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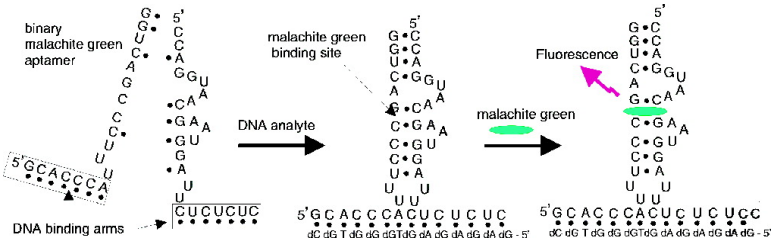

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Binary Malachite Green Aptamer for Fluorescent Detection of Nucleic Acids

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Sequence-specific detection of nucleic acids is crucial to disease diagnosis, genome study, and mRNA monitoring in living cells. Among the numerous methods for nucleic acid analysis of particular interest are those that provide immediate visible or fluorescent response after hybridization to complementary nucleic acid analytes, thus offering easy and instant detection of the specific DNA and RNA.[1,2] However, the selectivity of these methods is limited under physiological conditions, and this limitation hinders the applications in living cells.

The specificity of biopolymer recognition can be increased by separate binding of two ligands to a target biopolymer for the recognition event to occur only as a tripartite complex, that is, ligand 1−target biopolymer−ligand 2. Previously, we applied this concept for selective cross-linking of DNA polymerases.[3] In these experiments, a photoreactive primer was activated by energy transfer from a dNTP analogue in a tripartite complex DNA primer/template−DNA polymerase−dNTP. This approach allowed us to label only DNA polymerases in the presence of a large amount of other proteins in cellular extract.[4] I now report an extension of this idea to highly selective recognition of nucleic acids by creating a binary malachite green aptamer (biMGA) probe for fluorescent monitoring of nucleic acids.

The malachite green aptamer (MGA) is an RNA molecule that has submicromolar affinity to malachite green (MG), a triphenylmethane dye.[5] Upon binding, MGA increases the fluorescence of the dye >2000-fold (Figure 1A).[6] We used this aptamer previously to develop modular aptameric sensors for small molecules.[7] In the present work, MGA was separated into two strands, and nucleic acid binding arms were added to each strand through UU dinucleotide bridges, as depicted in Figure 1B. Furthermore, the inessential GAGA loop was removed. Stem I was shortened to three and Stem II to four base pairs in order to diminish the association of RNA strands in solution in the absence of nucleic acid analyte. In the presence of DNA complementary to the nucleic acid binding arms, the two RNA strands of the probe cooperatively hybridize to the adjacent positions of the target DNA and re-form MGA, which allows binding of MG and an increase in fluorescence (Figure 1C). Since each RNA strand of biMGA is bound to a relatively short analyte fragment, a single mismatched base pair substantially destabilizes such a hybrid, thus destabilizing the whole complex. Therefore, high selectivity of the probe should be expected. In this report, we demonstrate the ability of biMGA to perform both real-time DNA detection at room temperature in a buffer simulating physiological conditions (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) and discrimination abilities against a single nucleotide substitution in all possible positions of a 14 nucleotide DNA analyte.

In the absence of DNA analyte, the probe emits low background fluorescence (Figure 2, curve 1), whereas addition of 2 μM A14 increases the fluorescence intensity about 20 times (curve 2). The fluorescence was reduced to background level upon adding excess

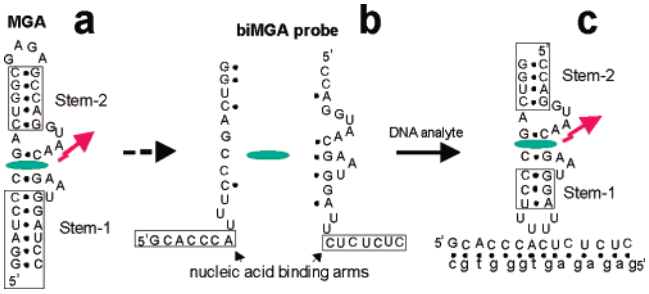

Figure 1. (a) Structures of MGA in complex with MG. (b) biMGA probe free in solution. (c) biMGA probe bound to complementary DNA. Ribonucleotides are represented in uppercase, whereas deoxyribonucleotides are in lowercase.

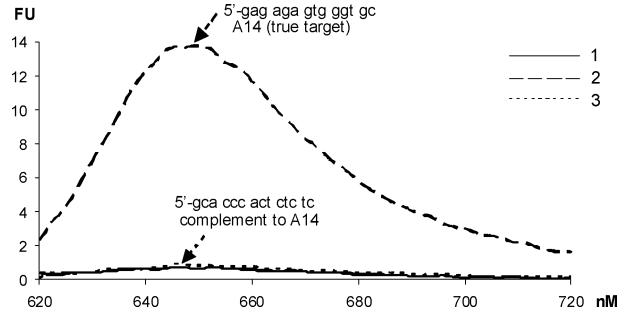

Figure 2. Binary malachite green aptamer probe increases its fluorescence upon hybridization to DNA analyte. The emitting spectra of MG (2 μM) and biMGA (1 μM) were recorded in the absence (1) or presence (2, 3) of 2 μM A14; curve (3) in the presence of 4 μM DNA competitor, which is complementary to A14.

amounts of DNA fully complementary to the analyte (curve 3). This confirmed the hypothesis that the increase in fluorescence is triggered by hybridization of the analyte binding arms to A14, and the structure of such a hybrid allows RNA strands to form an MG binding site.

The fluorescence intensity of the probe in the presence of fully matched target A14 was compared to the fluorescence intensities in the presence of oligonucleotides containing all possible single nucleotide substitutions (Table 1). The discrimination factor was calculated as a ratio of the fluorescence intensity of biMGA in the presence of A14 to fluorescence in the presence of mismatched oligonucleotides (after subtraction of background fluorescence). The discrimination factor was estimated as being higher than 20 for those oligonucleotides that trigger no increase in fluorescence above background. The probe reliably discriminates against mismatches at all positions of the analyte except substitution of T by A at the 8th position. The best discrimination was observed against mismatches located at internal positions of the two RNA/DNA hybrids. Thus, for 25 out of 42 oligonucleotides, the fluorescence intensity did not exceed the background. Particularly remarkable demonstrations of the specificity of the probe are a good discrimination

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Table 1. Discrimination Factors for Oligodeoxyribonucleotides Differing from A14 (5′-$g_1a_2g_3a_4g_5a_6g_7t_8g_9g_{10}g_{11}t_{12}g_{13}c_{14}$) by a Single Nucleotide[a]

substituted position	Substituted with			
	a	c	g	t
1	2.8 ± 0.4	2.4 ± 0.5		2.0 ± 0.2
2		13.7 ± 1.2	>20	5.5 ± 1.8
3	>20	>20		>20
4		>20	>20	>20
5	>20	>20		>20
6		15.3 ± 4.4	>20	5.5 ± 1.7
7	8.4 ± 2.0	6.0 ± 2.0		6.3 ± 2.2
8	0.7 ± 0.2	2.1 ± 0.6	2.0 ± 0.4	
9	>20	>20		>20
10	>20	>20		>20
11	>20	>20		>20
12	>20	>20	15.5 ± 3.6	
13	>20	>20		>20
14	7.0 ± 1.2		7.0 ± 1.8	4.5 ± 0.9

[a] The concentrations of all oligodeoxyribonucleotides were 2 μM; all other conditions were as described in the legend of Figure 2. The values are averages of four independent experiments.

(DF = 4.5 ± 0.9) of the T−G from C−G base pair at the 3′ terminal position of the DNA analyte (Table 1, last row, 5th column) and a discrimination A−U from G−U (DF > 20) at 4th position of A14 (Table 1, row 4, column 4).

Interestingly, an oligonucleotide containing T to A substitution at position 8 triggered higher fluorescence then A14 (DF = 0.7 ± 0.2). This may be due to base pair formation between the A8 of the deoxyoligonucleotide and one of the Us of the UU bridges. It is likely that such a complex favors RNA strands to form a stable MG binding site. The substitution of UU bridges with other linkers would allow an improvement in discrimination against T−A substitution at this position. It should be noted that if nucleotide recognition sites are separated by one or two nucleotides then the ensuing fluorescence signal of the complex is even higher than that found for the A14 DNA analyte (cf. Supporting Information). This result indicates that biMGA probes can be designed for selective recognition of not only adjacent fragments of nucleic acids but also fragments separated by a few nucleotides.

A general approach for distinguishing between mismatched and fully complementary nucleic acid duplexes is to destabilize the duplexes, causing them to become sensitive to a minor imperfection, such as a single base mispairing. For 13 and more nucleotide fragments, which are statistically required to uniquely define a particular site in a mRNA pool of the mammalian cell,[8] such destabilization can be succeeded at elevated temperatures or in the presence of denaturizing agents, such as formamide.[9] Although some of these methods have shown impressive results (for example, methods employing gold or fluorescent conjugated nanoparticles),[2] they are not fully applicable for highly specific monitoring of nucleic acids in living cells.

An alternative method for duplex destabilization is demonstrated in the case of molecular beacons (MBs)[10] and probes based on specific displacement hybridization (Yin-Yang probes).[11] In both cases, the probe design allows the formation of an alternative to the probe/analyte hybrid structures: stem loop in the case of MBs, or a duplex with a DNA competitor in the case of the Yin-Yang probe. The facilitated dissociation of the probe/analyte hybrid is achieved due to the reduction of the enthalpy component of the free energy of the probe/analyte dissociated state. This effect allows MBs and Yin-Yang probes to bind DNA more selectively than linear oligonucleotides.[10−12] At the same time, one comprehensive study revealed that MBs with a probe length of 17−19 bases possess good single mismatch discrimination properties only at 60−70 °C,[13] and another report demonstrated that MBs were not able to discriminate against single mismatches within a 20 nucleotide-long DNA analyte at room temperature.[14] Thus, the problem of selectivity still remains for the MB approach.

In the present work, a probe/analyte hybrid was destabilized by dividing the probe into two fragments. Due to the cooperative nature of the biMGA−DNA tripartite complex, it dissociates into three, rather than two, nucleic acid fragments, leading to a higher entropy gain in comparison to the conventional monolith probes. This reduction in free energy of the probe/analyte dissociated state enhances the dissociation process, especially in the presence of mismatch base pairing.

In conclusion, the design of the probe allows the hybridization event to be accompanied by an increase in fluorescence, which is easily and instantly detectable. The binary probe reliably discriminates 41 out of 42 possible single nucleotide substitutions in 14-mer DNA analyte with extremely high discrimination factors (>20) for more than half of the possible substitutions. These high discrimination abilities of the biMGA probe is demonstrated at room temperature in a buffer simulating physiological conditions. Moreover, biMGA consists entirely of unmodified RNA and thus can be expressed in living cells as a gene product. All these facts taken together make biMGA a promising instrument for highly selective fluorescent monitoring of nucleic acids in cell culture and in vivo.

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Supporting Information Available: Details of the experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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