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Boronic acid-mediated activity control of split 10-23 DNazymes

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Abstract: The 10-23 DNzyme is an artificially developed Mg^{2+} -dependent catalytic oligonucleotide that can cleave a RNA substrate in a sequence-specific fashion. In this study, we designed new split 10-23 DNzymes made of two non-functional fragments, the first one carrying a boronic acid group at its 5' end, while the other one is composed of a ribonucleotide at its 3' end. We demonstrate herein that the addition of Mg^{2+} ions leads to the assembly of the fragments, which in turn induces the formation of a new boronate internucleoside linkage that restores the DNzyme activity. A systematic evaluation has been performed that identified the most performant system. Our results highlight key features for an efficient control of DNzymes activity through the formation of boronate linkages.

The spontaneous and reversible reaction between boronic acids and cis-diols leading to the formation of cyclic boronate esters has been comprehensively investigated^[1] and has found widespread use for biomedical,^[2] sensing,^[3] materials,^[4] and supramolecular applications.^[5] This very unique reaction is notably characterized by the ability of boronic acids to operate in aqueous media, and by the reactivity of the boron atom, which can be either sp^2 - or sp^3 -hybridized depending on the addition of a Lewis base.^[6]

In this context, we recently developed reversible DNA- and RNA-templated ligation systems occurring through the reaction of two oligonucleotide fragments, the first one composed of a boronic acid group at its 5' end, while the second one carried a ribonucleotide at its 3' end (Figure 1A).^[7] The resulting ligated strand therefore differs from natural DNA by having a boronate internucleoside linkage instead of a phosphodiester. Inspired by these results we decided to evaluate if the formation of boronate esters at key positions of engineered split DNzymes could be used to control their activity (Figure 1B).

Like ribozymes, DNzymes are capable of supporting a wide variety of reactions.^[8] Yet, the most prominent and best studied representatives of the DNzyme family are RNA cleaving DNzymes.^[9] Composed of a catalytic core and substrate-recognizing domains RNA-cleaving DNzymes catalyze the phosphodiester bond cleavage of their RNA substrate with the aid of a divalent cation. Among them, the 10-23 DNzyme, which was named after the round and clone number of its respective *in vitro* selection, has been the most widely studied

RNA-cleaving DNzyme.^[10] Its catalytic core is composed of a loop flanked by two complementary substrate-recognition domains. Upon binding the substrate (which is a full oligoribonucleotide or an oligodeoxyribonucleotide with a single ribonucleotide at the cleavage site), the catalytically competent structure is formed in the presence of magnesium ions, followed by cleavage of a specific phosphodiester bond.^[11]

The 10-23 DNzyme catalytic core has been well studied through base or phosphate substitutions and deletions. The G_9 - G_{14} and C_{21} - G_{22} regions were demonstrated to be directly involved in forming the catalytic site.^[12]

Since DNzymes are easy to synthesize and to modify, many studies have been devoted to improve their stability and catalytic properties.^[12d, 13] In this context split-systems, which rely on dividing a DNzyme into a series of two or more independent fragments, able to assemble in the presence of a specific target, are emerging as novel biosensing tools for the modulation of DNzyme activity with high spatial and temporal resolution.^[14] Although split systems are yet easier to synthesize and carry less negative charges per strand, the design of efficient split systems can be a challenging task. The choice of the split site and the resulting stability and efficiency of the split DNzyme-substrate complex are key issues that need to be considered, in particular when application of the split system in diagnostics or therapy is planned.

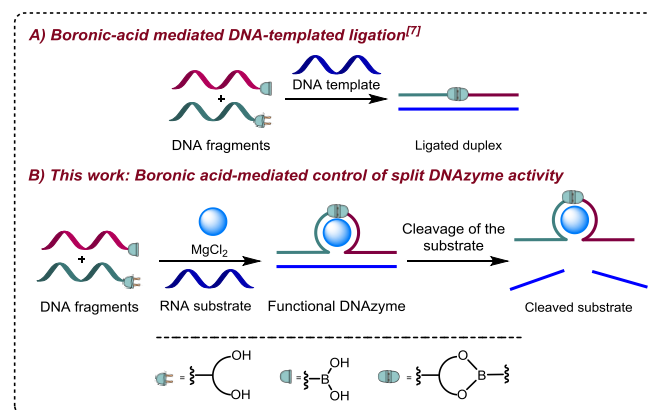


Figure 1. Schematic representation of boronic-acid mediated assembly of nucleic acids

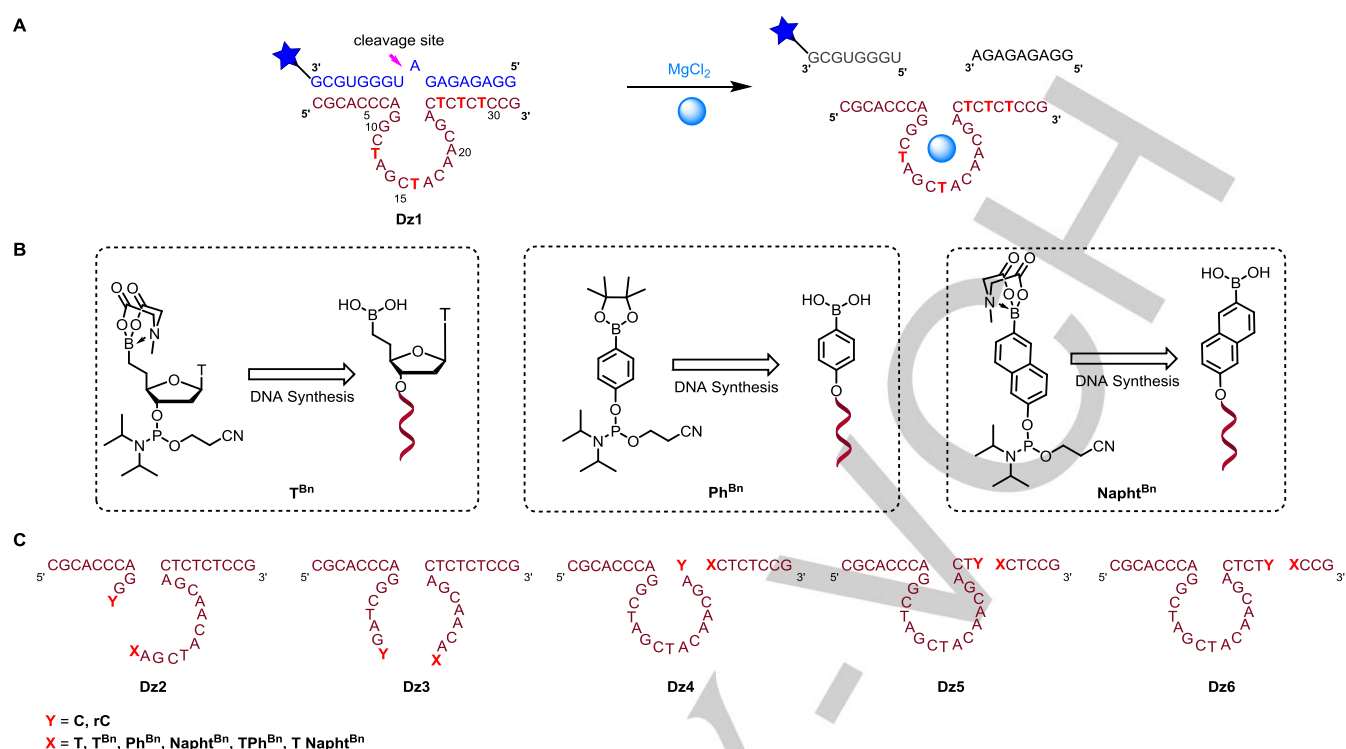


Figure 2. A) The secondary structure of wild-type 10-23 DNAzyme (**Dz1**). The pink arrowhead indicates the cleavage site and the blue star represents the fluorescent dye ATTO680. Thymidines that have been replaced with structures shown in panel B in individual experiments are highlighted in red. B) Phosphoramidite derivatives of boronic acid incorporated at various thymidine positions. C) Sequences of the split DNAzymes (**Dz2-Dz6**) employed in this study.

Most of the systems rely solely on the target-driven assembly of the fragments, which however, often results in unstable and/or poorly active systems. This drawback can be counterbalanced by the use of fragments modified with chemical functionalities that upon addition of the target will be brought in close proximity to allow covalent end-joining of the fragments. Ideally, this reaction occurs without prior chemical activation. Therefore, fragments design and experimental verification of the activity of the assembled structure are required for the development of split DNAzymes.

To achieve this control, the 10-23 DNAzyme was split in two fragments individually at all thymidine positions, located either in the catalytic loop or in the flanked arm. To monitor the performance of the different systems, the RNA substrate 5'-GGAGAGAGAUGGGUGCG-3' was fluorescently labeled with ATTO680 at its 3' extremity and the cleavage rates (k) of DNAzymes **Dz1-Dz6** (Figure 2C), were determined by quantification of the amount of cleaved RNA at eight different time points, analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) on a LICOR DNA sequencer (Figure 2A). Cleavage reactions were performed under single turnover conditions, hence binding of the substrate and release of the cleavage products were not needed to be considered.

The first fragment of each DNAzyme was modified at its 3'-extremity with a cytosine ribonucleotide, while the second fragment was modified at its 5'-extremity with a boronic acid moiety. Three phosphoramidite derivatives of boronic acid, namely **T^{Bn}**, **Ph^{Bn}** and **Napht^{Bn}** were prepared and incorporated individually at all thymidine positions of the 10-23 DNAzyme (Figure 2B and Figure 2C). The phosphoramidite of **T^{Bn}** was

prepared according to previously reported procedures from our laboratory.^[7d, 15] Phosphitylation of commercially available 4-hydroxyphenylboronic acid pinacol ester delivered the phosphoramidite derivative of **Ph^{Bn}**, while the phosphoramidite of **Napht^{Bn}** was obtained from 6-hydroxynaphthalene-2-boronic acid after protection of the boronic acid as a MIDA ester and phosphitylation (ESI). These derivatives were then incorporated at the 5'-end of the oligonucleotide sequences on an automated DNA synthesizer using conventional phosphoramidite chemistry. To further understand the effect of **Ph^{Bn}** and **Napht^{Bn}** modifications, we evaluated also the performance of split DNAzymes where these moieties were added after the T₁₂, T₁₆, T₂₅, T₂₇ and T₂₉ positions instead of replacing those final residues. In each case, the activity of the corresponding non-modified split DNAzymes was evaluated as control.

The Mg²⁺-dependent catalytic ability was first evaluated on the parent **Dz1**, which showed a high cleavage rate ($k = 0.237 \pm 0.045 \text{ min}^{-1}$) and a percentage of RNA cleaved (A) of around 90 % in accordance with previously reported data,^[16] while no reaction could be observed in the absence of Mg²⁺ ions (Table 1, entry 1, Figure 3A).

We started our study with the unmodified split **Dz2**, which showed no cleavage after 2 h of incubation. This result is coherent with previous studies, which demonstrated that non-bridging phosphate oxygens located between C₁₁ and T₁₂ are involved in direct coordination of Mg²⁺ ions (Table 1, entry 2).^[12c] Consequently, it was not surprising that the replacement of C₁₁ by a ribocytosine and T₁₂ by **T^{Bn}** did not restore the activity (Table 1, entry 3). It is interesting to note that, if the DNAzyme was split between C₁₅ and T₁₆ (**Dz3**), the enzyme is still active,

although a 6-fold reduction of the cleavage rate compared to **Dz1** was observed ($k = 0.039 \pm 0.003 \text{ min}^{-1}$, Table 1, entry 4).

Table 1. Observed rate constants and percentage of total RNA cleaved of 10-23 DNAzymes **Dz1** and split DNAzymes **Dz2-Dz6**.

Entry	DNAzyme	X	Y	$k \text{ [min}^{-1}]^a$	A (%)
1	Dz1	-	-	0.237 (± 0.045)	92
2	Dz2	C	T	inactive	-
3		rC	T ^{Bn}	inactive	-
4	Dz3	C	T	0.039 (± 0.003)	82
5		rC	T ^{Bn}	0.127 (± 0.011)	94
6		C	T ^{Bn}	0.048 (± 0.007)	83
7		rC	Nahpt ^{Bn}	0.050 (± 0.005)	88
8	rC	TNahpt ^{Bn}	0.068 (± 0.004)	83	
9	Dz4	C	T	0.005 (± 0.001)	34
10		rC	T ^{Bn}	0.094 (± 0.017)	69
11		rC	Ph ^{Bn}	0.021 (± 0.001)	41
12		rC	Nahpt ^{Bn}	0.026 (± 0.001)	45
13		rC	TPh ^{Bn}	0.045 (± 0.005)	55
14		rC	TNahpt ^{Bn}	0.021 (± 0.004)	39
15	Dz5	C	T	0.087 (± 0.012)	63
16		rC	T ^{Bn}	0.141 (± 0.052)	85
17		rC	Ph ^{Bn}	0.019 (± 0.003)	44
18		rC	Nahpt ^{Bn}	0.016 (± 0.006)	52
19		rC	TPh ^{Bn}	0.204 (± 0.058)	72
20		rC	TNahpt ^{Bn}	0.033 (± 0.004)	53
21	Dz6	C	T	0.059 (± 0.007)	48
22		rC	T ^{Bn}	0.211 (± 0.037)	71
23		rC	Ph ^{Bn}	0.045 (± 0.005)	62
24		rC	Nahpt ^{Bn}	0.052 (± 0.008)	62
25		rC	TPh ^{Bn}	0.085 (± 0.013)	74
26		rC	TNahpt ^{Bn}	0.057 (± 0.015)	79

^a Observed rate constants of DNAzymes under single-turnover conditions (2 h reaction at 25 °C, 50 mM Tris buffer (pH 8.6), 20 mM MgCl₂, 20 nM of substrate, and 2 μM of each **Dz** fragments). First-order rate constants (k) were fit to $[S]=A(1-e^{-kt})$, where $[S]$ is the fraction of substrate uncleaved at time t , and A is the percentage of total RNA cleaved after 2h of incubation.^[16]

However, replacing C₁₅ by a ribocytosine and T₁₆ by T^{Bn} significantly restored activity ($k = 0.127 \pm 0.045 \text{ min}^{-1}$, A = 94%), which implies that the two fragments can reconstitute more than half of the active enzyme by formation of a boronate internucleoside linkage instead of the natural phosphodiester (Table 1, entry 5). Interestingly, joining of the fragments occurs in a region where flexibility is important. A control experiment with the fragment carrying the terminal C₁₅ and the T^{Bn} modified fragment confirmed the requirement of having a diol system for boronate formation and to activate the split DNAzyme ($k = 0.048 \pm 0.007 \text{ min}^{-1}$, Table 1, entry 6). Among the other evaluated **Dz3**-based systems (Table 1, entries 7 and 8), only the one with a fragment bearing an additional Nahpt^{Bn} unit after T₁₆ displayed an activity higher than that of the non-modified split **Dz3** ($k = 0.068 \pm 0.004 \text{ min}^{-1}$) but still twofold lower than with the T^{Bn} modified fragment.

When the DNAzyme is split at the junction of the catalytic core and the flanking arm (**Dz4**), an almost complete loss of activity is observed ($k = 0.005 \pm 0.001 \text{ min}^{-1}$, Table 1, entry 9), most probably due to the incapacity of the DNAzyme to adopt its

active structure. As it was observed with **Dz3**, the replacement of C₂₄ by a rC residue and T₂₅ by T^{Bn} in **Dz4** (Table 1, entry 10, A = 69 %) restores the activity remarkably ($k = 0.094 \pm 0.017 \text{ min}^{-1}$). All other modifications increase the cleavage rate by varying degrees (Table 1, entries 11-14), the incorporation of TPh^{Bn} within the flanking arm being the best candidate after T^{Bn} ($k = 0.045 \pm 0.005 \text{ min}^{-1}$).

When the cut is positioned within the flanking arm between C₂₆ and T₂₇, the non-modified split DNAzyme **Dz5** (Table 1, entry 15) recovers some activity ($k = 0.087 \pm 0.012 \text{ min}^{-1}$), although only three base pairs of the 5'-fragment are complementary to the RNA substrate. Here again, the formation of a boronate internucleoside linkage significantly restores activity when C₂₆ and T₂₇ are replaced by a ribocytosine and T^{Bn} respectively (Table 1, entry 16, $k = 0.141 \pm 0.052 \text{ min}^{-1}$, A = 85 %). Surprisingly, in this specific configuration the highest cleavage rate is obtained, when Ph^{Bn} is added after T₂₇. Activity of this split variant is nearly as high as that of the parent **Dz1** (Table 1, entry 19, $k = 0.204 \pm 0.058 \text{ min}^{-1}$), although the percentage of total RNA cleavage is somewhat lower (A = 72%). The exact nature of the activity increase observed with Y = TPh^{Bn} in **Dz5** remains unclear. It is well known that the formation of boronate esters is favored with aromatic boronic acids compared to aliphatic ones.^[6] Hence, the rather high flexibility of the 3'-terminal nucleotides of the 5'-fragment in **Dz5** might be counterbalanced by the rigidity of the aromatic boronate ester, formed when Y = TPh^{Bn}. By contrast, when Ph^{Bn} replaces T₂₇ (Y = Ph^{Bn}), the activity is reduced by one order of magnitude (Table 1, entry 17, $k = 0.019 \pm 0.003 \text{ min}^{-1}$), most likely because the structure of the resulting DNAzyme becomes strained due to the missing nucleotide T₂₇.

Lastly, split DNAzyme **Dz6** is characterized by a cut between C₂₈ and T₂₉, such that five base pairs at the 3'-terminus of the 5'-fragment remain for binding to the substrate. Surprisingly, activity of this split variant is somewhat lower than activity of non-modified split **Dz5**, despite the two additional base pairs in **Dz6** (Table 1, entry 21, $k = 0.059 \pm 0.007 \text{ min}^{-1}$, A = 48 %). However, when C₂₈ and T₂₉ are replaced with rC and T^{Bn}, respectively, a remarkable increase of the cleavage rate is observed (Table 1, entry 22, $k = 0.211 \pm 0.037 \text{ min}^{-1}$), almost as high as for the parent **Dz1**. All other modifications did not increase the activity compared to the unmodified split **Dz6**. Just Ph^{Bn}, when added after T₂₉ slightly increased the cleavage rate (Table 1, entry 25, $k = 0.085 \pm 0.013 \text{ min}^{-1}$).

Important results have emerged from this study. The different non-modified split systems display various degrees of cleavage activity (Figure 3A). This was surprising, since one may expect that, if split into fragments, the catalytically competent conformation is difficult if not impossible to adopt. This would apply in particular to variants **Dz2**, **Dz3** and **Dz4**, where the split is located within the catalytic loop (Fig. 2C). Yet, activity was observed for the non-modified split variants of **Dz3** and **Dz4** (Table 1, Figure 3). Just **Dz2** showed high sensitivity when split in fragments, no activity was observed, neither for the non-modified split variant, nor for the modified variants with potential for boronate formation (Table 1, Fig. 3A). Thus, while many studies have been devoted to the analysis of functional sequence requirements within the catalytic core of the 10-23 DNAzyme,^[12] we here confirm and extend previous results^[16c, 17] that even if perturbed, non-modified split systems are able to maintain some level of functionality. Furthermore, we have

demonstrated that activity of split DNAzymes can be significantly restored by boronate ester formation, not requiring any kind of additional chemical or enzymatic activation.

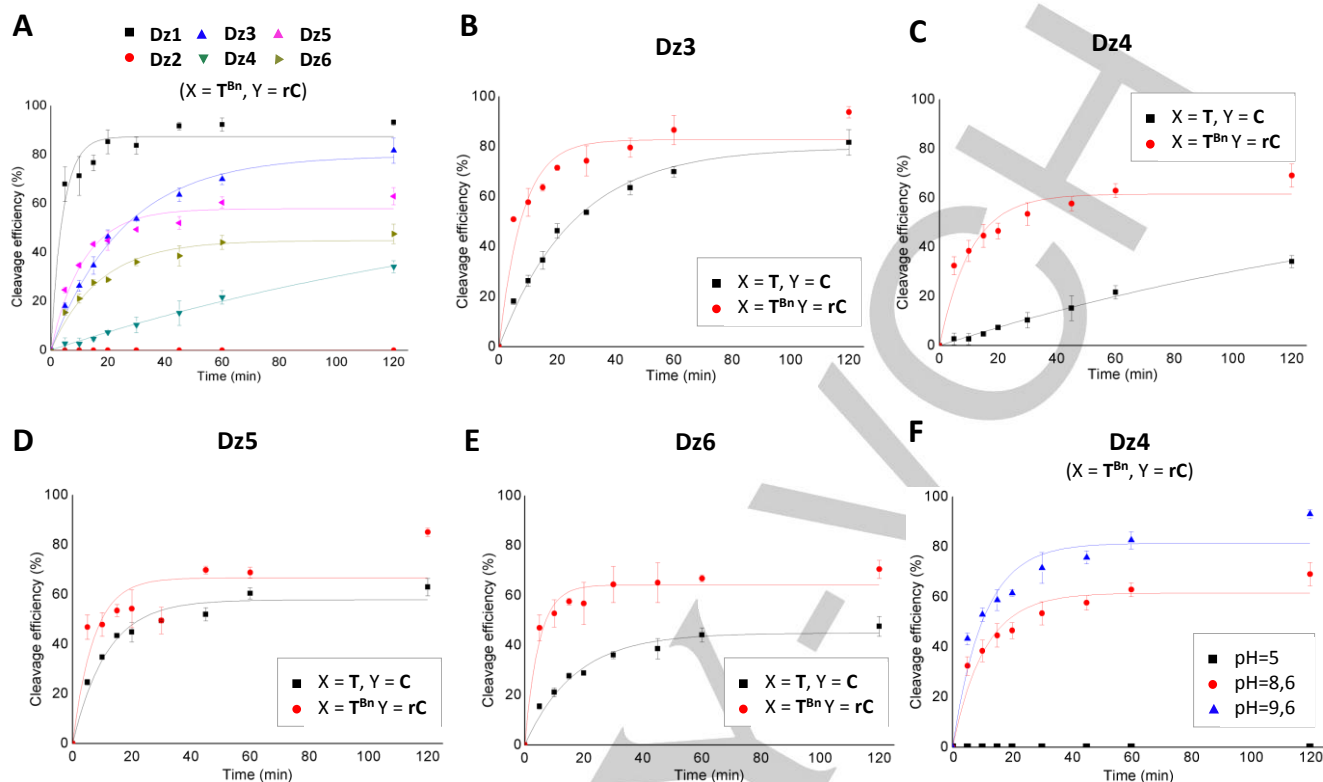


Figure 3. Time courses of cleavage reactions under single-turnover conditions analyzed by a LICOR DNA sequencer. A) Parent DNAzyme **Dz1** and split DNAzymes **Dz2-Dz6** modified at the 3'- and 5'-extremity with a ribocytosine and T^{Bn} respectively. DNAzyme activities of non-modified and modified split DNAzymes B) **Dz3**, C) **Dz4**, D) **Dz5**, E) **Dz6**. F) pH-dependency of **Dz4**.

This is a clear advantage over other split systems that, in order to become functional, rely on chemical or enzymatic ligation of the assembled fragments.^[14] While the potential of Ph^{Bn} and Napht^{Bn} remains limited, T^{Bn} stands out as an efficient bioisosteric analogue of natural thymidine, even when located in non-Watson-Crick base paired regions as demonstrated with **Dz3** (Figure 3B-E).

Although the formation of boronate esters can be observed in neutral aqueous media, it is well known that the association between boronic acids and *cis*-diols is favored at higher pH. This effect is explained by the formation of a hydroxyboronate complex resulting from the rehybridization of the boron atom from sp² to sp³ and a substantial release of angle strain around the boron center.^[18] Thus, we decided to evaluate the cleavage activity of **Dz4** from pH 5.5 to pH 9.6. At pH 5.5 neither the parent **Dz1** nor its split **Dz4** analogues (X = T or T^{Bn}, Y = C or dC) were found to be active. However, at pH 9.6 we were pleased to observe a slight increase of cleavage rate of the T^{Bn} modified split **Dz4** (Figure 3F, $k = 0.096 \pm 0.017 \text{ min}^{-1}$ at pH 9.6 vs $k = 0.094 \pm 0.017 \text{ min}^{-1}$ at pH 8.6). Moreover, this effect was accompanied by increase of the percentage of cleaved substrate from 65% to 95% after 2h of incubation, further demonstrating the ability of the noninvasive nature of borono-modified systems to control DNAzyme activity.

In conclusion, we report here a new concept for the control of activity of split DNAzymes, through the use of two fragments, one modified with a boronic acid at the 5'-end and the other with a *cis*-diol at the 3'-end. In the presence of an RNA substrate, the assembly of the two non-functional fragments brings the two moieties in close proximity and a boronate internucleoside linkage is formed spontaneously. This leads to stabilization of the DNAzyme structure and restored activity for cleavage of the RNA substrate. By varying the position of the boronate linkage we were able to characterize the key elements and the optimum split site for maintaining high levels of activity. In the long term, the reversible formation of boronate esters and the tunable boronic ester-boronate equilibrium may be a promising concept to control functional nucleic acids for the development of valuable biosensing platforms.

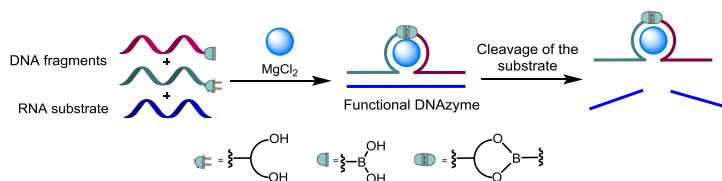
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Keywords: Split DNAzyme • RNA cleavage • Boronic Acid • Functional nucleic acid • autoligation

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Entry for the Table of Contents



Split, Join, Activate. Split DNAzymes made of two or more short nucleic acid strands are emerging as novel tools for biosensing applications. We report here, that when split into two non-functional fragments, the 10-23 DNAzyme activity could be restored by formation of a boronate internucleoside linkage.

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