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Evolved polymerases facilitate selection of fully 2'-OME-modified aptamers†

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RNA or DNA aptamers with 2'-OME-modifications have been pursued to increase resistance to nucleases, but have been difficult to identify because the OME groups ablate polymerase recognition. We recently reported evolution of the thermostable DNA polymerases SFM4-6 and SFM4-9, which enable the efficient "transcription" and "reverse transcription", respectively, of 2'-OME oligonucleotides. With these polymerases, we now report the first selection of fully 2'-OME modified aptamers, specifically aptamers that bind human neutrophil elastase (HNE). Two aptamers, 2mHNE-1 and 2mHNE-2, were isolated after five rounds of selection, and four more, 2mHNE-3–6, after an additional five rounds that included selection pressure for binding in the presence of serum. All six aptamers bind with reasonable affinity, which requires the 2'-OME substituents. Further characterization of one aptamer, 2mHNE-5, showed that unlike a previously reported natural anti-HNE aptamer, affinity for HNE is retained in the presence of high concentrations of salt or serum. The polymerases SFM4-6 and SFM4-9 should prove valuable for the production and further exploration of modified aptamers.

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Introduction

Aptamers, RNA or single-stranded DNA (ssDNA) oligonucleotides that bind to a specific target, are useful affinity reagents and potentially promising therapeutics.^{1–3} Aptamers are discovered by creating pools of oligonucleotides with randomized sequences, enriching the pools for members that bind to a desired target (*e.g.* selection), and then amplifying the enriched pools and subjecting them to additional rounds of selection. While both DNA and RNA aptamers may be developed relatively quickly and inexpensively, their use is limited by the inherent instability of natural oligonucleotides in biological solutions due to nuclease degradation.^{4,5} Thus, there is significant interest in the discovery of modified aptamers that are more resistant to nucleases, with 2'-OME and 2'-F substituents having received the most attention. In particular, 2'-OME substituents are desirable because they impart the highest level of nuclease resistance and are also relatively inexpensive as triphosphates. Moreover, while the discovery of aptamers with high affinity to desired targets is now routine, a second limitation in aptamer development is the identification of aptamers with high specificity, which is generally more challenging. This is particularly true with positively charged protein targets due to the dominance of non-specific electrostatic interactions with the negatively charged phosphate backbone.⁶ Modifications

such as 2'-OME substituents could potentially facilitate the formation of, or even directly engage in, more specific interactions, but this has not been extensively investigated.

Aptamers with 2'-modifications have historically been generated *via* post-selection modification of a natural aptamer,^{7,8} but their introduction often interferes with the selected activity. The straightforward inclusion of 2'-modifications in the selection process itself is challenging, because they generally interfere with polymerase recognition. While combinations of polymerases, polymerase mutants, and/or specific reaction conditions have been identified that allow for the inclusion of various types of modified sugars,^{4,9,10} the inclusion of 2'-OME modifications has proven particularly challenging. The only known, successful example using 2'-OME modifications in SELEX was provided by Burmeister *et al.*¹¹ who reported that with precisely optimized conditions, the Y639F/H784A/K378R triple mutant of T7 RNA polymerase can transcribe DNA into nearly fully 2'-OME modified oligonucleotides, which along with reverse transcription by ThermoScript reverse transcriptase and amplification of the natural DNA, allowed selection of modified aptamers that bind vascular endothelial growth factor (VEGF). However, chemical synthesis was used to supplement the transcribed library for selection and the transcription reactions required the addition of unmodified GTP, indicating that at least the majority of sequences could not be transcribed in their fully modified form. Reports of additional T7 RNAP mutants with improved recognition of 2'-OME substrates^{12,13} are notable, but no report of their use to select for modified aptamers has yet been reported.

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Table 1 Sequences of selected 2'-OMe aptamers and their affinities for HNE

Aptamer	Random region sequence	K_d (nM)
2mHNE-1	CCCTGTTCCCCCTCCGCCGCTTGACTCTCC	520 ± 50
2mHNE-2	CCCTGGCCGCCCTCTGGCGCGCTGATGT	180 ± 10
2mHNE-3	GCCCTACATCTGTGCGACTCCCCCCCCCGC	120 ± 7
2mHNE-4	CCCTTACTACTCTGCTCCCCCCCCCTCGGCTGC	45 ± 4
2mHNE-5	CCCTGTTGCTATCCCCCATCCCCGATTGC	110 ± 20
2mHNE-6	CCCGCTGTTCTCCCCCCCCGCTGAGATCCCTGT	110 ± 20

2mHNE-5 and DNA-I bound to HNE, but did not bind strongly to any of the other proteins (although both aptamers exhibited some affinity for lysozyme). However, under the high salt conditions, 2mHNE-5 specifically bound HNE while DNA-I did not, demonstrating that the 2'-OMe modified aptamer binds to HNE via more specific interactions.

To explore the ability of the aptamers to bind HNE in serum, we repeated the above described low-salt plate binding assays with 2mHNE-5 or DNA-I, but with increasing amounts of FBS in the wash buffer (Fig. 3A). The results clearly show that DNA-I

rapidly loses affinity for HNE as the percentage of FBS is increased. In contrast, 2mHNE-5 loses affinity more slowly and even retains the ability to bind HNE during washing with 100% FBS. We also directly measured binding in the presence of 80% FBS, and found that 2mHNE-5 clearly binds HNE better than does the DNA-I control (Fig. 3B).

To confirm that the 2'-OMe substituents do confer 2mHNE-5 with resistance to nuclease degradation, folded 2mHNE-5, HNE-5, or DNA-I was mixed with undiluted FBS (Fig. 4). After incubation at 37 °C, PAGE analysis revealed the degradation of DNA-I, with little aptamer remaining after 4 h. In contrast, even after 24 h, no degradation of 2mHNE-5 was observed, clearly



Fig. 2 Specificity of binding of 2mHNE-5 and DNA-I to different proteins in the presence of 150 mM NaCl (A) or 500 mM NaCl, 66 $\mu\text{g mL}^{-1}$ yeast tRNA (B). BSA, bovine serum albumin; THR, thrombin; PPE, porcine pancreas elastase; CHY, chymotrypsin; LYS, lysozyme. Data are the average and s.d. of three independent determinations.



Fig. 3 Binding of 2mHNE-5 and DNA-I to HNE in the presence of increasing concentrations of FBS in the wash buffer (A) or in the presence of 80% FBS (B). Data are the average and s.d. of three independent determinations.





Fig. 4 Stability of 2mHNE-5, HNE-5, and DNA-I in FBS. Data are the average and s.d. of three independent determinations.

demonstrating that the 2'-OMe modifications do indeed provide significant stabilization against nuclease degradation. HNE-5 was degraded even faster than DNA-I, likely due to the absence of secondary structure, suggesting that the 2'-substituents of 2mHNE-5 are required for proper folding.

Conclusions

There has been both academic and industrial interest in developing 2'-OMe aptamers,^{4,11,19} but their identification has historically been challenging, because aptamer selections rely on polymerase recognition, and the modified triphosphates are not well recognized. In fact, to our knowledge, prior to this work no selections have been performed in which the modified libraries were produced using only 2'-OMe triphosphates. The directed evolution of SFM4-6 and SFM4-9 now allows for the "transcription" and "reverse transcription" of fully modified aptamers using only 2'-OMe modified triphosphates and we have used them here to select 2'-OMe modified aptamers that bind HNE. Importantly, the OMe substituents of the selected aptamers are required for high affinity and specific HNE binding. Likely, both the affinity and specificity afforded by the modifications could be increased further by additional diversification and selection, including negative selection against binding related proteins. Finally, the availability of SFM4-6 and SFM4-9 should enable the facile selection of other 2'-OMe aptamers and the exploration of the effects of the added OMe groups beyond imparting the aptamers with resistance to nucleases.

Conflicts of interest

There are no conflicts to declare.

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