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Effect of 3′-end capping of aptamer with various 2′,4′-bridged nucleotides: Enzymatic post-modification toward a practical use of polyclonal aptamers

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ABSTRACT

The capping of the 3′-ends of thrombin binding aptamers (TBAs) with bridged nucleotides increased the nuclease resistances and the stabilities in human serum. The binding abilities of the aptamers were not affected by the capping. The capping could be simply executed via a one step enzymatic process using 2′,4′-bridged nucleoside 5′-triphosphate and terminal deoxynucleotidyl transferase.

Researchers have created a variety of nucleic acid aptamers with the systematic evolution of ligands by exponential enrichment (SELEX) method[1–3] and studied them in order to apply them as research tools, biosensors, therapeutic agents and so on. With this technique, nucleic acid aptamers can be selected from a library of single-stranded oligonucleotides, differing in nucleotide sequence. In general, after being selected from an initial library, individual aptamers are isolated from an enriched pool by cloning and researchers determine their sequences. For practical use, nuclease resistance is a necessary property of aptamers. Therefore, in order to gain stability in cell cultures and in vivo, isolated monoclonal aptamers should be modified with proper substituents, based on their sequence information and the correlations between modifications and activities, which may involve costly and monotonous processes. If an enriched pool, functioning as the polyclonal family, could gain nuclease resistance without cloning, sequencing and the aforementioned modifications, the enriched pool, that is, the polyclonal aptamer[4], could be used as a polyclonal antibody.

Until now, 2′-*O*,4′-*C*-methylene bridged/locked nucleic acid (2′,4′-BNA[5,6]/LNA[7]) and its analogues[8–12], have been developed, and some of them showed excellent nuclease resistances. Recently, we reported the convenient conferring of nuclease resistance on nucleic acid by using 2′,4′-bridged nucleoside-5′-triphosphates[13,14] and terminal deoxynucleotidyl transferase (TdT).[15] We were easily able to add the 2′,4′-bridged nucleotides to the 3′-ends of oligodeoxy-ribonucleotides (ODNs) with an enzymatic reaction. After the addition, the nuclease resistances of the ODNs were enhanced, depending on the chemical structures of the bridged moieties. In this work, we have designed and synthesized a new 2′,4′-bridged nucleoside-5′-triphosphate with a 2′-CH(Ph)OCH_2-4′ bridged linkage, **QTP**, expecting that the bulkiness of the phenyl group would interfere with the nuclease degradation of the ODN. Furthermore, the substrate properties of **QTP** for TdT and the characterizations of thrombin binding aptamers (TBAs) capped with various 2′,4′-bridged nucleotides for nuclease resistances, biostabilities in human serum and binding affinities to the target were investigated.

First, we studied the incorporation of the triphosphate **QTP** during the enzyme reaction with TdT, using a 26-mer single-stranded ODN with 5′-FAM-GGC GTT GAG TGA GTG AAT GAG TGA GT-3′ (ODN1); the 5′-end of ODN1 was labelled with 6-carboxyfluorescein (6-FAM) for detection. Syntheses and characterizations of **QTP** and its intermediates are described in detail in Supplementary data. As we have reported[8], ODNs capped with **K**, **L** and **M**-type bridged nucleotides were quantitatively produced using **KTP**, **LTP** and **MTP** (see Figure 1), respectively, after incubating the reaction mixture

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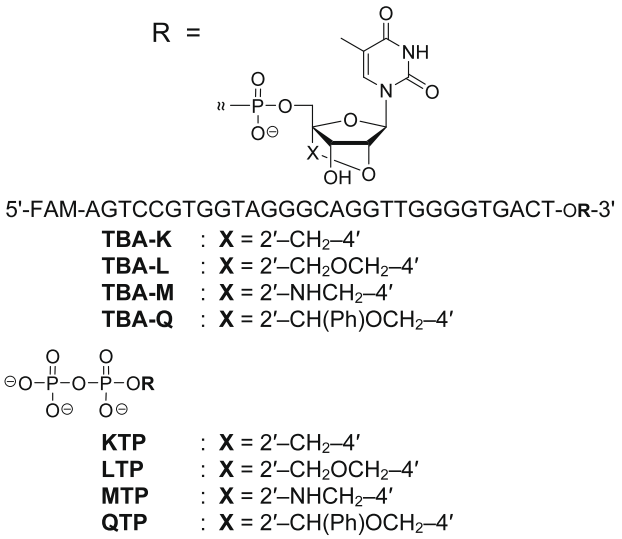

Figure 1. Chemical structures of 2′,4′-bridged nucleotides used in this experiment.

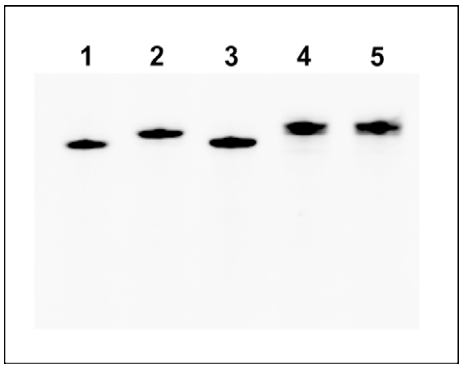

Figure 2. Representative gel images of reactions using the 2′,4′-bridged nucleoside triphosphates with TdT. The reaction mixtures for the positive control with **LTP** and TdT (0.2 U/μL) incubated for 1 h (lane 2), those containing **QTP** and TdT (0.2 U/μL) incubated for 1 h (lane 3), **QTP** and TdT (10 U/μL) incubated for 2 h (lane 4), and **QTP** and TdT (10 U/μL) incubated for 3 h (lane 5). ODN1 migrated only in lane 1.

containing 0.4 μM of ODN1, 200 μM of **KTP**, **LTP** or **MTP**, 0.2 U/μL of TdT and the reaction buffer (an enzyme at 1 × concentration) for 1 h at 37 °C. However, the use of **QTP** did not provide the corresponding

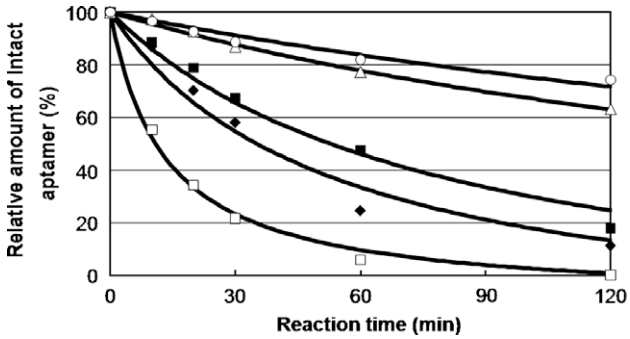

Figure 4. The time course of the degradation of thrombin binding aptamers (TBAs) by phosphodiesterase I; reaction using TBA1 (open squares), TBA-K (closed diamonds), TBA-L (closed squares), TBA-M (open triangles), and TBA-Q (open circles). The *x*-axis indicates the reaction time (min), and the *y*-axis represents the relative amount of intact TBAs (%). Total quantities of the products were set at 100% in each reaction mixture.

ODN capped with a single **Q**-type bridged nucleotide under the same conditions. If we increased the enzyme concentration to 10 U/μL and extended the incubation time for a few hours, the addition reaction was almost completed (see Fig. 2). We then assessed the nuclease resistance of the capped ODN for snake venom phosphodiesterase I, which has a strong 3′–5′ exonuclease activity. It was about 736-fold more stable than uncapped ODN1 and 51-, 19- and 2.3-fold more stable than the other capped ODNs with **K**, **L** and **M**-type bridged nucleotides, respectively, under the above reaction conditions (see Supplementary data Table S1). These results indicated that the bulky phenyl group on the bridged linkage affected the addition reaction with TdT but improved resistance for phosphodiesterase I.

The addition of the **Q**-type bridged nucleotide at the 3′-end greatly enhanced the nuclease resistance; however, ODN1 is not an oligodeoxyribonucleotide with a special function, such as antisense ODNs[16], decoy ODNs[17] or DNA aptamers.[18] Therefore, we then performed experiments with the same conditions using a thrombin binding aptamer[19], 5′-FAM-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′ (TBA1) and its capped aptamers, TBA-K, TBA-L, TBA-M and TBA-Q, instead of ODN1 (see Fig. 1). As shown in Figure 3, the band of the intact TBA1 disappeared after 120 minutes of incubation (lane 6, image A) while the bands of the intact TBA-M and TBA-Q were thick (lanes 6, images D and E). Compared to the decay curves of intact aptamers in Figure 4, the capped aptamers were most stable in the order TBA-Q > TBA-M > TBA-L > TBA-K in the solution containing the nuclease. The initial reaction rates

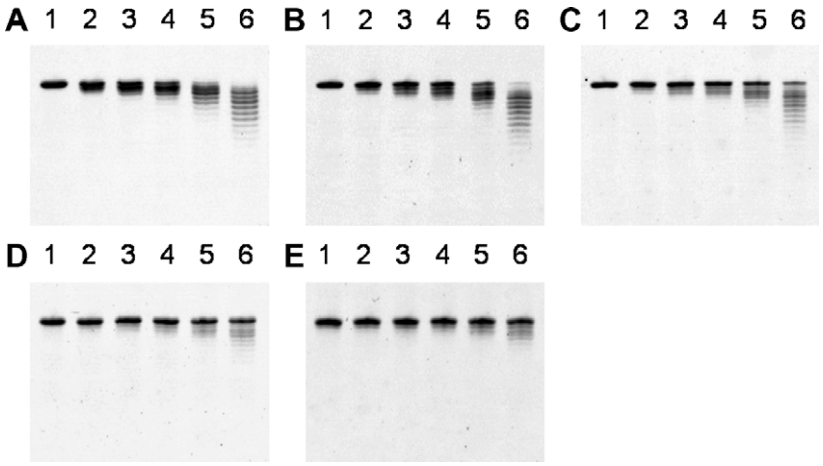

Figure 3. Representative gel images of degradation of TBAs with phosphodiesterase I. Reactions using TBA1 (image A), TBA-K (image B), TBA-L (image C), TBA-M (image D), and TBA-Q (image E). The reaction mixtures at 0, 10, 20, 30, 60, and 120 min are in lanes 1–6, respectively.

Table 1
Degradation of thrombin binding aptamers with snake venom phosphodiesterase I

Thrombin binding aptamers	v_0 (%/min)[a]	Relative rate
TBA1	8.5	27
TBA-K	2.4	7.5
TBA-L	1.6	5.0
TBA-M	0.46	1.5
TBA-Q	0.32	1

[a] Initial rates of degradation v_0 were calculated by the fitting of their decay curves, respectively.

Table 2
Degradation of thrombin binding aptamers in 80% v/v human serum

Thrombin binding aptamers	v_0 (%/min)[a]	Relative rate
TBA1	3.5	4.0
TBA-K	2.4	2.7
TBA-L	1.3	1.5
TBA-M	0.88	1
TBA-Q	1.1	1.2

[a] Initial rates of degradation v_0 were calculated by the fitting of their decay curves, respectively.

estimated from the decay curves of TBA1, TBA-K, TBA-L, TBA-M and TBA-Q were 8.5, 2.4, 1.6, 0.46 and 0.32, respectively (see Table 1). The capping effect of TBA1 was smaller than that of ODN1 presumably because the aptamer formed a specific conformation, that is, G-quadruplex,[20,21] which would have decreased the accessibility of the nuclease to the aptamer. However, the capping effect with bridged nucleotides clearly reflected in the nuclease resistances and the correlations between the chemical structures of the bridged linkages and the nuclease resistances were extremely consistent with the case of ODN1.

Next, we examined the biostabilities of these capped TBAs in human serum containing various types of nucleases. As shown in Figure 5, TBA-M and TBA-Q were apparently more stable than the others; more than 20% of the intact TBA remained in the cases of these two aptamers while the intact TBA was completely gone in the case of the uncapped TBA1 (see Supplementary data Figure S1) after incubating in 80% v/v human serum for 8 hours at 37 °C. The relative rates of the degradation showed that TBA-M and TBA-Q were about 4.0 and 3.3-fold more stable than TBA1 (see Table 2), but on the other hand, TBA-Q was more stable than TBA-M in solution containing phosphodiesterase I. The difference in the stabilities was small, but we observed this tendency had good reproducibility, indicating that it may be due to the difference of the substrate specificities between phosphodiesterase I and 3′–5′ exonuclease contained in human serum and not to any measurement errors. The capping effect for the stability in human serum compared to that in phosphodiesterase I solution was significantly smaller because of the action of endonuclease contained in the serum. However, the effect on the nuclease resistance was still clearly observed, indicating that the action of 3′ to 5′ exonuclease would be significantly dominant compared to that of any other types of nuclease in human serum. Recently, Peng et al. reported an effort of enhancing the nuclease resistance of a thrombin binding aptamer in the sequence d($G_2T_2G_2TGTG_2T_2G_2$) by modification with

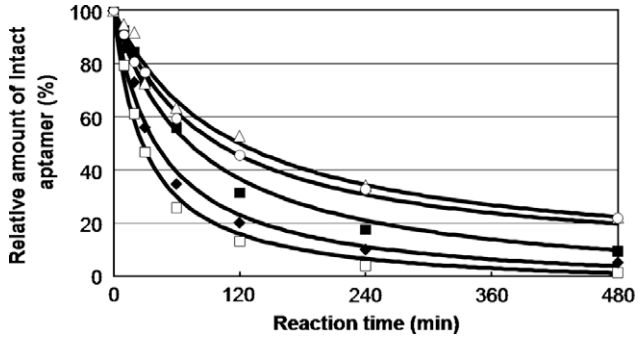

Figure 5. The time course of degradation of thrombin binding aptamers (TBAs) in 80% v/v human serum; reaction using TBA1 (open squares), TBA-K (closed diamonds), TBA-L (closed squares), TBA-M (open triangles), and TBA-Q (open circles). The *x*-axis indicates the reaction time (min), and the *y*-axis represents the relative amount of intact TBAs (%). Total quantities of the products were set at 100% in each reaction mixture.

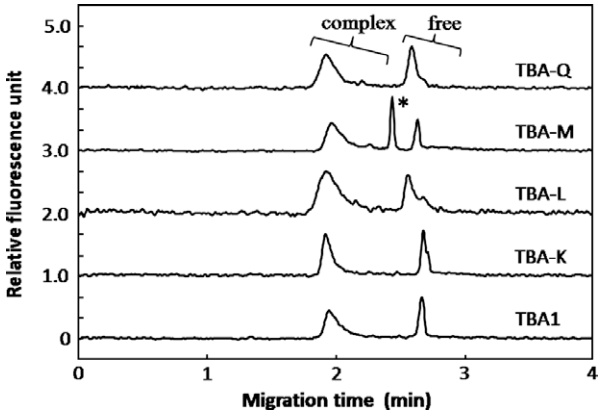

Figure 6. Representative capillary electropherograms for individual thrombin-TBA complexes. The *x*-axis indicates the migration time (min), and the *y*-axis represents the relative intensity of signals from emission of 5′-FAM detected at 520 nm (excited at 488 nm). The peak with the asterisk (*) corresponds to TBA-M dissociated from thrombin during CE, which might conform a particular structure. The concentrations of TBAs and thrombin were 1 nM and 4 nM. The sample buffer and running buffer were 20 mM Tris–HCl (1 mM $MgCl_2$) at pH 7.4 and 100 mM sodium borate at pH 8.35, respectively. Experimental conditions: separation voltage, +12 kV; injection 0.5 psi × 7.8 s.

2′-deoxy-2′-fluoro-D-arabinonucleotide.[22] Some modified aptamers exhibited increased nuclease resistances (4–7-fold) in 10% v/v fetal bovine serum at 37 °C without losing their binding abilities. However, gaining stability in the serum required at least four replacements with the modified nucleotide. Furthermore, some modified aptamers almost lost their binding abilities completely depending on the numbers and positions of the replacement points. Thus, conferring nuclease resistance on aptamers by post-modification[23] is not very easy in general.

Finally, we measured the binding affinities of capped TBAs to human thrombin using non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)[24] in order to assess the capping effect on the binding ability of the aptamer[25] (Fig. 6). The K_d values for the target-TBA interactions were determined from the biding saturation curves of TBA with different concentrations of thrombin as seen in Figure S3. The K_d values of TBA1, TBA-K, TBA-L, TBA-M and TBA-Q for the target were 0.25 ± 0.06, 0.18 ± 0.04, 0.27 ± 0.05, 0.21 ± 0.06 and 0.28 ± 0.09 nM, respectively; those affinities were found to be almost the same level.

In conclusion, the capping of the 3′-ends of thrombin binding aptamers with bridged nucleotides increased the nuclease resistances 3.6–27-fold and the stabilities in human serum 1.5–4.0-fold. Also, the binding abilities of the aptamers were not affected by the capping. The capping could be simply executed via a one step enzymatic process using 2′,4′-bridged nucleoside 5′-triphosphate and TdT. The merit of this method is in developing a method that will enable us to modify and use aptamers without isolating them from the polyclonal family. The chemical structure of the bridged linker should be improved further. Particularly, it is interesting that

phosphodiesterase I nucleases in human serum may have different substrate specificities, as shown in the experiment using TBA-M and TBA-Q. The bulkiness of the bridged ring structure likely enhanced the biostabilities; however, enlarging the ring structure would make its triphosphate analogue poorer as a substrate for TdT. Molecular design based on the steric structure of the substrate-binding site in TdT and the nuclease should lead to optimized chemical structures of the analogue in future.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.028.

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