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COMMUNICATION

Dye-functionalized Phosphate-binding Macrocycles: From Nucleotide to G-quadruplex Recognition and “turn-on” Fluorescence Sensing

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A novel strategy to design “turn-on” fluorescent receptors for G-quadruplexes of DNA is presented, which relies on the connection of phosphate binding macrocycles (PBM) with naphthalimide dyes. A new PBM-dye family was synthesized and evaluated in terms of binding and detection of nucleotides and DNA G-quadruplexes of different topologies.

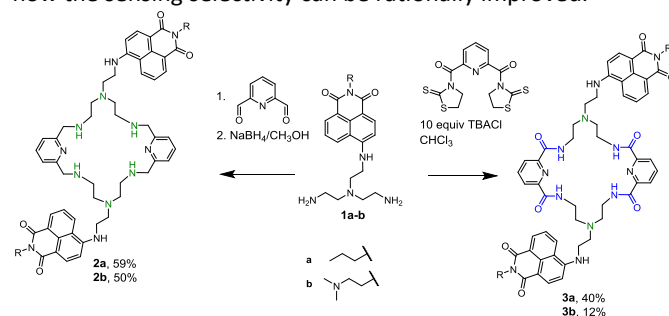
Recognition and sensing of G-quadruplex (G4) structures of DNA and RNA with synthetic receptors have attracted increasing attention in recent years.¹ Indeed, about 1% of the human genome has the potential to fold into G4 structures, and G4-forming sequences are enriched in functional genic components such as promoters, CpG islands, and 5' and 3' untranslated regions (UTRs), as well as in nongenic functional regions such as replication origins and transcription enhancers.² High density of G4 structures has been found in the telomeres at the end of chromosomes,³ and inhibition of telomerase by stabilization of G4 structures has long been proposed as potential cancer therapy.⁴

A large number of small-molecule fluorescent probes have been developed to detect G4 fragments in DNA and RNA structures.^{1a, 1b, 5} Among them are metal complexes,⁶ tweezers-like molecules⁷ and macrocyclic compounds.⁸ “Turn-on” probes are most desirable, since they provide high contrast for visualization of G4 structures in cells.⁹ However, the discovery of new probes is mostly based on the modification of well-established G4 ligands with fluorescence dye scaffolds,^{9f, 10} or on environment-sensitive fluorescence dyes. Systematic approaches to discover new fluorescent probes detecting G4s through supramolecular interactions are still rare and represent a new wave in this field.^{9d} In particular, probes that

are able to distinguish between different G4 topologies through modulation of a fluorescent signal are highly desirable. Although several such probes have been reported, they have limited freedom for structural modification and further optimization of sensing and binding properties.¹¹

Recently, we have found that phosphate binding macrocycles endowed with fluorescent reporter groups show selectivity for guanosine phosphates, as well as for tetranucleotide 5'-d(GGGG)-3' under acidic conditions (pH 3.6), in which the receptors are fully protonated.¹² Thus, we sought new designs to construct receptors showing “turn-on” response under physiological condition.

Herein, we exploit the PBM-dye design strategy to discover new fluorescent receptors for binding and “turn-on” sensing of DNA oligonucleotides forming G4 structures. We show that fine-tuning of the receptor structure enables a selective detection of parallel G-quadruplexes of DNA. Specifically, we elucidate how fluorescence response of receptors changes from simple nucleotides to more complex G4 architectures and how the sensing selectivity can be rationally improved.



Scheme 1. Synthetic route to target macrocycles.

Naphthalimide dyes have been proven to function as efficient DNA intercalators.¹³ The presence of the attached aminoalkyl groups that carry a positive charge under neutral conditions provides strong binding to a negatively charged DNA backbone. With this idea in mind, we synthesized four new macrocyclic structures **2a–3b** by varying terminal substituents on the naphthalimide dye and the number of amine groups in the macrocyclic ring. The macrocyclic core is responsible for

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phosphate recognition, while a pH-sensitive naphthalimide dye is expected to form π - π interactions with guanine residues.

The receptors were synthesized starting from 4-bromo-1,8-naphthalic anhydride by reactions with two different primary amines, 1-aminopropane (**a**) and 2-(dimethylamino)ethylamine (**b**). In the next step, tris(2-aminoethyl)amine was attached to give compounds **1a** and **1b** (Scheme 1).¹⁴ These compounds served as starting materials for the macrocyclization reactions to obtain **2a–b** and **3a–b** (ESI[†]).

Initially, the fluorescence response of the receptors was studied in the presence of simpler analytes such as nucleoside triphosphates (ATP, GTP, CTP and UTP) under neutral conditions (10 mM NaAsO₂Me₂, 100 mM KCl buffer, pH 7.2). As UTP and TTP show similar binding properties, we used the more accessible uridine derivative. As a rule, addition of nucleoside triphosphates to receptors led to a fluorescence enhancement, which was the strongest for polyammonium receptors **2a** and, particularly, **2b** (2- to 9-fold). Among the four nucleotides, addition of ATP resulted in maximal fluorescence enhancement (9-fold for **2b**), which is in agreement with the general behavior of naphthalimide-functionalized macrocycles reported by us earlier.^{12, 15} Supposedly, protonation of receptors at their amine functions upon binding to ATP results in hindering of the photo-induced electron transfer (PeT) between the naphthalimide fluorophore and the tertiary amine group, which leads to a fluorescence recovery. Conversely, the addition of GTP quenched the fluorescence of **3a**, as well as the fluorescence of **1a–b** (Fig. 1). According to spectrofluorometric titrations (SI, Figs. S8–S12), the receptors have moderate affinity for nucleotides in the order of 10³ M⁻¹ (Table 1). Since polyammonium receptors **2a** and **2b** are at least 4- to 5-fold protonated at pH 7.2, they demonstrated a 1:2 binding stoichiometry according to the fitting analysis. Macrocycles **3a** and **3b** showed relatively low fluorescence changes upon nucleotide addition, so that the binding constants could not be obtained, except the stabilities with ATP (**3a**: log *K* = 3.48, **3b**: log *K* = 3.89).

Interesting conclusions about the effect of substituents **a** and **b** can be inferred from the analysis of Fig. 1: (i) for a “turn-on” response with nucleotides, the macrocyclic structure of the receptors is essential, and (ii) the fluorescence enhancement (FE) is stronger with increasing number of amine groups present in the structure of the receptor. For instance, **2b** having the largest number of amine groups, shows the strongest FE in the presence of nucleotides. The dimethylamine group connected to naphthalimide appears to be responsible for the notable fluorescence enhancement upon interaction with GTP. Therefore, the receptors bearing dimethylamine groups (**1b**, **2b** and **3b**) were selected for binding studies with tetranucleotides 3'-d(NNNN)-5', where N = G, A, C, or T. As can be seen in Fig. 2, a considerable fluorescence enhancement for **3b** (more than 3-fold) was observed in the presence of d(GGGG), but not in the presence of other tetranucleotides. This behaviour is in marked contrast to the interaction of **3b** with GTP, which was not accompanied by fluorescence changes.

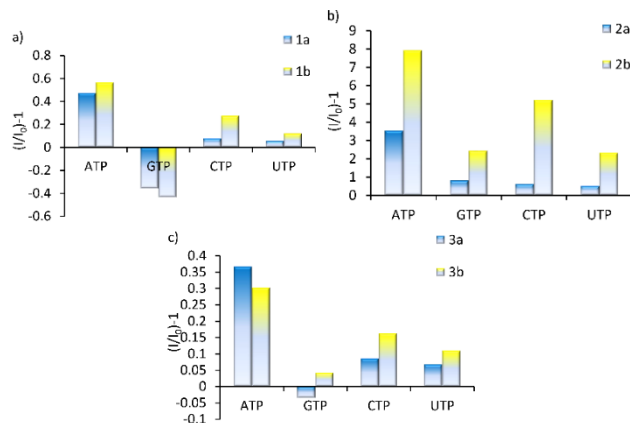


Figure 1. Fluorescence enhancement/quenching in the presence of 100 equiv of nucleotides for (a) **1a–b**, (b) **2a–b** and (c) **3a–b**. Conditions: 10⁻⁵ M receptors in a 10 mM NaAsO₂Me₂ buffer (5% DMSO), 100 mM KCl, pH 7.2, λ_{ex} =450 nm, λ_{em} =460–650 nm.

Table 1. Binding constants (log*K*) determined by fluorescence titrations in a 10 mM cacodylate buffer (pH 7.2) containing 0.1M KCl.

Receptor/ Nucleotide	ATP	GTP	CTP	UTP
1a	3.55(1) ^[a]	3.64(2)	4.54(2)	4.72(2)
1b	3.76(2)	3.85(1)	3.25(2)	4.74(2)
2a	log <i>K</i> ₁₂ = 6.43(3)	log <i>K</i> ₁₂ = 6.53(3)	log <i>K</i> ₁₂ = 6.08(3)	log <i>K</i> ₁₂ = 6.11(3)
2b	log <i>K</i> ₁₂ = 6.51(4)	log <i>K</i> ₁₂ = 6.91(3)	log <i>K</i> ₁₂ = 6.50(3)	log <i>K</i> ₁₂ = 6.51(3)
3a	3.48(2)	– ^[b]	– ^[b]	– ^[b]
3b	3.89(3)	– ^[b]	– ^[b]	– ^[b]
	AAAA	GGGG	CCCC	TTTT
1b	4.76(2)	4.92(1)	– ^[b]	– ^[b]
2b	5.37(4)	5.23(5)	4.46(5)	– ^[b]
3b	5.46(4)	5.53(3)	– ^[b]	– ^[b]

[a] The standard deviation of the measurements is shown in log units of the last decimal in parenthesis. [b] Changes are too small for accurate assessment of binding affinity.

Circular dichroism spectroscopy revealed that in a K⁺-rich buffer, d(GGGG) displayed a positive peak at 260 nm characteristic for a parallel G-quadruplex architecture, that was absent in the absence of K⁺ cations (ESI[†], Fig. S26). Indeed, a related hexanucleotide d(TG₄T) was the first parallel-stranded G-quadruplex whose structure was fully characterized.¹⁶ Thus, the exceptional fluorescence properties of **3a** in the presence of d(GGGG) can be attributed to the interaction of the receptor with a G-quadruplex structure rather than with the unstructured tetranucleotide.

The analysis of titrations performed with d(GGGG) (Fig. 2d) reveals that FE increases in the row **1b** < **2b** < **3b**. In addition, a hypsochromic shift was observed for all three compounds (Fig. 2, a–c). According to our previous work, this shift indicates that the “turn-on” effect originates from the complexation-induced protonation of amine groups and intercalation.¹² In receptor **3b**, only the tertiary amines of the macrocyclic scaffold are not protonated at pH 7.2. Thus, the binding-induced protonation of these amines is suggested to be the origin of the

fluorescence increase. A comparison of FE factors (I/I_0) suggests that the higher the negative charge of the sequence, the stronger the overall enhancement.

Building on the hypothesis that fluorescence enhancement of **3b** is specific for interaction with G-quadruplex structures, the response of the macrocycle family was studied with respect to eight G4-DNA structures of different folding topologies (parallel: *myc22*, *Pu24T*, *KRAS*; antiparallel: *hras1*, *22CTA*, *hTel21T18*; hybrid: *25TAG* and *24TTG*), as well as single-stranded (*dT₂₂*) and double-stranded (*ds26*) oligonucleotides of comparable length (ESI†, Table S1). The response of the polyamine macrocycles **2a–b** to G4s was identical to that observed for the starting naphthalimide polyamines **1a,b**. Thus, in Fig. 3a, only the response for receptors bearing dimethylamine groups is shown. The fluorescence of compound **1b** did not change in the presence of oligonucleotide analytes, indicating that naphthalimide fluorophore does not interact with oligonucleotides in the absence of a macrocyclic receptor. Remarkably, the fluorescence of **2b** and **3b** increased considerably in the presence of oligonucleotides. The fluorescence of the polyamine macrocycle **2b** increased 4-fold to 9-fold in the presence of G4-quadruplex structures, as well as in the presence of a single-stranded oligonucleotide *dT₂₂*, indicating that DNA binding of this macrocycle was not structure-specific. Among all receptors, the fluorescence of the amido-amine receptor **3b** was selectively enhanced in the presence of parallel G4 structures (6–9-fold), but much weaker in the presence of other quadruplex topologies (2–4-fold) and less than 2-fold in the presence of double-stranded DNA.

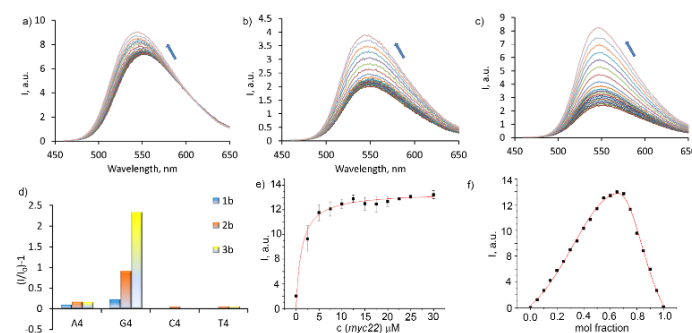


Figure 2. Fluorescence changes of (a) **1b**, (b) **2b** and (c) **3b** in the presence of 5'-d(GGGG)-3'. (d) Fluorescence enhancement histogram obtained after saturation of receptors with tetranucleotides (6 equiv.) (e) Fluorescence intensity changes of **3b** together with the fitting curve in the presence of increasing amounts of *myc22* G4-DNA. (f) Job plot of fluorescence intensity for mixtures of **3b** with *myc22* G4-DNA. Error bars represent the standard deviation from three independent titrations. Conditions: receptors with concentrations of 10^{-5} M in a 10 mM NaAsO₂Me₂ buffer (5% DMSO), 100 mM KCl, pH 7.2.

According to the titration of *myc22* (Fig. 2, e-f), two molecules of **3b** are bound to *myc22* with identical dissociation constants $K_{11} = K_{12} = 1.09 \mu\text{M}$, which can be rationalized by binding of one receptor molecule to each of the two external G-tetrads. Similar to the receptor-triphosphate interactions, it is proposed that binding to higher negatively charged oligonucleotide results in protonation of tertiary amino groups

of the macrocyclic scaffold, which in turn leads to a fluorescence enhancement. As such, it is likely that **3b** is found in a 4-fold protonated state in the complex with the oligonucleotide, with positive charges located at the two tertiary amine groups and the two dimethylamine groups. The proposed sensing mechanism is shown in Fig. 3c

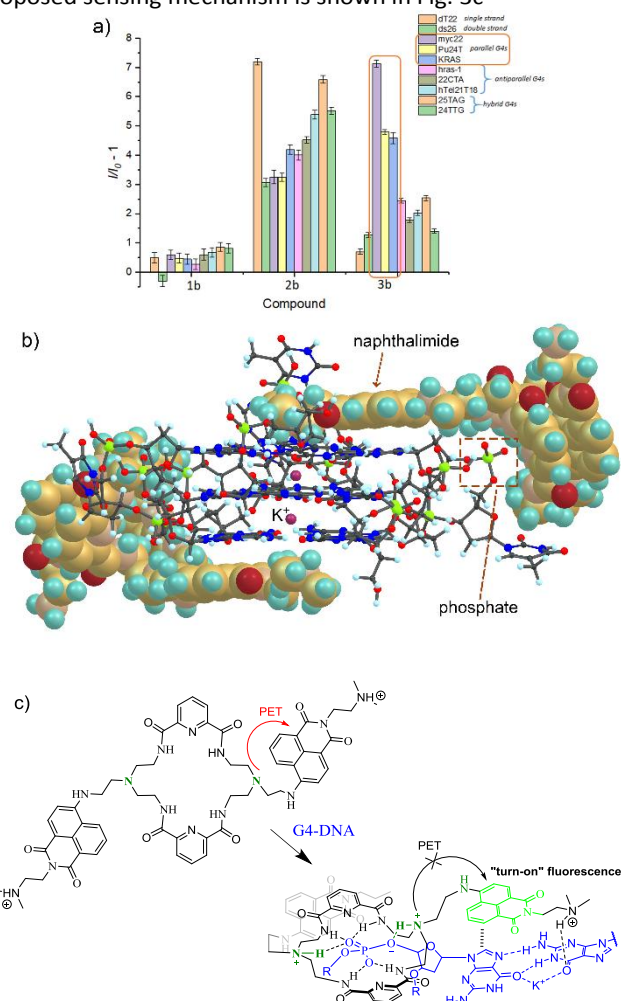


Figure 3. a) Fluorescence response towards RNA and DNA sequences for compounds **1b**, **2b** and **3b**. Conditions: oligonucleotide (10 μM), receptor (5 μM), $\lambda_{\text{ex}} / \lambda_{\text{em}} = 440/544\text{nm}$; buffer: 5% DMSO, 10 mM NaAsO₂Me₂, 100 mM KCl, pH 7.2. b) Optimized structure of the 2:1 complex of receptor **3b** with a model G-quadruplex structure d(G₃TG₄TG₃TG₃). The atoms of the receptor are highlighted with as Van der Waals spheres. c) Schematic representation of the “turn-on” sensing mechanism during the recognition of a G-quadruplex.

The obtained data show that the receptor–nucleotide complex should be stabilized by several non-covalent interactions: macrocycle-phosphate electrostatic and hydrogen bonding interactions, guanine-naphthalimide stacking, and electrostatic interactions between the dimethylamino groups and G-quadruplex. To determine how receptors are bound to *myc22* we used a simplified G-quadruplex structure d(G₃TG₄TG₃TG₃) in a parallel fold and modelled its interaction with two molecules of the receptor in the conformation found in our previous work for the sulfate complex.¹² Possible geometries of the 2:1 complex were optimized by a parametrized model involving dispersion interactions with the help of “Priroda”

software package.¹⁷ We found that in the optimized structure the receptors are bound to the sterically most accessible phosphate residues. One naphthalimide moiety of each receptor was found to stack with each of the external G-tetrads, whereas the second naphthalimide residue formed stacking interactions with pyridine rings of the macrocycle (Fig. 3b). Additional stabilization was indeed provided by the protonated dimethylamine groups coordinated to guanine oxygen atom. Two receptors are bound from above and below to the quadruplex structure. This fact explains the observed 2:1 binding stoichiometry for *myc22*.

In conclusion, we have synthesized a new family of G4-binding receptors based on PBM-dye architecture. While the amine macrocycles **2a** and **2b** have demonstrated a strong fluorescence enhancement with all kinds of mono- and oligonucleotides, they possess low binding and sensing selectivity. Amido-amine receptor **3b** bearing dimethylamine groups has been proved to be the most selective in terms of fluorescence enhancement in the presence of parallel G4-topologies.^{11c, 18} Interestingly, tetranucleotide 3'-d(GGGG)-5' forms a parallel G-quadruplex in a buffered solution containing K⁺ and can serve as a good model to discover new "turn-on" probes. We believe that a proper choice of macrocycles, spacers and dyes may address challenges associated with the degree of fluorescence response, selectivity for a particular G4 topology, and absorbance window. Studies of new systems in cell imaging application are in progress.

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Conflicts of interest

There are no conflicts to declare.

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