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Boronic acid-modified DNA that changes fluorescent properties upon carbohydrate binding†

Xiaochuan Yang, Chaofeng Dai, Angie Dayan Calderon Molina and Binghe Wang*

A long wavelength boronic acid-modified TTP (NB-TTP) has been synthesized and enzymatically incorporated into DNA. Such DNA shows intrinsic fluorescent changes upon carbohydrate addition.

In addition to their critical roles in storing genomic information, DNA molecules are also very important in the development of aptamers,[1] nanoelectronic assemblies, nanosensors, nanoclusters, nanocomputing,[2] and DNA rotary machines, to name a few.[3] DNA functionalization brings in additional properties for broadened applications. For example, gold-nanoparticle functionalized DNA assemblies have been used in sensor development with extraordinary sensitivity;[3] and side-chain functionalized DNA has been used in aptamer selection with improved affinity and properties.[4,5]

Our lab has had a long-standing interest in using the boronic acid moiety, which is known to interact with diols/carbohydrates, fluoride, cyanide, and other nucleophiles/Lewis bases, as a recognition group for sensing and other applications.[6,7] Recently, we have reported the synthesis of a quinoline boronic acid-modified thymidine triphosphate (QB-TTP, Fig. 1),[8] its enzymatic incorporation into DNA, and discussed the feasibility of using it in DNA aptamer selection. Along this line, we desire to incorporate a fluorescent boronic acid into DNA that has the following properties: (1) the excitation and emission wavelengths are longer than the λ_{max} of nucleobases; (2) shows fluorescent property changes upon carbohydrate binding; (3) is stable under PCR conditions; (4) is stable under copper-mediated "click" conditions;[9,10] and (5) is relatively easy to synthesize. Such boronic acid-modified DNA may have applications in carbohydrate and glycoprotein sensing as well as the construction of other DNA assemblies with unique properties.[7] For example, nucleic acid and peptide-templated multi-boronic acid compounds are artificial lectin mimics; boronic acid-containing compounds have been used in the preparation of polymers with reversible properties; boronic acid-modified proteins can be used as lectin mimics with improved affinity and for protein purification; and boronic acid-containing polymers have been used for the development of glucose-sensitive insulin release systems. Beyond simple sensing, incorporation of fluorescent boronic acid-modified thymidine into genomic DNA may allow for the probe of changes in the nuclear environment,

nuclear imaging, and cell division genomic DNA tracing. Herein, we report the very first side chain functionalized naphthalimide-based boronic acid-modified TTP (NB-TTP, Fig. 1) that by itself shows significant intrinsic fluorescent property changes upon carbohydrate binding and retains this ability even after incorporation into DNA.

4-Amino-1,4-naphthalimide was chosen as the fluorophore after careful consideration and evaluation.[11–14] Many boronic acids that change fluorescent properties have been reported. However, not all fluorescent boronic acids retain their fluorescent properties after incorporation into DNA. Such is the case with quinolineboronic acid, which loses its ability to show significant fluorescent intensity changes (over 40-fold) in response to sugar binding after DNA incorporation.[8] Furthermore, when we applied the criteria set out above, the naphthalimide-based boronic acid moiety in Fig. 1 seemed to represent the best choice because of its relatively long excitation and emission wavelengths, stability, and ease of synthesis. For example, under constant irradiation at 490 nm for one hour, the fluorescence of boronic acid **7** (Scheme 1) did not show noticeable changes (Fig. S1, ESI†). Thus, the problem of photo bleaching faced by many fluorophores[15,16] should not be a major issue in this case. In this design, CuAAC (copper(I)-catalyzed azide-alkyne cycloaddition),[17] which has been studied in previous boronic acid synthesis,[8,18,19] was used to conjugate the thymidine triphosphate moiety with the boronic acid moiety. This was done because of the difficulty in performing the triphosphorylation reaction in the presence of a boronic acid moiety (hygroscopic) and the difficulty in installing a boronic acid group in the presence of a triphosphate. The synthesis of the boronic acid moiety is described in Scheme 1 and the synthesis of M-TTP (Fig. 1) has been described previously.[8]
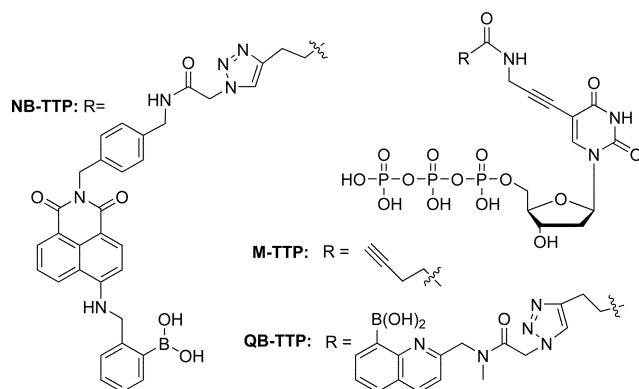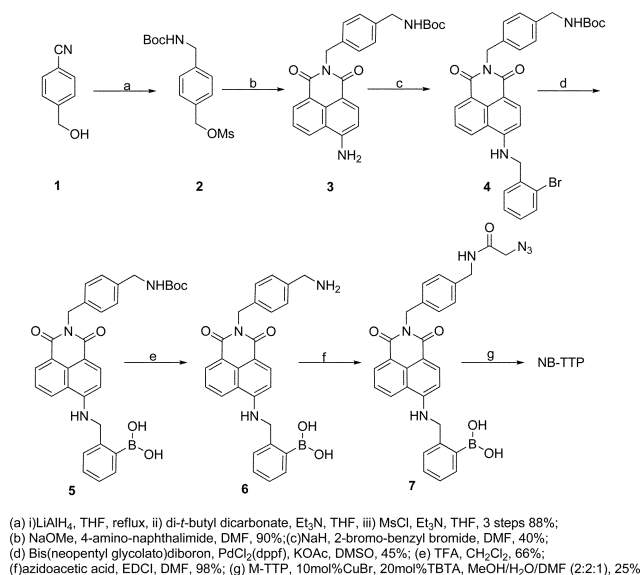
Department of Chemistry and Center for Biotechnology and Drug Design, Georgia State University, Atlanta, Georgia 30302-4098, USA. E-mail: wang@gsu.edu; Fax: +1 4044135543; Tel: +1 4044135545
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Fig. 1 M-TTP, QB-TTP and NB-TTP.

(a) i)LiAlH₄, THF, reflux, ii) di-t-butyl dicarbonate, Et₃N, THF, iii) MsCl, Et₃N, THF, 3 steps 88%;
(b) NaOMe, 4-amino-naphthalimide, DMF, 90%;(c)NaH, 2-bromo-benzyl bromide, DMF, 40%;
(d) Bis(neopentyl glycolato)diboron, PdCl₂(dppf), KOAc, DMSO, 45%; (e) TFA, CH₂Cl₂, 66%;
(f)azidoacetic acid, EDCI, DMF, 98%; (g) M-TTP, 10mol%CuBr, 20mol%TBTA, MeOH/H₂O/DMF (2:2:1), 25%

Scheme 1 Synthesis route of NB-TTP.

During the experiment, it was observed in steps (e) and (f) (Scheme 1) that the solubility of boronic acids **6** and **7** in methanol was very low if the solution was acidic and treatment with K_2CO_3 brought the boronic acids into solution. The synthesized NB-TTP has a solubility of about 170 μM in water as determined using UV. This solubility is high enough for most enzymatic reaction requirements.

Since the NB-TTP compound was designed for its ability to change fluorescent properties, we studied its binding with a model sugar, fructose. As shown in Fig. 2a, the fluorescence intensity of NB-TTP increased upon D-fructose addition in a concentration dependent fashion (λ_{ex}: 490 nm). The K_a was determined to be 73 ± 5 M^{-1} (see ESI for details†). Such results demonstrate that even after conjugation with a nucleobase (thymidine), the naphthalimide-based boronic acid maintains its ability to change fluorescent properties upon sugar binding.

Next we studied whether this NB-TTP could be incorporated into DNA in an enzyme-catalyzed reaction. In this study, NB-TTP was incorporated into a DNA sequence of a 14-nt primer (Table 1) using a 21-nt template (Template 0, Table 1) through Klenow fragment catalyzed primer extension.[20] The primer and template sequences were designed in such a way that the first base to be incorporated is a T, which led to a "none-or-all" case in the extension. In the control reaction,
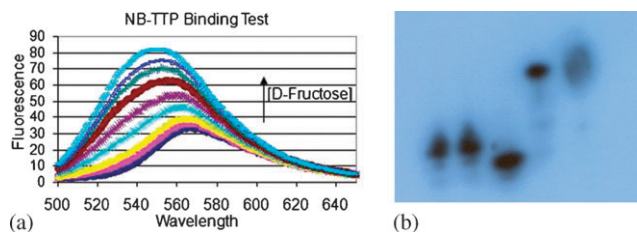

Fig. 2 (a) Fluorescent binding test of NB-TTP with D-fructose. (b) Primer extension analyzed on 15% PAGE: (1) primer only; (2) reaction without enzyme; (3) reaction without dNTPs; (4) reaction with natural dNTPs; (5) reaction with NB-TTP and other dNTPs.

Table 1 Designed sequences for NB-TTP-DNA construction

Primer	3′-GGTCGTTGGGCGAT-5′
Template 0	5′-GGTTCCACCAGCAACCCGCTA-3′
Template 1	5′-GGTTCCAACCAGCAACCCGCTA-3′
Template 2	5′-GGTTCACACCAGCAACCCGCTA-3′
Template 3	5′-GGTTACCACCAGCAACCCGCTA-3′
Template 4	5′-GGTATCCACCAGCAACCCGCTA-3′
Template 5	5′-GGATTCCACCAGCAACCCGCTA-3′
Template 6	5′-GAGTTCCACCAGCAACCCGCTA-3′
Template 7	5′-AGGTTCCACCAGCAACCCGCTA-3′

full extension of the primer using natural dNTPs yielded a DNA with m/z of 6520.4 (calculated $[M+H]^+$: 6519.3) as the major product peak in MALDI (Fig. S2.1, ESI†). The minor peak of m/z 6850.6 could have resulted from a non-specific extension with an extra nucleotide. In the NB-TTP (replacing TTP) reaction, primer extension yielded a DNA with m/z of 7142.8 as the major product peak (Fig. S2.2, ESI†) and a new peak of m/z: 7160.6 after treatment with H_2O_2 (Fig. S2.3, ESI†). Each was assigned as the deboryl-ated (calc. $[M+H-HBO_2]^+$: 7142.5) and oxidative deborylated NB-TTP-DNA sequence (calc. $[M+H-HBO]^+$: 7158.5), respectively. Thus, it was concluded that the boronic acid of NB-TTP was intact after incorporation into the DNA sequence and deborylation happened during the ionization process, which is common with arylboronic acids.[21]

The DNA from primer extension using NB-TTP was studied using gel electrophoresis. The 14-nt primer was labeled with ^{32}P at the 5′-end using γ-^{32}P-ATP and T4 kinase (Fig. 2b, Lane 1). Negative control reactions without the Klenow fragment (Fig. 2b Lane 2) and without dNTPs (Fig. 2b, Lane 3) showed no full length DNA sequence. On the other hand, positive control reactions in the presence of both the enzyme and natural dNTPs (Fig. 2b, Lane 4) showed full length DNA. The shorter length of DNA in Lane 3 without dNTPs could result from the $3' \rightarrow 5'$ exonuclease activity of Klenow fragment.[20] Primer extension using NB-TTP and three other natural dNTPs gave a full length of DNA sequence (Fig. 2b, Lane 5). Its smear band could be due to the inter-action of the boronic acid moiety with the gel matrix or the DNA conformation disrupted by the large naphthalimide moiety. Thus, both mass spectrometry and gel electrophoresis results indicated that NB-TTP was recognized by DNA polymerase as a substrate and incorporated into DNA.

After incorporating NB-TTP into the DNA sequence, we were interested in studying whether sugar addition to the DNA solution would still cause fluorescence property changes. Since dNTPs and oligonucleotides are known to quench fluorescence,[22–24] the retention of NB-TTP's fluorescent property in DNA was uncertain. Fructose in the concentration range of 1 to 100 mM was used as a model sugar. Fig. 3 shows that the fluorescence intensity of NB-TTP-DNA increased by about 1.5-fold upon fructose addition at its highest concentration used (100 mM) (λ_{ex}: 490 nm), which demonstrated that the fluorescent properties of NB-TTP were retained. Again, this is the very first example of side-chain functionalized DNA showing intrinsic fluorescent property changes upon sugar binding and such changes do not rely on FRET.
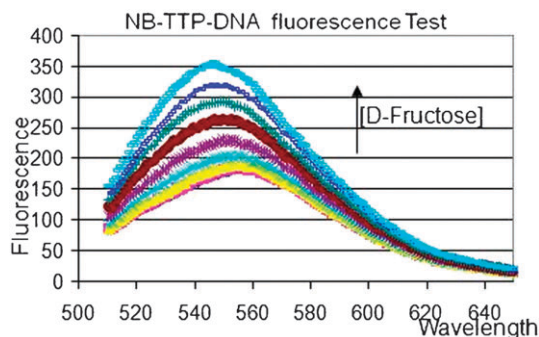
Fig. 3 Fluorescent binding tests of NB-TTP-DNA with D-fructose.

Since fluorophores can self-quench through stacking and DNA can also modify the fluorescent properties of a fluorophore if intercalation occurs, we were also interested in examining whether spacing between two boronic acid-modified T would affect the intrinsic fluorescent properties of the fluorophore. Therefore, Templates 1–7 were designed in such a way that two A bases at the 5′-end were separated by 0–6 bases in between while keeping the rest of bases the same (Table 1), which would lead to DNA sequences with two boronic acid-modified T separated from 0 to 6 bases.

The same model sugar, fructose, was chosen for the fluorescent test. Double stranded NB-TTP-DNA sequences were generated through primer extension reactions with a primer/template ratio of 1 : 1 and no heat denaturing before the binding test. The 21-nt template denoted as Template 0 was used as the control (Table 1). From the testing results (Fig. S3.1, ESI†), it can be seen that all boronic acid-modified DNA showed fluorescent intensity changes upon sugar addition. Such results indicate that having more than one fluorophore in the DNA sequence did not affect its ability to fluoresce. The same studies were conducted using the ssDNA. Again, the boronic acid fluorophore retained its ability to change fluorescence (Fig. S3.2, ESI†). It should be noted that the fructose studies were meant to test the intrinsic ability for the fluorophore in question to change fluorescence. This is not a "sensor" for fructose or any other sugar.

A novel fluorescent boronic acid conjugated TTP analogue (NB-TTP) was successfully synthesized. NB-TTP was readily recognized by the Klenow fragment and incorporated into DNA. NB-TTP modified DNA showed fluorescence intensity increases upon binding with fructose. Spacing two boronic acids in various positions does not fundamentally affect its ability to change fluorescence upon sugar binding. The boronic acid-modified thymidine will be very useful for the preparation of DNA-based sensing and molecular recognition applications and for genomic DNA incorporation to probe specific properties in the nucleus.

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