

# Investigating Modified Oligonucleotides as Robust, Programmable Release Agents

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## ABSTRACT

While LNA has been examined as a therapeutic agent in various antisense scenarios, we are exploring LNA as a programmable biomaterials assembly and disassembly tool under isothermal conditions. In brief, we employ displacement strategies in which primary hybridization partners (linking particles together) are replaced by secondary targets (driving disassembly) in order to program the release of nanoparticles from LNA-linked colloidal assemblies. In order to better elucidate the displacement activity, in the current study we directly interrogate suspensions of microspheres functionalized with LNA-based primary duplexes using flow cytometry immediately following the introduction of the secondary target. By eliminating wash steps, we can thus directly assess displacement events *as they occur on colloidal surfaces*. Time-dependent release profiles are generated to measure the observed displacement rate constant for both natural and modified oligonucleotide analogs.

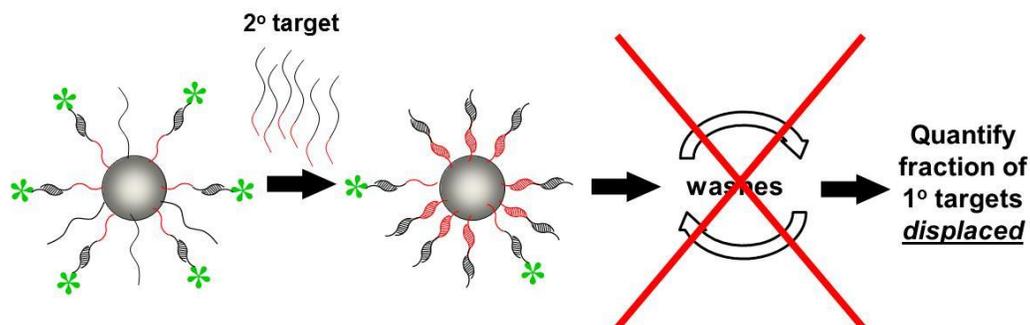
## INTRODUCTION

DNA has been successfully employed as a programmable, thermally reversible materials assembly tool in the absence of cells.<sup>1-3</sup> One of the key limitations, however, to extending this successful approach to biomaterials stems from the susceptibility of DNA to nuclease cleavage. Furthermore, the elevated temperature conditions employed (typically, above 50 °C) to thermally dissociate DNA-based bridges are likely to compromise the viability of cells and cause denaturation of proteins present. Locked nucleic acids (LNA), on the other hand, are promising nucleic acid analogs in a variety of biomedical

applications due to their reportedly low cytotoxicity effects and superior nuclease resistance over natural oligonucleotides.<sup>4</sup> While LNA has been examined as a therapeutic agent in various antisense scenarios, we are exploring LNA as a programmable biomaterials assembly and disassembly tool under isothermal conditions.

In brief, we employ displacement strategies<sup>5</sup> in which primary hybridization partners (linking particles together) are replaced by secondary targets (driving disassembly) in order to program the release of nanoparticles from LNA-linked colloidal assemblies<sup>6</sup>. Importantly, flow cytometry was employed in our first report<sup>6</sup> to quantify duplex formation on microspheres of various candidate sequences following post-hybridization wash steps to remove any excess or weakly associated oligonucleotide targets on the microsphere surface. In the current study, however, we modified our experimental approach by directly sampling suspensions with a flow cytometer immediately following the introduction of the secondary target as illustrated in **Scheme 1**. By eliminating wash steps, we can thus directly assess displacement events *as they occur on colloidal surfaces*.

This *in situ* approach allows us to better compare the competitive displacement of DNA or LNA primary targets by a longer DNA or LNA secondary target. In addition, time-dependent release profiles can be generated to measure the observed displacement rate constant for these natural and modified oligonucleotides. Importantly, any thermal dissociation of weak primary hybridization partners can also be readily taken into account with this approach by using noncomplementary sequences as the secondary target strands. This work demonstrates that LNA serves as a promising recognition-based tool for programmable assembly and disassembly of colloids serving, for example, as multifunctional therapeutic and diagnostic agents.



**Scheme 1.** Graphic illustration of the high throughput experimental approach used in the current flow cytometry studies in which conventional wash steps (to remove excess or unassociated FITC-labeled targets) are excluded in order to measure target displacement events as they occur on microsphere surfaces.

**Table 1.** List of the function and nomenclature of various DNA and LNA sequences<sup>a</sup>

Function	Nomenclature
immobilized DNA probe	<b>A20</b> = 3'-TAGTCGGCGTTAGGTTTTTT-5'
immobilized LNA-DNA mixmer probes	<b>L<sup>3</sup>A20</b> = 3'-TA <sup>L</sup> GTC <sup>L</sup> GGC <sup>L</sup> GTT <sup>L</sup> AGG <sup>L</sup> TTTTTT-5'
	<b>L<sup>3</sup>A20-flip</b> = 3'-TTTTTTA <sup>L</sup> GTC <sup>L</sup> GGC <sup>L</sup> GTT <sup>L</sup> AGG <sup>L</sup> T-5'
soluble, FITC-labeled LNA 1° targets	<b>L<sup>3</sup>B9F</b> = 5'-AT <sup>L</sup> CAG <sup>L</sup> CCG <sup>L</sup> C-3'
	<b>L<sup>3</sup>M9F</b> = 5'-AT <sup>L</sup> CAC <sup>L</sup> CCG <sup>L</sup> C-3'
soluble, unlabeled DNA 2° target	<b>B15U</b> = 5'-ATCAGCCGCAATCCA-3'
soluble, unlabeled LNA-DNA 2° mixmer target	<b>L<sup>3</sup>B15U</b> = 5'-AT <sup>L</sup> CAG <sup>L</sup> CCG <sup>L</sup> CAA <sup>L</sup> TCC <sup>L</sup> A-3'

<sup>a</sup>Sequences that contain LNA residues are indicated by the “L<sup>3</sup>” prefix in the sequence nomenclature; the “3” superscript denotes the frequency of LNA substitution, at every third base. The location of LNA bases is marked by an “L” superscript to the right of a base.

## MATERIALS AND METHODS

### Sequences and Microspheres

A list of sequences used in the current study is provided in **Table 1**. DNA sequences and LNA-DNA “mixmers” were purchased from Integrated DNA Technologies (IDT) and Exiqon. Aminated probes were couple to 1.1 μm carboxylated polystyrene microspheres (Bangs Laboratories) using well-known EDAC-based conjugation. All primary targets are FITC-labeled; however, secondary or competitive targets remain unlabeled.

### In situ measurements of primary target release

Flow cytometry was performed on a Becton Dickinson (BD) LSRII flow cytometer. Average fluorescence intensity value was converted into duplex density values using QuantumFITC-5 MESF standards (Bangs Laboratories). For these *in situ* competitive displacement studies, suspensions of microspheres with FITC-labeled primary duplexes were introduced to a solution of unlabeled secondary targets strands, immediately vortexed and then introduced to the flow cytometer. After a brief equilibration time (~15 s), three readings were taken within one minute, and then every minute thereafter during a 30-minute experimental timeframe.

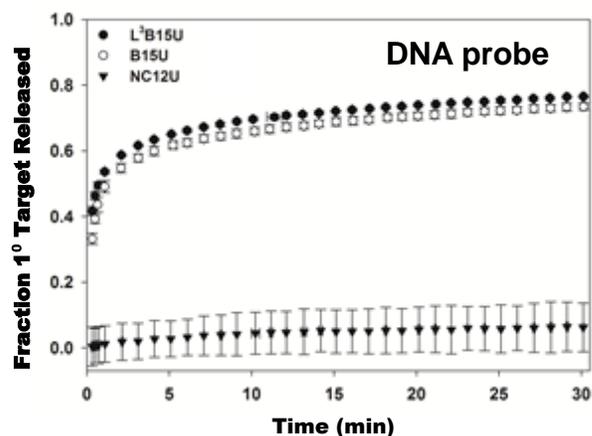
In brief, these *in situ* measurements entail monitoring the changes in primary duplex density due to either thermal dissociation (if relevant for a given primary target) or due to displacement of the primary target by a secondary target. Notably, since different probe-primary target sequences yielded different initial duplex density values, all time-dependent profiles are plotted as the fraction of primary duplexes released or displaced.

## RESULTS AND DISCUSSION

In these studies, we quantitatively assess the effects of LNA content in the probe, primary target and secondary target sequences on the release of nine base-long primary targets. Here, *in situ* measurements of fraction of primary targets released in the presence of 15 base-long DNA and LNA-DNA mixmer targets are shown for both DNA-functionalized microspheres (**Figure 1**) as well microspheres functionalized with LNA-DNA mixmer strands (**Figure 2**).

### Primary target release from DNA-functionalized microspheres

As shown in **Figure 1**, there is extensive and nearly equivalent release of nine base-long LNA-DNA primary targets from DNA-functionalized microspheres by both LNA and DNA secondary targets. In contrast, there is little

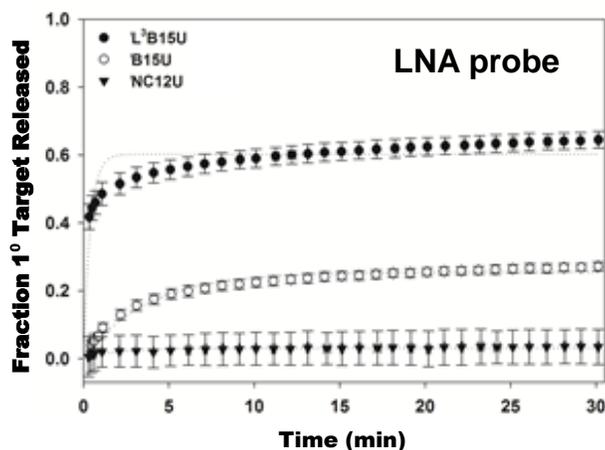


**Figure 1.** *In situ* measurements of the fraction of fluorescently labeled 9 base-long L<sup>3</sup>B9F LNA primary target released from DNA-functionalized microspheres in the presence of unlabeled, 15 base-long L<sup>3</sup>B15U LNA (closed circles), B15U DNA (open circles), or noncomplementary NC12U DNA (closed triangles) secondary targets.

target release in the presence of noncomplementary secondary targets. Collectively, these results indicate that target release can be attributed to displacement activity rather than thermal dissociation events. Intriguingly, while separate results (not shown) indicate that LNA-DNA mixmers typically exhibit stronger hybridization activity than their DNA analogs, both the timing and extent of displacement activity is nearly identical for both DNA and LNA-DNA secondary targets.

### Primary target release from LNA-functionalized microspheres

In contrast to release profiles shown in **Figure 1** for DNA-functionalized microspheres, there are distinct differences in the release profiles for LNA-functionalized microspheres shown in **Figure 2**. Extensive release of primary target occurs in the presence of LNA secondary target. Only modest release, however, is achieved in the presence of DNA secondary target. Similar to the control discussed previously for **Figure 1**, minimal target release is achieved in the presence of noncomplementary secondary target. Thus, as before, primary target release from these LNA-functionalized microspheres is due almost exclusively to competitive displacement, not thermal dissociation. Moreover, this work demonstrates the ability to tune the release of targets through choice in the modified nucleic acid content of probe and target strands.



**Figure 2.** *In situ* measurements of the fraction of fluorescently labeled 9 base-long L<sup>3</sup>B9F LNA primary target released from LNA-functionalized microspheres in the presence of unlabeled, 15 base-long L<sup>3</sup>B15U LNA (closed circles), B15U DNA (open circles), or noncomplementary NC12U DNA (closed triangles) secondary targets.

In order to assess displacement rate constants, release profiles were first converted into displacement profiles using the following equation:

$$f_d = (f_t - f_{r,NC}) / (1 - f_{r,NC}) \quad (1)$$

in which primary target displaced by competitive targets,  $f_d$ , are calculated by taking into account any primary target dissociation that occurs in the presence of noncomplementary secondary target,  $f_{r,NC}$ .

The observed displacement rate constant,  $k_d$ , is then calculated using the following equation:

$$f_d = f_\infty (1 - \exp(-k_d t)) \quad (2)$$

in which  $f_\infty$  is the total primary target displaced by competitive targets at the end of the experimental timeframe of 30 min, and  $t$ , is time in seconds.

Curve-fits of the time-dependent displacement profiles (data not shown, but profiles are nearly identical to the release profiles shown in Figure 1 and Figure 2) revealed a range of  $k_d$  values from  $3 \times 10^{-3} - 4 \times 10^{-2} \text{ s}^{-1}$ , depending on the combination of probe, primary and secondary target sequences. Thus, this work demonstrates the ability to tune the timing and extent of target displacement through choices in the modified nucleic acid content of probe and target strands.

## CONCLUSIONS

Motivated by our prior success using LNA as an isothermal colloidal assembly and disassembly too, we use flow cytometry in an unconventional manner in the current study to measure *in situ* displacement activity on microspheres for various DNA and modified oligonucleotides. We generally find that the timing and extent of displacement activity depends on the content of modified nucleotides.

## REFERENCES

1. Biancaniello, P.L., Kim, A.J. & Crocker, J.C. Colloidal interactions and self-assembly using DNA hybridization. *Physical Review Letters* **94**, (5) DOI 10.1103/Physrevlett.94.058302 (2005).
2. Milam, V.T., Hiddessen, A.L., Crocker, J.C., Graves, D.J. & Hammer, D.A. DNA-driven assembly of bidisperse, micron-sized colloids. *Langmuir* **19**, (24) 10317-10323 (2003).
3. Mirkin, C.A., Letsinger, R.L., Mucic, R.C. & Storhoff, J.J. A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature* **382**, (6592) 607-609 (1996).
4. Kaur, H., Babu, B.R. & Maiti, S. Perspectives on chemistry and therapeutic applications of Locked

Nucleic Acid (LNA). *Chem. Rev.* **107**, (11) 4672-4697 (2007).

5. Baker, B.A., Mahmoudabadi, G. & Milam, V.T. Strand displacement in DNA-based materials systems. *Soft Matter* **9** 11160-11172 (2013).
6. Eze, N.A. & Milam, V.T. Exploring locked nucleic acids as a bio-inspired materials assembly and disassembly tool. *Soft Matter* **9**, (8) 2403-2411 (2013).