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Evaluation of Endcapped DNA Modules for pRNA Capture and Functionalization

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Abstract

The ability of packaging RNA (pRNA) from the phi29 DNA packaging motor to form nanoassemblies and nanostructures has been exploited for the development of the nascent field of RNA nanotechnology and subsequent applications in nanomedicine. For applications in nanomedicine, it is necessary to modify the pRNA structure for the conjugation of active molecules. We have investigated endcapped double-stranded DNA segments as reversible capture reagents for pRNA. These capture agents can be designed to allow the conjugation of any desired molecule for pRNA functionalization. The results of model studies presented in this report show that 5- to 7-nucleotide overhangs on a target RNA can provide efficient handles for the high affinity association to capped double-stranded DNA.

Keywords

pRNA; RNA nanotechnology; RNA capture; Endcapped oligonucleotides

The emerging field of RNA nanotechnology was ignited by the discovery by Guo and coworkers that packaging RNA (pRNA) from the phi29 bacteriophage DNA packaging nanomotor can be re-engineered and manipulated to form a variety of nanoassemblies and nanostructures.^{1–6} Wild type pRNA spontaneously self-assemble into a hexameric ring via complementary base pairing interactions between loop regions within the pRNA structure.^{3,7,8} The pRNA monomer contains two functional domains (Figure 1A): the intermolecular-interacting domain comprised of a right hand and left hand loop and the 5'/3' double-stranded helical DNA-translocation domain.^{4,5,8} It has been shown that these two domains fold independently of one another and modification of the one domain does not affect the function of the other.^{3–5,9,10}

The two interlocking left and right loops of the intermolecular-interacting domain can be engineered for bottom-up nanoparticle assembly and has been modified to produce a variety of nanostructures and shapes including rods, triangles, twins, tetramers, and 3D arrays up to several micrometers in size.^{1,4,5} The 3'/5'-paired DNA translocation domain of the pRNA

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Supporting Information Available: Experimental procedures and thermal profiles for experiments 3–7. This material is available free of charge via the Internet at <http://pubs.acs.org>

can be re-engineered and modified without affecting the conformation of the intermolecular binding domain and its ability to form multimeric nanostructures.^{3,10–12} Guo and coworkers have fabricated pRNA monomers with functional RNA sequences (e.g. ribozymes, aptamers and siRNA) and small molecules (e.g. Folic acid, fluorophores) at the 5'/3' translocation domain.^{2,6,9,10} These properties of pRNA are the basis for the design and fabrication of multifunctional pRNA nanoparticles of the types shown in Figure 1B (dimer model shown), for use in nanomedicine.

In order to take full advantage of the potential of pRNA nanotechnology, it is important to develop methods for the attachment of functional molecules to pRNA. The wild type pRNA contains a 3' overhang with the sequence 3'-AAU followed by a double-stranded stem (Figure 1A). We believe that there is a way to rapidly and reversibly add modules to the 3'/5'-pRNA overhang that are small, have high affinity and specificity for the pRNA overhang, are simple to synthesize, and can link virtually any kind of molecule (including small ligands, peptides, lipids, carbohydrates, and fluorophores). Bergstrom and coworkers have utilized a method for stabilizing short segments of double-stranded oligonucleotides by covalently linking the 3' and 5' ends of the nucleic acid with a simple spacer.^{13–15} The simplest spacer is the C12 hydrocarbon (**S2**) shown in Figure 1D. Nucleic acid duplexes containing just four base pairs and linked by a spacer at one end have melting temperatures (T_m values) in the range 41 to 62 °C (the same duplexes without the spacers are unstable at room temperature, i.e. $T_m < 25^\circ\text{C}$). Importantly, we also determined that the endcaps protect double-stranded nucleic acids from degradation by exonucleases.

In the present study, oligodeoxyribonucleotides containing a 5 bp duplex segment capped at one end by a stabilizing spacer and having a 5 nt or 7 nt single stranded overhang at the other end (Figure 1C, capture modules 5 and 7), would be used as reagents for the capture and modification of the 3'/5' terminal region of a modified pRNA. Modified bases such as the commercially available C5 substituted thymine, bearing an alkyne moiety (Fig 1D, M1), may be incorporated into the capture module. This modification will allow the conjugation of fluorophores, affinity probes, and targeting ligands through a bio-orthogonal 3+2 cycloaddition reaction between the alkyne on the modified base and an alkyl azide on the conjugate moiety.

In order to determine the feasibility of our pRNA capture strategy, the model system shown in Figure 1C was developed and tested. The truncated pRNA models are stabilized by a well established RNA tetraloop (5'-UUCG-3') known to form RNA hairpins with remarkable stability (T_m values $> 70^\circ\text{C}$).^{16,17} The DNA modules were constructed by using a hexaethylene glycol linker (**S1**) or a C12 hydrocarbon (**S2**) which confers significant stability to short sequences.

The ability of the endcapped DNA capture modules to stably hybridize with the truncated pRNA models were investigated by conducting melting temperature experiments. The RNA hairpin models- RNA5 and RNA7, had T_m values of 81.5 °C and 74.4 °C respectively (Table 1). The higher T_m value for RNA5 is most likely due to the higher C•G content in the stem region of RNA5 compared to that of RNA7. The DNA capture modules, DNA5-**S1** and DNA7-**S1**, were determined to have T_m values of 64.6 °C and 65.8 °C respectively (Table 1). Spacer **S1** was used in the initial experiments because the hexaethylene glycol linker is well established as one of the first linkers used to stabilize duplex structure and is commercially available.^{18,19}

We anticipated that these structures would be sufficiently stable such that the first dissociation event would be the dissociation of the RNA from DNA when equimolar quantities of complementary RNA and DNA modules are heated (Scheme 1). Hence, at least

two transitions ($A \rightarrow B$ and $B \rightarrow C$) should be readily observed in the thermal profile from which approximate T_m values for the first dissociation event ($A \rightarrow B$, T_{m1}) can be determined. The second transition ($B \rightarrow C$, T_{m2}) is expected to involve the melting of the RNA hairpin and the endcapped DNA hairpin. These two events are indistinguishable in the thermal profile in some cases.

Thermal melting profiles were obtained for the hybridization of equimolar (1 μ M) concentrations of RNA5 + DNA5-**S1** and RNA7 + DNA7-**S1** (Figure 2). The anticipated transitions were observed in both cases with the T_m values for the first dissociation events determined to be 43.2 °C and 61.1 °C for capture module 5 and capture module 7 respectively. For biological and therapeutic applications it is important to have structures that are stable at 37 °C. The T_m values indicate that both capture module 5 and capture module 7 would have sufficient thermal stability under physiological conditions, with capture module 7 exhibiting significantly greater stability.

Control experiments were conducted to assess the level of enhancement provided by the endcapped 5 bp stem used in capture module 7 when compared to modules having a 1 bp stem (DNActrl-**S1**) or a 1 nt overhang with no endcap (DNActrl-dC). The T_m value obtained for the first dissociation event with DNActrl-**S1** was found to be 44.1 °C (Table 2, exp 3 and supplementary Figure S3) whereas the T_m value for DNActrl-dC was found to be 41.2 °C (Table 2, exp 4 and supplementary Figure S4). The observed T_m values indicate that the endcapped 5 bp stem significantly enhances the ability of the DNA capture module to stably hybridize with the RNA target. These results suggest that 'pre-organization' of the 5 bp duplex stem in the capture module by the endcap plays a role in augmenting the hybridization stability of the RNA capture system.

Having established the feasibility of the system using spacer **S1** in the DNA capture modules, experiments were conducted using spacer **S2** (C12 spacer), which more closely matches the average distance between the terminal 3'-O and 5'-O of B-DNA duplexes between the terminal base pairs, previously shown to be approximately 16.2 Å.¹³ Spacer **S2**, when used as the endcap in DNA capture module 7 resulted in a T_m value of 67.2 °C for the melting of the 5 bp duplex stem (Table 1), a value slightly higher than spacer **S1**. The first dissociation event when hybridized to RNA7 was determined to be 47.4 °C ((Table 2, exp 5 and supplementary Figure S5)). This T_m value, despite being sufficient for physiological conditions, is significantly lower than that obtained with spacer **S1**. This difference in observed hybridization stability may be due to the differences in length, flexibility and hydrophobicity of the spacers. These are attributes that are important to consider in the future design of endcaps for stabilizing DNA capture modules.

It was of interest to determine the level of tolerance for mismatches or imperfect target sequences. Imperfect sequences and mismatches are potential issues to consider in the proposed strategy since one of the main methods of preparing full-length (117 nt) pRNA is *in vitro* transcription using T7 RNA polymerase. T7 RNA polymerase lacks proofreading ability and is known to produce aberrant RNA transcripts and extra bases at the 3' terminus.²⁰ Another potential application for the use DNA capture modules is in the capture of microRNA (miRNA) or other small RNA that present short overhanging regions. Mismatch discrimination would be an important concern in these applications. A simple approach was used for these studies in which the previously synthesized RNA models (RNA5 and RNA7) and DNA capture modules (5 and 7) were mismatched such that RNA7 was hybridized with DNA5-**S1** and *vice versa* (Scheme 1-III and IV).

Hybridization of equimolar concentrations of RNA5 and DNA7-**S1** would produce an equilibrium comprising three possible structures shown in Scheme 1-III. The T_m value

obtained for the first dissociation event with these structures was found to be 36.2 °C ((Table 2, exp 6 and supplementary Figure S6)) indicating that the net result of this interaction is less stable than the exact complement. Combination of RNA7 with DNA5-S1 produces a structure with two unpaired nucleotides (Scheme 1-IV) and results in a T_m value for the first dissociation event of 32.6 °C (Table 2, exp 7 and supplementary Figure S7). Both mispaired structures are unstable under physiological conditions and show that the overall system relies on high fidelity of base pairing.

The results presented here indicate that the endcapped DNA capture module system for the functional modification of pRNA is a potentially feasible strategy. The successful application of this strategy would require further optimization in order to create the most versatile and effective capture and functionalization system. The studies of the control modules DNACtrl-S1 with 1 bp stem and DNACtrl-dC with no endcap are instructive for the design of an optimized system. These control modules, although less stable than DNA7-S1, displayed stability under physiological temperatures. These observations suggest that it may be possible to design even simpler modules with shorter stems or by using stabilizing nucleotides (e.g. LNA) in the single stranded overhang to enhance hybridization to the pRNA overhang. It would also be necessary to conduct studies at different concentrations in order to assess the concentration dependence of hybridization. It is evident from the study of the C12 hydrocarbon endcap, that the nature and length of the endcap are important considerations in the design. This observation is supported by previous work conducted in the Bergstrom lab on the evaluation of spacers for use as dsDNA endcaps.^{13–15} The endcapped duplex stem in the capture modules confers high affinity and specificity for hybridization to overhanging sequences at the termini of pRNA over hybridization to the same sequence within the RNA. This specificity is due to the ‘pre-organization’ of the duplex stem region by the endcap. The DNA capture module system can therefore be widely applied to the capture of any nucleic acid structure bearing overhanging sequences at their termini.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structures of pRNA and endcapped DNA capture module system. (A) Wild type pRNA model showing functional domains. (B) Engineered pRNA dimer showing potential modifications for use in nanomedicine applications. (C) Model system for assessing the feasibility of the endcapped DNA capture module strategy. (D) Structures of endcaps (S1 and S2) and modified base (M1) for use in DNA capture modules.

Figure 2. Thermal melting profile and 1st derivative plots for (A) RNA5 + DNA5-S1 (table 2, exp 1) and (B) RNA7 + DNA7-S1 (table 2, exp 2). The states a, b and c corresponds to structures A, B and C in scheme 1.

I. RNA5 + DNA capture module 5

II. RNA7 + DNA capture module 7

III. RNA5 + DNA capture module 7

IV. RNA7 + DNA capture module 5

Scheme 1.

Anticipated change in structure and hybridization of the endcapped DNA based RNA capture model system (A $\rightarrow$ B $\rightarrow$ C) with increasing temperature.

Table 1

Mass Spectrometry (MALDI-TOF-MS) and melting temperature (T_m) analysis of endcapped DNA capture modules and truncated pRNA hairpin models

Name	Sequence/Structure	(M-H) ⁻ Calculated	(M-H) ⁻ Observed	^a T_m (°C)
DNA5-S1	GTCAG-3' CAGTCGCGTG-5'	4953.21	4953.3	64.6
DNA7-S1	GTCAG-3' CAGTCGCGTGCC-5'	5531.7	5531.4	65.8
DNActrl-S1	G-3' CGCGTGCC-5'	3059.1	3059.2	N/A
DNA7-S2	GTCAG-3' CAGTCGCGTGCC-5'	5451.57	5454.0	67.2
DNActrl-dC	3'-CGCGTGCC-5'	2385.6	2385.0	N/A
RNA5		6026.4	6027.1	81.5
RNA7		6703.0	6702.6	74.4

S1 = Hexaethylene glycol spacer; S2 = C12 Hydrocarbon spacer,

^amelting of hairpin

Table 2

Melting temperatures for endcapped DNA capture module hybridization to truncated pRNA models

Experiment	Oligonucleotides	aT_{m1} (°C)	bT_{m2} (°C)
1	DNA5-S1 + RNA5	43.2	66.2, ^c 84.6
2	DNA7-S1 + RNA7	61.1	[*] 72.1
3	DNACtrl-S1 + RNA7	44.1	[*] 69.6
4	DNACtrl-dC + RNA7	41.2	[*] 68.7
5	DNA7-S2 + RNA7	47.4	62.0, ^c 71.7
6	DNA7-S1 + RNA5	36.2	69.6, ^c 84.1
7	DNA5-S1 + RNA7	32.6	[*] 72.2

^aFirst dissociation event (Melting of DNA-RNA hybrid)^bSecond and^cthird dissociation events (melting of RNA hairpin and endcapped DNA)^{*}Second and third dissociation events cannot be differentiated