Design and Synthesis of Nucleic Acid-binding Naphthyridine Conjugates for Fluorescence Biosensing

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論　文　内　容　要　旨

The chemistry of DNA-binding drugs and/or ligands is of ongoing interest due to their promising functions and biological activities toward analytical, biological, and medicinal applications. In our laboratory, DNA-binding fluorescent ligands have been developed for analytical applications, including single-nucleotide polymorphism (SNP) typing, affinity-labeling in aptamer assays, and microRNA detection. These ligands show selective binding to the target nucleobase opposite an abasic site (AP site) in DNA duplexes, which accompanies the fluorescence signalling of ligand. In this work, I devote myself to develop more powerful AP site-binding ligand for the analysis of nucleobase recognition in nucleic acid. First, in order to minimize the

variation of environmental, probe concentrations, and light intensity on the fluorescence response of these AP site-binding ligands, I design a new kind of ratiometric ligands which shows more accurate analysis for target analytes. Second, I develop a new generation of on-off like ligands with light-up property which shows high selectivity and strong affinity for analysis of nucleobase in nucleic acid.

Chapter 1: Introduction

This chapter is introduction of background of related studies and objective of this research.

Chapter 2: Design and synthesis of ratiometric fluorescent ligands for analysis of SNPs

AP site-binding ligands are developed into the ratiometric fluorescent ligands, **C2**, for which ATMND (2-amino-5,6,7-trimethyl-1,8-naphthyridine) is conjugated with (4-(*N,N*-dimethylaminosulphonyl)-1,2,3-benzoxadiazole) DBD. The fluorescence response of **C2** to 21-mer AP site-containing DNA duplexes (5'-GCA GCT CCC GXG GTC TCC TCG -3'/3' -CGT CGA GGG CNC CAG AGG AGC-5', X = AP site; Spacer C3, N = G, C, A or T) was examined. **C2** exhibits a selective fluorescent response to pyrimidine over purine nucleobases, and the response is the most effective to thymine over other the three nucleobases. When binding with thymine (N = T), the fluorescence of ATMND moiety is effectively quenched while the emission from DBD moiety is significantly enhanced. It is likely that the DBD moiety is located at the hydrophobic microenvironment of the DNA duplex, which would be responsible for the observed fluorescence enhancement. Indeed, the examination of the energy-minimization of DNA-**C2** complex reveals that the DBD moiety is protruding into the hydrophobic minor groove of the DNA duplexes.

Then the binding-induced response of **C2** allows the ratiometric fluorescence signalling for the detection of the target nucleobase, for which we utilize the change in the ratio of the emission intensities (F_{585} / F_{420}). Upon binding to thymine (N = T), the value of F_{585} / F_{420} increases by a factor of 27 and this enables the highly selective detection of thymine over the other three nucleobases. Moreover, the ratiometric response of **C2** was applicable to the naked-eye detection of thymine-related single-base mutation. Finally, **C2** was applied to the analysis of single-base mutation in 107-meric DNAs (K-ras gene, sense strand, condon 12) which could be applicable to analysis of transversion from pyrimidine bases to purine bases. (or vice versa)

In summary, conjugation with an environmentally sensitive fluorescent dye to probe hydrophobic grooves of the DNA helix was developed in this chapter. **C2** exhibits a ratiometric response with selectivity for pyrimidine base. The ratiometric method is applicable to the analysis of PCR products. So ratiometric fluorescent ligand was successfully developed.

Chapter 3: Design and synthesis of on-off like fluorescent signaling ligands

In combination with intercalator thiazole orange (TO) and AP site-binding ligand, AClDMND-C10-TO was developed which indicates two binding mode with DNA, AClDMND moiety acts as hydrogen bonding with target nucleobase opposite an AP site in DNA duplex and TO functions as intercalating binding between base pairs of nucleic acid.

First, the characteristic of AClDMND-C10-TO and 21-meric AP site-containing DNA duplexes was investigated by UV-visible and fluorescence spectroscopy. The UV-visible spectra showed that when the base opposite the AP site is cytosine, the absorption increased remarkably due to the intercalation of TO between base

pairs. Fluorescence experiment also indicates the quenching at AClDMND moiety and enhancing at TO moiety. These results suggested that the effective binding of AClDMND to target nucleobase opposite the AP site and intercalation of TO between DNA base pairs occurs. Fluorescence titration experiments revealed that the binding affinity of AClDMND-C10-TO for cytosine in the AP site-containing DNA duplex reaches up to 10^8 M^{-1} (1:1, binding constant K_{11}), which is two orders of magnitude higher than the AClDMND moiety itself. For comparison, the other ligand features that AClDMND moiety is connected through benzothiazole nitrogen is synthesized, named as AClDMND-C10-TOz. However, the ligand showed poor property compared to the AClDMND-C10-TO that linked through quinoline nitrogen, AClDMND-C10-TO. The reason was probably due to the quinoline and benzothiazole moiety thread different groove of DNA helix when TO intercalates into DNA base pairs. More interesting, in case of target nucleobase is cytosine, AClDMND-C10-TO/DNA shows green emission under UV irradiation while AClDMND-C10-TOz/DNA exhibits almost no fluorescence.

In summary, chloro-substitued naphthyridine-TO conjugate was synthesized, in which on-off like response was obtained with a high selectivity for cytosine and binding affinity reaches to nM level. Base discrimination can be seen by naked eyes (DNA: 500 nM). So we concluded that fluorescent ligand with improved signalling and binding properties was successfully developed.

Chapter 4: Use of AClDMND-C10-TO for microRNA (miRNA) detection

Based on the above results, AClDMND-C10-TO was applied for RNA detection, for which DNA and RNA probe were utilized. The results indicate that AClDMND-C10-TO shows higher affinity for cytosine ($K_{11} = 4.5 \times 10^7$ M^{-1}) in the DNA probe/RNA target hybrids compared to that of RNA probe/ RNA target duplexes ($K_{11} = 0.07 \times 10^7$ M^{-1}). The reason is most likely due to the difference in conformations between DNA/RNA hybrids and RNA/RNA duplexes. DNA/RNA hybrids tend to adopt B-form conformation while RNA/RNA duplexes employ A-form conformation, and B-form structure would provide more space for intercalation of TO moiety between base pairs. Thus, I design a DNA probe for miRNA (let-7 family members) detection, and fluorescence experiment revealed that AClDMND-C10-TO was able to discriminate let-7d from the other let-7 family members with a binding affinity up to 10^7 M^{-1}.

In this chapter, we have demonstrated that use of AClDMND-C10-TO for miRNA detection. By utilizing a DNA probe, the ligand exhibited strong binding affinity and high selectivity for cytosine in RNA. Due to the high specificity of this ligand, as low as nM miRNA can be accurately determined.

Chapter 5: Conclusion

In this work, nucleic acid-binding ligands have been developed with high selectivity and strong binding affinity. This new type of ligand exhibits higher detection sensitivity over traditional AP site-binding ligands by two orders of magnitude and high speficity for target nucleobases. These kinds of ligands can be potentially applied to miRNA detection in the total RNA sample. Moreover, this proposed method does not require any modified or labelled DNA probes, which should significantly reduce the cost and simplify the experimental procedure.

論文審査の結果の要旨

本論文は、「蛍光性色素を連結したナフチリジン誘導体の設計と合成、また、それらの遺伝子解析への応用」に関する研究成果について報告したものである。

第1章では、序論として、遺伝子解析の現状、特に一塩基多型検出やマイクロRNA検出に関する現状をまとめるとともに、本研究の目的について述べている。

第2章では、ナフチリジン骨格（ATMND: 2-amino-5,6,7-trimethyl-1,8-naphthyridine）と疎水場環境応答性色素（DBD: 4-(N,N-dimethylaminosulphonyl)-1,2,3-benzoxadiazole）を、アルキル基を介して連結したリガンドを合成し、脱塩基部位含有DNA二重鎖（5'-GCA GCT CCC GXG GTC TCC TCG -3'/3' -CGT CGA GGG CNC CAG AGG AGC-5', X = AP site; Spacer C3, N = G, C, A or T）への結合力や結合選択性、また、蛍光応答特性について評価している。その結果、ATMNDとDBDをエチレン基で連結したリガンド（**C2**）が、ピリミジン塩基（チミン、シトシン）に対して優れた結合選択性（解離定数 K_d = 150 nM for T）を発現しうることを見出している。また、ATMNDとDBDに由来する蛍光応答を利用することで、二波長蛍光強度比解析が可能で、検出限界は2.1 nM（for T）に達している。さらに、実試料に準じた107-meric PCR（polymerase chain reaction）産物の解析が可能であることを示しており、実用レベルの検出機能が得られていると判断できる。

第3章では、ナフチリジン骨格と蛍光性色素チアゾールオレンジ（TO）を、アルキル基（C_{10}）を介して連結したリガンド（**AClDMND-C10-TO**）を設計・合成し、その結合特性と蛍光応答特性を評価している。ここでは、ナフチリジン骨格に電子求引基（クロロ基）を導入し、ナフチリジン骨格のプロトネーション部位を制御することで、シトシンに対するほぼ特異的な結合選択性を達成している（K_d = 6.7 nM）。さらに、結合に伴ってon-off的な蛍光応答（TO基由来）が得られており、極めて実用性の高いDNA検出リガンドの開発を達成している。

第4章では、第3章で合成したリガンド（**AClDMND-C10-TO**）をマイクロRNA検出に適用した結果について述べられている。ここでは、DNAプローブを用いることで、**AClDMND-C10-TO**の結合力と蛍光応答特性が飛躍的に向上することを見出しており、RNA/DNAハイブリッド二重鎖への解離定数は23 nMに達している。さらに、**AClDMND-C10-TO**をlet-7ファミリーメンバーの検出に適用した結果、標的メンバー（let-7d）を特異的に検出することが可能であることを示している。

第5章では、本研究で得られた知見を総括している。

以上の研究成果は、論文提出者が自立して研究活動を行うに必要な高度の研究能力と学識を有することを示している。したがって、王春霞君提出の博士論文は博士（理学）の学位論文として合格と認める。