shown in Figure 5 that the non-selective background CL was minimized by the last addition of $Cu(OAc)_2$ to the reaction mixture. In the case of Target (**1X**), one nucleotide difference at the spacer site of the target sequence was not discriminated, suggesting that the nucleotide at the spacer between the binding sites had no effect on the CL activity (Fig. 6A). In contrast, when one nucleotide difference was inserted in the Probe**2** binding site, only the full-matched target with G at X (Target **1T(G)**) produced higher a CL response (Fig. 6B). This result indicates that this probe can discriminate the presence of a single nucleoside mismatch base. From the UV melting experiments, only the full-matched target produced a clear melting behavior, indicating that the selectivity in the CL response to the one nucleotide difference of Target **1T(X)** is due to the formation of hybridized complexes between the probe ODNs and the target DNA.

3. Conclusion

In this study, we have synthesized the β-ketoenamine-conjugated ODN probe for complexation with Cu(II) ion. The alkyl linker length between the β-ketoenamine part, the probe ODN and the spacer nucleotide number of the target sequence between the binding sites of the two probes have been optimized. It has been successfully demonstrated that the β-ketoenamine-conjugated ODN probes exhibit a catalytic activity for the luminol CL with a sequence selectivity. This study has provided a new entry into the design of the ODN probes with signal-amplifying machinery for the sensitive detection of nucleic acids without amplification procedures.

4. Materials and methods

4.1. General

The ^1H NMR (400 MHz) and ^{13}C NMR (125 MHz) spectra were recorded by Varian UNITY-400 spectrometers. The ^{31}P NMR (161 MHz) spectrum was recorded using 10% phosphoric acid in D_2O for the internal standard at 0 ppm. The UV–vis spectra and T_m measurement were obtained using a BECKMAN COULTER DU-800. The high-resolution mass spectra were recorded by an Applied Biosystems Mariner System 5299 spectrometer using bradykinin and angiotensin as the internal standard. The MALD-TOF/MS spectra were recorded by a BRUKER DALTONICS Microflex instrument.

4.1.1. (*R*)-3-{(2-Acetyl-3-oxobut-1-en-1-yl)amino}-2-hydroxy propyl 4-methylbenzenesulfonate (4)

To a solution of **1** (201 mg, 1.0 mmol) in pyridine (5.0 mL) was added *p*-toluenesulfonyl chloride (229 mg, 1.2 mmol) at 0 °C. After

stirring for 24 h at the same temperature, the reaction was quenched by saturated aqueous $NaHCO_3$ and extracted with organic solvents (chloroform/isopropanol 4:1, v/v). The organic layer was washed with brine and dried over anhydrous $MgSO_4$, and then the solvent was concentrated under reduced pressure. The crude product was purified by silica-gel column chromatography (chloroform/methanol = 15:1) to give **4** (310 mg, 0.87 mmol, 87%) as an orange oil. ^1H NMR ($CDCl_3$, 400 MHz) δ 2.26 (br s, 1H), 2.33 (br s, 6H), 2.44 (s, 3H), 3.37–3.40 (m, 1H), 3.50–3.54 (m, 1H), 4.02 (s, 3H), 7.35 (d, 2H, J = 8.24 Hz), 7.75 (d, 1H, J = 12.4 Hz), 7.77 (d, 2H, J = 8.24 Hz), 11.0 (br s, 1H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.7, 51.8, 68.4, 70.0, 111.9, 128.0, 130.1, 132.2, 145.5, 161.1. FTIR (neat): 3357, 1629, 1200 cm^{-1}. HRMS (ESI) m/z: calcd for $C_{16}H_{22}NO_6S$ $(M+H)^+$ 356.1162, found 356.1156.

4.2. Synthesis of the oligonucleotides

All oligonucleotides were synthesized on a 1 μmol scale by an ABI 394 DNA/RNA synthesizer with the standard β-cyanoethyl phosphoramidite chemistry. All amidite reagents and CPG supports were synthesized or purchased from Glen Research. After synthesis of the 5′-modified or 3′-modified ODNs, they were cleaved from the CPG resin in 28% ammonium hydroxide solution. The crude product was purified by RP-HPLC (column: nacalai tesque COSMOSIL 5C18MS-II 10 × 250 mm, solvent: A; 0.1 M TEAA, B; CH_3CN, and B concn 10–40%/20 min linear gradient, 3 mL/min flow rate, detected at 254 nm). These ODNs were treated with 10% acetic acid and washed with Et_2O, and then the water phase was lyophilized under reduced pressure to remove the DMTr-protecting group.

4.3. General procedure of synthesis of ligand conjugated ODN probes. The synthesis of Probes1 and 2

The corresponding ODNs were treated with TCEP (3.0 equiv) in water for 1 h for conversion to the thiol group. These thiol-modified ODNs were then added to the solution of **4** (30 equiv) in the mixture of DMF and sodium bicarbonate buffer/DMF (9:1) at 50 °C for 6 h. The resulting mixture was purified by RP-HPLC (column: nacalai tesque COSMOSIL 5C18MS-II 10 × 250 mm, solvent: A; 0.1 M TEAA, B; CH_3CN, and B concn 10–40%/20 min linear gradient, 3 mL/min flow rate, detected at 254 nm). The structures of all ODNs were confirmed by MALDI-TOF MS measurement. The MALD/MS data of the synthesized probes: Probe**1** (n = 1), 3′ CGACCCGCAATT-(C3)-ligand, calcd: 3926.4; found: 3925.6. Probe**1** (n = 2): 3′ CGACCCGCAATT-(C6)-ligand, calcd; 3969.9; found: 3968.4. Probe**2** (n = 1), 5′ GTTCGCCTCTTT-(C3)-ligand, calcd: 3915.2; found: 3914.2. Probe**2** (n = 2), 5′ GTTCGCCTCTTT-(C6)-ligand, calcd: 3953.2; found, 3952.9.

4.4. UV–vis measurement

The solution of copper acetate (final concn 25 mM) was added to the H_3BO_3–NaOH buffer (final concn 50 mM) in the presence of ligand **2** (final concn 25 mM) at pH 9 or 12. The spectrum was then obtained at 400–800 nm for the d–d band.

4.5. Chemiluminescence (CL) measurement

Method A for Figure 5: The reaction was initiated by the addition of luminol sodium (final concn 25 μM) and ascorbic acid (final concn 25 μM) to a reaction mixture which was mixed with the target DNA (final concn 1 μM), Probe**1** (final concn 1 μM), Probe**2** (final concn 1 μM), copper acetate (final concn 5 μM), and H_3BO_3–NaOH buffer (final concn 50 mM at pH 12) in a final reaction volume of 50 μL.

Method B for Fig. 6: The reaction was initiated by the addition of copper acetate (final concn 5 μM) to a reaction mixture which was mixed with the target DNA (final concn 1 μM), Probe**1** (final concn 1 μM), Probe**2** (final concn 1 μM), luminol sodium (final concn 25 μM), ascorbic acid (final concn 25 μM), and H_3BO_3–NaOH buffer (final concn 50 mM at pH 12) in a final reaction volume of 50 μL. The luminescence was measured by a luminometer produced by PROMEGA.

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Supplementary data

Supplementary data (NMR spectra, crystal structure and data) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.007.

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