



A binary Cy3 aptamer probe composed of folded modules

Kei Endo, Yoshikazu Nakamura *

Department of Basic Medical Sciences, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

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ABSTRACT

Aptamers are short single-stranded DNA or RNA sequences that are selected in vitro based on their high affinity to a target molecule. Dye-binding aptamers are promising tools for real-time detection of not only DNA or RNA sequences but also proteins of interest both in vitro and in vivo. In this study, we aimed to isolate an RNA aptamer to Cy3, a widely used, membrane-permeant, and nontoxic fluorescent cyanine dye. Extensive selection of affinity RNA molecules to Cy3 yielded a unique sequence aptamer named Cy3_apt. The selected Cy3_apt was 83 nucleotides long and successfully shortened to 49 nucleotides long with increased affinity to Cy3 by multiple base changes. The shortest Cy3_apt is composed of two separate hairpin modules that are required for the affinity to Cy3 as monitored by the surface plasmon resonance (SPR) assay. Also, the fluorescence of Cy3 increased on binding to Cy3_apt. The two modules of Cy3_apt, when detached from each other, functioned as a binary aptamer probe. We demonstrate that the binary Cy3_apt probe is applicable to the detection of target oligonucleotides or RNA–RNA interaction by tagging with target sequences. This binary probe consists of two folded modules, referred to as a folded binary probe.

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The concept of using single-stranded nucleic acids (aptamers) as affinity molecules for protein or compound binding was initially described in 1990 [1–3]. The concept is based on the ability of short (20–80 mer) sequences to fold, in the presence of a target, into unique three-dimensional structures that bind to the target with high affinity and specificity. Aptamers are generated by a process that combines combinatorial chemistry with in vitro evolution, known as SELEX (systematic evolution of ligands by exponential enrichment),¹ from a complex library of randomized sequences of typically 10^{14} different molecules [4–8]. Because of high affinity and selectivity, aptamers are applicable to both basic and applied sciences as reagents for affinity purification [9–11] and therapeutic treatments (reviewed in Ref. [8]) [12–14]. Importantly, targets of aptamer can be small (e.g., chemical compounds) or large (e.g., proteins), and they can be simple (e.g., purified proteins) or complex (e.g., protein complexes or cell surface receptors); therefore, aptamers are also recognized as a useful biosensor element. Up to now, a variety of aptamer-based sensors have been reported (reviewed in Refs. [15,16]).

We have created several aptamers to cellular proteins, including human translation initiation factors [4,5,17], and extracellular pro-

teins, including cytokines [14] and receptors [6]. One of these aptamers, the eIF4A-binding aptamer 4Aapt, was used to develop a unique biosensor probe [18]. This method is based on fluorescent protein complementation regulated by the interaction of the two domains of eIF4A, with each being fused to split fragments of the enhanced green fluorescent protein (EGFP). We have also developed a semiquantitative measurement system of eIF4A at nanogram levels within whole cell lysates using a surface plasmon resonance (SPR) assay with a 4Aapt-immobilized sensor chip [19].

Aptamers have also been generated to dyes and fluorophores such as malachite green [20] and sulforhodamine B [21,22]. These aptamers are composed of unmodified nucleotides and, thus, are applicable to sensitive real-time detection of nucleic acid or small molecules by fusing to the complementary sequences or secondary aptamers to target molecules [23–25]. To reduce background fluorescence and increase detection specificity, a binary aptamer probe was designed by Kolpashchikov from the malachite green aptamer (MGA) [23]. MGA is unique in that it dramatically increases the fluorescence of the dye [26]. MGA has a stem-loop structure containing internal bulged loops [20]. These double-stranded sequences were separated into two single-stranded sequences, each of which has no affinity to malachite green and was tagged to sequences complementary to nucleic acid analytes. This probe is referred to as a binary MGA probe and provides immediate fluorescent response after hybridization to complementary nucleic acid analytes, thereby offering easy and instant detection of the specific DNA and RNA [23]. Recently, Sando and coworkers reported a light-up DNA aptamer that triggers fluorescence of an otherwise

* Corresponding author. Fax: +81 3 5449 5415.

E-mail address: nak@ims.u-tokyo.ac.jp (Y. Nakamura).

¹ Abbreviations used: SELEX, systematic evolution of ligands by exponential enrichment; EGFP, enhanced green fluorescent protein; SPR, surface plasmon resonance; MGA, malachite green aptamer; DTT, dithiothreitol; nt, nucleotides; PCR, polymerase chain reaction; RT, reverse transcription; PAGE, polyacrylamide gel electrophoresis.

nonfluorescent Hoechst derivative, and they manipulated a binary aptamer probe that detects DNA analyte according to Kolpashchikov's design principle [27].

The choice of chromophore is critical for widespread practical applications of aptamer probes to live imaging in living cells. Ideally, the chromophore should have good solubility in aqueous solutions, cell permeability, substantial fluorescence enhancement/shift on binding to the target, and a high degree of bioorthogonality against other biological components. Although MGA has been successfully applied to a binary aptamer probe, it remains uncertain whether malachite green is the best choice for intracellular imaging. It is worth mentioning that malachite green generates singlet oxygen very efficiently on irradiation, is used to achieve targeted damage of messenger RNA constructs [20], and may lead to the undesired behavior of cells during the imaging process and severe limitations in experimental setups. Therefore, an alternative chromophore that is less toxic and well-suited for live cell imaging is desirable.

In this study, we aimed to isolate an aptamer to cyanine dye Cy3. Cy3 is a widely used, membrane-permeant, and nontoxic dye, but its aptamer has yet to be reported in the literature. The selected aptamer to Cy3, referred to as Cy3_apt, is composed of two structurally separable modules, each of which has no affinity to Cy3 by itself and exhibits affinity to Cy3 on being properly arranged in a tertiary configuration. Based on this property, each module of Cy3_apt was used separately to construct a binary Cy3 aptamer probe.

Materials and methods

Chemicals

Chemicals used in this study were purchased from commercial suppliers as indicated if necessary. The oligonucleotides (listed in [Supplemental Table S1](#) in the [supplementary material](#)) were purchased from Operon Biotechnologies. SH resin was prepared by incubating Thiopropyl Sepharose 6B (GE Healthcare) in binding buffer (20 mM Na-Hepes [pH 7.0], 100 mM NaCl, and 5 mM MgCl₂) containing 50 mM dithiothreitol (DTT) for 90 min, followed by washing with the same binding buffer. The resulting SH resin was incubated with 570 μ M Cy3 maleimide mono-reactive dye (GE Healthcare) overnight and washed with the binding buffer, giving rise to the Cy3-immobilized resin. Cy3 resin (200 μ l) was packed into Econo-Column (Bio-Rad) for each SELEX process.

Selection of Cy3 aptamer

The preparation of a pool of 10¹⁴ different RNA molecules randomized over 50 nucleotides (nt, N50 RNA pool) and affinity RNA selection–amplification were carried out essentially as described previously [4,17]. For the first-round selection, 0.3 nmol of a single-stranded DNA pool (5'-CTCTCATGTGCGCCGTTA-N50-CGTCATTGTGTCCCTATAGTGAGTCGTATTA-3') was amplified by polymerase chain reaction (PCR) with the forward primer Fwd1 (5'-T AATACGACTCACTATAGGGACACAATGGACG-3') and the reverse primer Rev83 (5'-CTCTCATGTGCGCCGTTA-3') and then was subjected to in vitro transcription. Then 15 μ g of the RNA pool in binding buffer (500 μ l) was denatured at 85 °C for 10 min and immediately cooled to 4 °C. The denatured transcripts were first passed through Cy3-free SH resin (negative selection) and charged to the Cy3 resin column (positive selection). The bound RNAs were washed with 10 column volumes of binding buffer and then eluted with 5 column volumes of binding buffer containing 7 M urea (first to eighth rounds) or 1 mM Cy3-COOH (ninth round). The eluted RNA was purified, amplified by reverse transcription (RT)-PCR, and sub-

jected to the next round of selection. After nine rounds of selection, the PCR product was cloned into a pGEM-T Easy vector (Promega) and sequenced.

SPR analysis

SPR analysis was carried out using a BIAcore 2000 instrument (GE Healthcare). Cy3-maleimide was immobilized on the sensor chip CM5 using a Thiol Coupling Kit (GE Healthcare) according to the manufacturer's instructions. Essentially following the instructions, cystamine was immobilized on the chip via amine coupling following the thiol coupling of Cy3-maleimide. Aptamer RNAs were then injected onto the chip for the indicated time period, and after dissociation of bound aptamers the sensor chip was regenerated by washing with 2 M urea. SPR experiments were performed in the same binding buffer as used during SELEX at 25 °C and a flow rate of 10 μ l/min.

Fluorescence measurements

Binding buffer (2.5 μ l) containing Cy3-monofunctional Hexanoic Acid Dye (Toronto Research Chemicals) and 2.5 μ l of solution of various concentrations of RNAs were mixed and incubated for 5 min at room temperature. The fluorescence intensity of Cy3 and Cy5 was measured by a CytoFluor 4000 fluorescent plate reader (Applied Biosystems), with appropriate filter sets described in figure legends.

Construction of derivatives from Cy3_apt

A plasmid-containing Cy3_apt fragment obtained after the SELEX procedure was referred to as pCy3_apt. Plasmids used as a template for in vitro transcription in this study were created by primer PCR mutagenesis with a set of primers and a template listed in [Supplemental Table S2](#) or by PCR amplification with that listed in [Supplemental Table S3](#) (see [supplementary material](#)) followed by cloning into pGEM-T Easy. PCR fragments that were amplified with an appropriate primer set listed in [Supplemental Tables S2](#) or [S3](#) using cloned plasmids as a template were purified through polyacrylamide gel electrophoresis (PAGE) and subjected to in vitro transcription. Fragments 1–74, 1–71, and 1–68 were amplified with Rev74, Rev71, and Rev68, respectively, together with forward primer and pCy3_apt as a template. Δ 3'GCG was amplified with Fwd11 A13G, Rev71 A67U-G71C-C72G, and pCy3_apt* as a template. Δ 33–42 and Δ 47–56 were directly transcribed from synthetic oligonucleotides listed in [Supplemental Table S4](#) (see [supplementary material](#)). p Δ 33–42 was created via TA cloning with a primer set of Fwd11 and Rev74 and Δ 33–42 as a template.

Construction of binary Cy3_apt probe and its application to in vitro detection of oligonucleotides and RNA–RNA interaction

To keep orientation between 3' termini of domain I and 5' termini of domain II similar to Cy3_apt*, target binding arms were conjugated to the opposite terminus of these domains when Cy3_apt* was used for detecting oligonucleotides. For the same reason, in the case of monitoring RNA–RNA interaction, domain I and domain II were appended to 5' and 3' termini of a pair of complementary RNA sequences, respectively. Templates for in vitro transcription are listed in [Supplemental Table S4](#).

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