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http://hdl.handle.net/2324/25988

バージョン：accepted
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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 H2O2 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.

Keywords: Copper(II) complex, Chemiluminescence, Oligonucleotide sensing, Signal-amplification

1. Introduction

The sensitive detection of nucleic acids plays an essential role in gene diagnosis and biological research for both extra- and intracellular events. 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. 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. The alternative approach utilizes the target nucleic acid as the template to catalyze the chemical reactions 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. 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. 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.

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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.
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 (probe1) or at the 3′ end (probe2) through an appropriate linker (Figure 1B). It was expected that the two probes would be assembled on the target nucleic acid to form a dicopper complex, thus inducing the catalytic activity for the CL of luminol (Figure 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 H₂O₂ (Figure 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 (Figure 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,¹²,¹³ 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 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 hr, a peak around 12 min of the 5′-thiol-modified ODN disappeared, and a new peak corresponding to the conjugated product (Probe2 (n=2)) appeared around 13 min. After purification by HPLC, the conjugated structure was confirmed by MALDI-TOF MS measurements.

![Figure 2](image)

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).

![Figure 3](image)

Figure 3. The HPLC chart of the conjugation reactions. HPLC conditions: column; shiseido CAPCELL PAK C18, 4.6 x 250 mm, Flow rate; 1.0 ml/min, Buffer; A 0.1 M TEAA, B CH₃CN, B conc. 10% to 30%/20 min linear gradient, column oven; 35°C, UV-detector; 254 nm.

![Scheme 1](image)

Scheme 1. The synthesis of the ligand-conjugate ODN probes (1 and 2) and the target sequence. (a) p-TsCl, pyridine, 0°C, 81%. (b) (1) Thiol-modified ODNs, NaHCO₃, aq./DMF (9/1), 50°C, 6 hr.

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 (Figure 4).¹¹ These results suggested that the CL experiments with the ODN-ligand conjugates should be carried out at pH 12.

![Figure 4](image)

Figure 4. (A) UV-spectra of 10 mM ligand (1) in a borate buffer (H₂BO₃-NaOH) contains the 10 mM Cu(OAc)₂ at pH 9 or pH 12. (B) Expanded UV-spectra.

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, Probe1 (n=2) and Probe2 (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 Probe1 (n=1) and Probe2 (n=2) would have an equivalent alkyl linker length with that of Probe1 (n=2) and Probe2 (n=1), they showed a low activity for the CL reaction. In the case of the combination of Probe1 (n=2) and Probe2 (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 Probe1 (n=2) bearing the C6 alkyl linker and Probe2 (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 Probe1 (n=2), Probe2 (n=1) and Target (1T) were measured in the presence or absence of Cu(OAc)$_2$. 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)$_2$.

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 $^1$H 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$_2$O 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 MALDI-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-hydroxypropyl 4-methylbenzenesulfonate (4)
To a solution of I (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 hr at the same temperature, the reaction was quenched by saturated aqueous NaHCO3 and extracted with organic solvents (chloroform:isopropanol 4:1, v/v). The organic layer was washed with brine and dried over anhydrous MgSO4, 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 (CDCl3, 400 MHz) δ 2.26 (bs, 1H), 2.33 (bs, 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 (bs, 1H). 13C-NMR (CDCl3, 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 C16H28NO5S (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 amide 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 x 250 mm, solvent: A; 0.1 M TEAA, B; CH3CN, to a reaction mixture which was mixed with the target DNA (final conc. 1 μM), Probe1 (final conc. 1 μM), Probe2 (final conc. 1 μM), copper acetate (final conc. 5 μM) and H2BO3·NaOH buffer (final conc. 50 mM at pH 12) in a final reaction volume of 50 μL.

Method B for Figure 6: The reaction was initiated by the addition of copper acetate (final conc. 5 μM) to a reaction mixture which was mixed with the target DNA (final conc. 1 μM), Probe1 (final conc. 1 μM), Probe2 (final conc. 1 μM), and ascorbic acid (final conc. 25 μM) and H2BO3·NaOH buffer (final conc. 50 mM at pH 12) in a final reaction volume of 50 μL. The luminescence was measured by a luminometer produced by PROMEGA.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (S) from Japan Society for the Promotion of Science (JSPS), CREST from Japan Science and Technology Agency. We acknowledge Dr. Kenji Yozu at BRUKER AXS for the X-ray study.

Supplementary Material

NMR spectra, crystal structure and data associated with this article can be found in the online version.

References and notes