論 文 内 容 要 旨

氏　　名	张 钰爽（ZHANG, Yushuang）	提出年	平成 26 年
学位論文の 題　　目	Study on analytical methods based on DNA/RNA-small ligand interactions (DNA/RNA-小分子リガンド相互作用に基づく分析法の開発)		

論 文 目 次

論 文 内 容 要 旨

(1) Development of abasic site-containing DNA duplex-based aptamers for theophylline's detection [1]

Aptamers, short nucleic acids with significant binding ability, have emerged as promising candidates for molecular recognition events to various ligands. Our group has reported a fluorescent signaling aptamer that targets a common drug, theophylline.[2] In this DNA duplex aptamer, an abasic site (AP site) was utilized as an active cavity for binding events (cf. Fig. 1), and a fluorescent adenine analogue, 2-aminopurine, was incorporated into the duplex so as to flank the AP site. However, the need of the modification with 2-aminopurine makes the assay expensive, and the poor photostability of 2-aminopurine is also problematic.

In this work, a modification-free AP site-containing DNA duplex aptamer (AP-aptamer) is developed for the detection of theophylline. The assay is based on the competitive binding of theophylline with riboflavin at the AP site. As shown in Fig. 1, in the absence of theophylline, riboflavin binds to the receptor nucleobase opposite the AP site, and this is accompanied by fluorescence quenching. Upon addition of theophylline, the competitive binding of theophylline with riboflavin occurs, which results in the effective fluorescence restoration due to the release of riboflavin from the AP site.

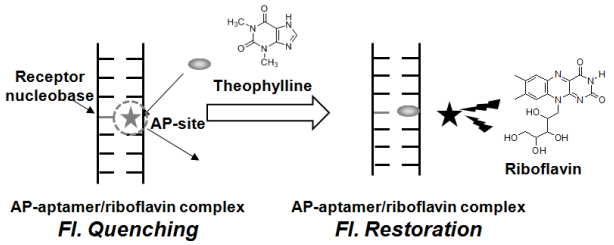

Fig.1. Competitive binding of theophylline with a fluorescent ligand at an AP site in a DNA duplex aptamer.

Riboflavin/23-mer AP aptamer (5'-TCT GCG TCC AGX GCA ACG CAC AC-3'/5'-GTG TGC GTT GCC CTG GAC GCA GA-3'; X: the AP site (Spacer C3, a propylene residue)) system is found to be effective for the detection of theophylline with a high selectivity over caffeine, theobromine and other substrates. In serum samples, a linear response to theophylline can be obtained in the concentration range from 10 μM to 33 μM (50 μM to 163 μM in original serum samples), which covers the monitoring of serum theophylline concentration in the therapeutic range (60 μM to 100 μM). This system is thus expected to be applicable to practical use.

(2) Development of a highly selective ligand binding with cytosine opposite an abasic site in DNA duplexes[3]

Detection of single-nucleotide polymorphisms (SNPs) in human genome has become increasingly important in the fields of genetics, molecular diagnostics and cancer research. Our group has reported that a naphthyridine derivative (AMND, Fig. 2)[4] was capable of selectively binding to cytosine (C) opposite an AP-site in DNA duplexes, and the binding-induced fluorescence quenching was applicable to the analysis of the C- related single base mutation. However, AMND showed an insufficient selectivity for C/T discrimination: the difference in the binding affinity of AMND for C over T is only two- or three-fold.[3,4]

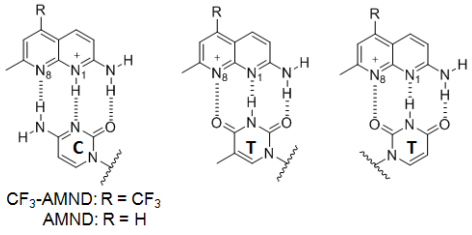

Fig.2. Binding modes of AMND/CF_3-AMND with C and T (2 possible patterns).

In this work, AMND is modified for highly selective detection of C by introducing of CF_3 group to the naphthyridine ring. The binding constant of the resulting CF_3-AMND (Fig. 2) for C ($K_{11}/10^5$ M^{-1} (n = 3): 7.1 ± 0.2) is found to be more than 50-fold larger than those for the other three nucleobases ($K_{11}/10^5$ M^{-1} (n = 3): T, 0.14 ± 0.016; G, 0.16 ± 0.029; A, <0.10). The high selectivity might be caused from more favorable protonation at the N1 position than the N8 position due to the electron-withdrawing effect of the CF_3 group, which then enables the effective pseudo-base pairing with C through hydrogen bonding (Fig. 2).

Furthermore, CF_3-AMND is applicable to the analysis of single-base mutation in 107-mer DNAs (K-ras gene; codon 12, sense strand) amplified by asymmetric PCR, with highly selectivity for C. This will facilitate the accurate analysis of C-related single-base mutation in practical use.

(3) Development of peptide nucleic acid-thiazole orange conjugates for discrimination of terminal mismatches in DNA/RNA

In general, detection of mismatches of DNA/RNAs relies on the method based on the thermal stability of duplex hybridization. A mismatch near or at the terminus of a duplex is less destabilized than an internal mismatch, so as to be difficult for terminal mismatch detection. Recently, our group has developed a peptide nucleic acid conjugated with thiazole orange (PNA-TO) for siRNA imaging in living cells.[5] This ligand was capable of binding to 3'-overhanging nucleotides with a good selectivity for perfect match sequences over other mismatched ones. For the binding of this ligand, PNA unit recognizes the terminal two nucleobases via complementary hydrogen-bonding, and TO unit works as a fluorescent probe by intercalation into the duplex (cf. Fig.3).

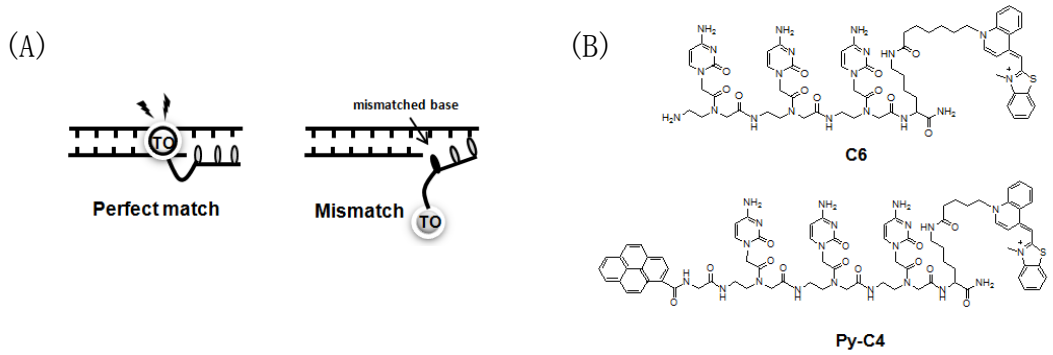

Fig.3. (A) Illustration of PNA-TO binding to terminal nucleobases; (B) Structures of representative PNA-TO ligands (C6 and Py-C4).

In this work, PNA-TO conjugates are developed for the detection of a single nucleobase mismatch at the terminal positions of DNA/RNAs (Fig. 3). All of the synthesized PNA-TO conjugates show a good selectivity at 3' end, but the selectivity is moderate for terminal mismatches at 5' end. To improve the binding selectivity at 5' end, pyrene as a π-stacking cap has been introduced into the N-terminal of PNA-TO. The resulting Pyrene-PNA-TO shows a specific binding ability for perfect match over terminal mismatches, including the most disturbing position, the very 1st terminal mismatch. In addition, Pyrene-PNA-TO is applicable to microRNA detection to recognize let-7d out of let-7 family with LOD as low as 0.9 nmol in 50 μL sample. It's thus expected that our method is promising for terminal mismatch detection and the ligand design would have flexibility for any kind of terminal sequences.

Reference

[1] Y. Sato, Y. Zhang, et al., *Chem. Eur. J.*, **2012**, 18, 12719.

[2] M. Li et al., *J. Am. Chem. Soc.*, **2009**, 131, 2448.

[3] Y. Sato, Y. Zhang, et al., *Org. Biomol. Chem*., **2012**, 10, 4003.

[4] K. Yoshimoto et al., *J. Am. Chem. Soc.*, **2003**, 125, 8982. ; Y. Sato et al., *Nucleic Acids Res.*, **2009**, 37, 1411.

[5] T. Sato et al., *Chem. Commun.*, **2015**, 51, 1421.

論文審査の結果の要旨

本論文は、核酸と小分子との相互作用に基づく新規分析法の開発を目的として、「薬剤を標的とする DNA アプタマー」、「一塩基多型検出のための蛍光性 DNA 結合リガンド」、また、「miRNA 検出を目的とするペプチド性 RNA 結合リガンド」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、アプタマー開発や一塩基多型検出法、また miRNA 検出法に関する最新の研究動向をまとめるとともに、本研究の目的について記述している。

第２章では、テオフィリン（気管支喘息の治療薬）を標的とする DNA アプタマーについて述べられている。一般に、既存のアプタマーは、RNA を利用するものが主流であるが、化学的に不安定であることが RNA アプタマーの問題で、汎用性のある分析試薬としての活用には難がある。これに対して、本研究では、DNA 二重鎖中に設けた AP site を結合サイトとして利用することに着目し、さらにリボフラビンを蛍光マーカーとして利用する競合アッセイ系を構築することで、テオフィリンを標的基質とするアプタマーを開発することに成功している。

第３章では、ナフチリジンを基本骨格とする蛍光性 DNA 結合リガンドに関して、その結合特性ついて述べている。ここでは、ナフチリジン骨格に電子求引基（トリフルオロメチル基）を導入し、ナフチリジン骨格のプロトン化部位を制御することで、極めて高選択的なシトシン結合リガンドの開発が可能であることを示している。さらに、実試料解析にも適用できることを示しており、実用性の高い一塩基多型検出リガンドの開発を達成している。

第４章では、ペプチド核酸（PNA）を基本骨格とするリガンドを設計・合成し、RNA との結合機能について評価した結果について述べている。一般に、標的１本鎖核酸の末端近傍に存在する一塩基の違いを検出することは容易ではないが、PNA を利用することで高選択的な識別が可能であることを明らかにしている。さらに、実在 miRNA 配列を対象とした検討では、有用な検出限界が得られており、実用への応用・展開が期待できる。

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、张 钰爽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。