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COMMUNICATION

A Method for Selecting Modified DNAzymes Without the Use of Modified DNA as a Template in PCR

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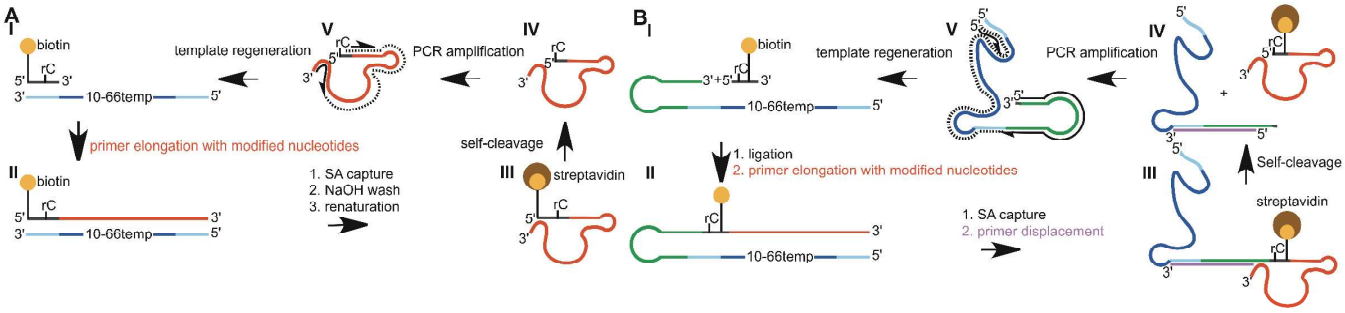
Modified DNAzyme selections typically depend on recopying catalytically active modified DNA (mDNA) into cDNA in a PCR amplification step. However mDNA is often a poor template in PCR. Herein we propose a selection method in which the catalytically active, mDNA strand is covalently linked to the unmodified DNA template strand from which it was polymerized. Following selection, the unmodified DNA template is amplified in a PCR instead of the mDNA. This method circumvents the PCR amplification of mDNA.

The selection of chemically modified DNAzymes (mDNAzymes) from synthetically modified dNPTs represents an exciting endeavour in synthetic chemical biology whereby modified DNA (mDNA) often shows improved catalytic properties compared to unmodified DNA and RNA[1] with applications in catalysis, sensing, and medicine. Modified DNAzymes are identified by '*in vitro* selection' which entails the synthesis of mDNA starting from a cDNA library (**I-II**), the removal of template DNA in a NaOH wash step, the renaturation of mDNAzymes into catalytically active structures (**III**), selection of catalysts (**IV**), the recovery of catalytically active sequences via a PCR step in which mDNA is copied into its cDNA (**V**), and the enzymatic resynthesis of the enriched mDNA library from this cDNA before the start of the next round of selection (as shown in Scheme 1A).

Numerous reports e.g. refs 41-57 in Hollenstein *et al*,[2] refs 18-27 in Lam *et al*.,[3] and others[4] explored the enzymatic synthesis of mDNA from many modified dNTPs and showed the potential for exponential amplification of mDNA into cDNA.[5] This large number of reports underscores an enduring interest in augmenting the chemical and catalytic functionality of *Nucl.* acid aptamers and catalysts. Yet far fewer reports have ever disclosed a selected catalyst or aptamer. For example, Eaton and co-workers used this process in order to select a pyridyl-modified RNA Diels-Alderase and an imidazole-modified transaminase. [1b, 1c] Benner and co-workers selected a propargylamine-modified ATP-binding aptamers.[6] Joyce *et al*. selected an imidazole-modified DNAzyme that chelated Zn^{2+} for efficient RNA cleavage.[7] Perrin *et al.* selected DNAzymes modified with amines, imidazoles and guanidines that catalyse transphosphorylative RNA cleavage.[1e, 2-3, 8] Gold *et al*. selected modified aptamers.[9] In all of the above, mDNA was directly copied into unmodified cDNA for PCR amplification. Of these, a few reports showed that not all modified nucleotides meet preconditions for *in vitro* selections: the modified nucleotides have to be a substrate of at least one polymerase and the resulting mDNA must function as a template in PCR.[10] Additionally, variations in both the incorporation rate of modified dNTPs and the amplification efficiency of mDNA can result in significant bias for certain sequences and negatively affect the sequence space coverage in an *in vitro* selection experiment.[8e, 11] Moreover, slight chemical variations on a modified nucleoside can also result in drastically restricted sequence space from which catalysts are selected.[8e] Finally, structural rigidity is thought to favour tight-binding aptamers and thermostable DNAzymes, but is liable to disfavour the requisite unfolding that must precede PCR amplification and read-out. Hence any selection is likely to reflect a compromise between structural rigidity and flexibility.

Despite the ensemble of these concerns that would suggest insurmountable barriers to success, Hollenstein *et al.* used three modified dNTPs that present ammonium ions, guanidinium ions, and imidazoles to select mDNAzymes with M^{2+}-independent ribophosphodiesterase activity.[8e] Yet difficulties in selecting modified catalysts can be attributed to the general resistance of mDNA to undergo semiconservative recopying in the first step of PCR. To address such limitations, specialized polymerases may be evolved, although this is time-consuming and may have to be done individually for each new set of modified nucleotides.[10a, 12] Alternatively, modified dNTPs can be designed not only for efficient incorporation but also to provide modified templates that may be amplified with similar efficiency to unmodified DNA.[5] Another way of circumventing difficult amplification of mDNA is with the design of a method in which the mDNA is not directly amplified but instead are linked to the cDNA, which serves both as a template for mDNA synthesis and for PCR readout. Elegant examples of such proof-of-concept methods designed for the selections of mDNA aptamers are found in the elegant works of Chaput, Liu and Krauss.[13] Such new developments derive conceptually from the early precedent of ribosome display whereby a translated peptide phenotype was then covalently linked to its mRNA genotype via a puromycin trap.[14] Here we detail a method selecting RNA-cleaving mDNAzymes, in which the mDNA catalyst is covalently linked to the cDNA template from which it is synthesized such that upon catalytic cleavage, the cDNA is liberated for PCR amplification (Scheme 1B).



Scheme 1 The improved *in vitro* selection procedure for ribophosphodiester bond cleaving modified DNAzymes. 10-66temp represents the template sequence of Dz10-66. The modified DNA library is indicated in red. The displacement primer is shown in pink.

To begin, a primer with a biotin-dT downstream of the embedded ribose is ligated to the 3′-terminus of a single-stranded DNA template (**I**). Once ligated, the product is elongated in the presence of modified dNTPs (**II**). Following mDNA synthesis, the construct is captured on streptavidin beads to remove the non-ligated templates while the hairpin is denatured using NaOH. Following this step, the strand is renatured rapidly in the presence of a "displacement primer". This so-called "displacement primer" is designed to bind the loop region to disfavour complete refolding of a long hairpin loop (**III**). Unable to refold into the hairpin, the mDNAzyme is expected to adopt a conformation that undergoes self-cleavage (**IV**). In contrast to existing selection methods, self-cleavage liberates the cDNA template strand from the streptavidin beads. This cDNA can directly be amplified by PCR (**V**). After re-ligation to the hairpin loop, the next round of selection can be initiated. The main advantage of this method is that instead of amplifying mDNA, cDNA is amplified in PCR.

In order to validate this method, we introduced the self-cleaving mDNAzyme Dz10-66, which had originally been selected by standard mDNAzyme selection protocols.[7] Dz10-66 is the first M^{2+}-independent DNAzyme with three modified nucleosides, $dUTP^{guan}$ (guanidine) $dATP^{his}$ (imidazole) and $dCTP^{am}$ (amino) (SI Figure 1), that afforded multiple turnover RNA cleavage under physiological conditions and at 37 °C. Self-cleavage at a single embedded ribophosphodiester bond is especially fast (k_{obs} > 0.60 min^{-1}).

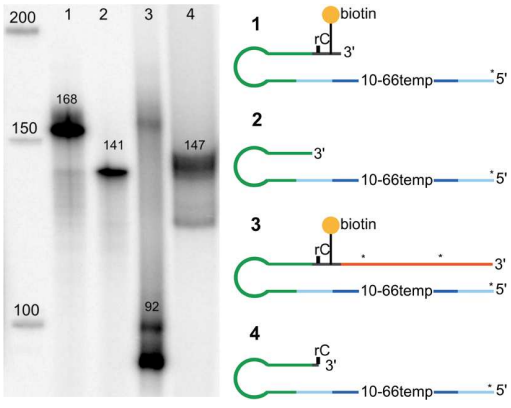


Figure 1 Dz10-66 in the new selection system: 1) The ligation product of the biotinylated primer and the Dz10-66 template, 2) The unligated Dz10-66 template (5′-labelled), 3) The ligation product elongated with $dUTP^{guan}$, $dCTP^{am}$, $dATP^{his}$, dGTP and α-^{32}P-dGTP, 4) The supernatant of the beads after incubation with elongation product and NaOH treatment. The numbers above the bands indicate the length (in nucleotides) of the products.

The ligation of the template sequence of Dz10-66 to a primer containing an embedded ribonucleotide and a biotin tag (**1**) and the elongation of the ligation product using $dUTP^{guan}$, $dATP^{his}$, $dCTP^{am}$ and dGTP (**2**) are shown in Figure 1. The self-cleavage of Dz10-66 after renaturation in the presence of the displacement primer and incubation with a suitable buffer is shown in Figure 2. Internal labelling of the elongated product with α-^{32}P-dGTP allows the observation of the increase of cleaved product on the beads expressed in % of the total amount of cleavage product vs. time. When the same construct is prepared with unmodified DNA, or upon omission of the displacement primer, no cleavage is observed (data not shown).

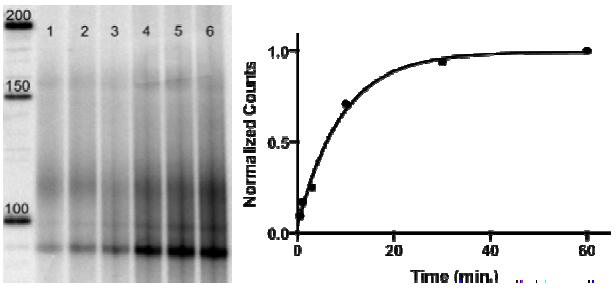


Figure 2 Time course of Dz10-66 self-cleavage when incorporated in the construct depicted in Scheme 1B. Lanes 1-6 correspond to the self-cleavage of the construct on streptavidin beads after 0.5, 1, 3, 10, 30 and 60 minutes. The autoradiographic density corresponding to the cleavage product (y-axis) is plotted against the time (x-axis). The data were fitted to a first-order exponential decay function

Self-cleavage activity of Dz10-66 indicates that the construct folds into the secondary structure required for catalysis. Yet the observed self-cleavage rate constant is slightly depressed (k_{obs}=0.11 min^{-1}) compared to the original Dz10-66 (k_{obs} ≥ 0.6 min^{-1}). This is likely due to the fact that the unmodified template may transiently hybridize with the mDNAzyme. This feature may ultimately provide a level of stringency that will enable selection of catalyst with higher rates. Collectively, these results demonstrate the viability of linking a catalytically active mDNAzyme to its unmodified template. Hence, this method should be suitable for the selection of new mDNAzymes that cleave ribophosphodiester bonds or which form new linkages.

To further expand on this finding, we replaced the template sequence of Dz10-66 with an N20 or N40 randomized region (Figure 3-**I**) to investigate whether mDNA libraries can be synthesized using this method. The ligation products of N20 and N40 template sequences and the primer containing a single embedded ribonucleotide and a biotin tag were subjected to a

primer extension step using two different sets of modified nucleotides, containing dUTP^{ph} (phenol) and dATP^{his} (imidazole) or dUTP^{ph} (phenol) and dCTP^{am} (amino) (Figure 1-SI), respectively (Figure 3-**II**). The construct was 5′-labelled on the template portion while the mDNA was internally labelled using α-^{32}P-dGTP via primer extension. The elongated material is captured on streptavidin beads, and the constructs are cleaved at the ribophosphodiester bond using NaOH (Figure 3-**III**). The cleavage products reveal the successful elongation of the construct with the modified nucleotide mixes both against an N20 and an N40 region with the formation of full-length product (72 and 92 nucleotides long respectively).

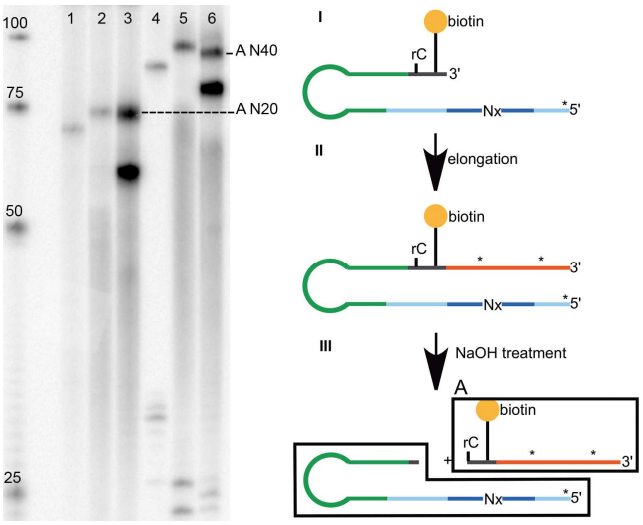


Figure 3 The elongation of the constructs containing an N20 (lanes 1-3) or an N40 randomized region (lanes 4-6) with natural dNTPs (lanes 1 and 4), the nucleotide mixes containing the dUTP^{ph} and dATP^{his} (lanes 2 and 5), or dUTP^{ph} and dCTP^{am} (lanes 3 and 6), after treatment with NaOH. The scheme on the right shows the experimental route to obtain the sequences visible on the gel. 'A' indicates the full-length mDNA elongation product after NaOH treatment. Radioactive labeling is indicated by stars. Biotin is drawn as a yellow circle.

Conclusions

Several recent proof-of-concept studies have linked cDNA templates to aptamers composed of mDNA or TNA. Yet to date, there has been no report of such in the context of a self-cleaving mDNA catalyst (mDNAzyme). Here we have validated a new method mDNAzyme selection. In this approach, the difficult PCR amplification of mDNA is circumvented. This method should empower others to select mDNAzymes that catalyse a wide range of bond-forming or -breaking reactions by covalently attaching an organic molecule to either a DNA primer or linking it to an affinity tag such as biotin (Scheme 1SI). The development of more efficient selection procedures where mDNA is used for catalysis and cDNA templates are used for encoding should pave the way for rapid and efficient generation of active mDNAzymes.

Given the extensive interest in exploring the use of modified nucleotides for expanding the functionality of DNAzymes, we wish to share these findings with those seeking to avoid PCR amplification of mDNA. Results of functional selections using this method will be reported in due time.

Notes and references

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