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LNA-modified oligonucleotides effectively drive intramolecular-stable hairpin to intermolecular-duplex state

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Abstract

Sequence-specific hybridization of antisense and antigene agent to the target nucleic acid is an important therapeutic strategy to modulate gene expression. However, efficiency of such agents falls due to inherent intramolecular-secondary-structures present in the target that pose competition to intermolecular hybridization by complementary antisense/antigene agent. Performance of these agents can be improved by employing structurally modified complementary oligonucleotides that efficiently hybridize to the target and force it to transit from an intramolecular-structured-state to an intermolecular-duplex state. In this study, the potential of variably substituted locked nucleic acid-modified oligonucleotides (8mer) to hybridize and disrupt highly stable, secondary structure of nucleic acid has been biophysically characterized and compared with the conventionally used unmodified DNA oligonucleotides. The target here is a stem–loop hairpin oligonucleotide—a structure commonly present in most structured-nucleic acids and known to exhibit an array of biological functions. Using fluorescence-based studies and EMSA we prove that LNA-modified oligonucleotides hybridize to the target hairpin with higher binding affinity even at lower concentration and subsequently, force it to assume a duplex conformation. LNA-modified oligonucleotides may thus, prove as potential therapeutic candidates to manipulate gene expression by disruption of biologically relevant nucleic acid secondary structure.

Keywords: Locked nucleic acid; Hairpin trapping; Therapeutic oligonucleotide

Since long oligonucleotides are being considered as excellent therapeutic candidates capable of modulating gene expression by acting either at the level of DNA (antigene agent) or RNA (antisense agent). Most of such gene-silencing approaches involve 'sequence-specific hybridization' of the synthetic oligonucleotide with the target DNA or RNA that subsequently interferes with specific biological functions of the target nucleic acid [1]. However, for applications in intact cells, signal and background issues still need to be addressed before the full potential of these methods is realized. A major concern pertinent to this is the propensity of nucleic acids, particularly RNAs, to adopt multiple secondary structures that are implicated in their specific biological functions. Disruption of these secondary structures by subsequent hybridization to a therapeutic oligonucleotide serves as a key to modulate gene expression. The oligonucleotide competes with the intramolecular state of nucleic acid and drives it to undergo a change to an intermolecular-duplex state. However, in spite of the absolute complementarity of the oligonucleotide to the target nucleic acid, the highly structured state of the target often renders it inaccessible for hybridization [2]. Hybridization of therapeutic oligonucleotide with the target nucleic acid becomes further challenging when the target is either smaller in size or is present in low quantity. Furthermore, susceptibility of the unmodified oligonucleotides to endogenous nucleases can significantly limit their hybridization efficiency, which may result in signals unrelated to oligonucleotide–target hybridization. However, by employing nuclease-resistant modified nucleotide such

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as locked nucleic acid, degradation of these therapeutic oligonucleotide can be substantially reduced.

A locked nucleic acid (LNA) is a nucleic acid analog containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA-mimicking sugar conformation [3]. This conformational restriction is translated into unprecedented hybridization affinity towards complementary DNA/RNA without loss of specificity, which makes fully modified LNAs, LNA/DNA mixmers, or LNA/RNA mixmers uniquely suited for mimicking and targeting nucleic acid secondary structures *in vitro* or *in vivo* [4,5]. Superior specificity of these short synthetic LNA probes raises the hope of distinguishing small differences in sequences, ultimately leading to the selective targeting of even short-length, minority miRNAs and siRNAs against a large background of cellular RNAs/DNA. Furthermore, this exceptionally high specificity can be exploited to target even highly structured-nucleic acids that are usually inaccessible to conventionally used unmodified DNA oligonucleotides [6–14]. Incorporation of LNA nucleotides into the probe also drastically improves its nuclease stability [8,15].

In the current study we assess the potential of LNA-modified oligonucleotides to compete with highly stable, hairpin-structured state of the nucleic acid, in comparison with unmodified DNA probes. The target in our study consists of a stem–loop hairpin oligonucleotide—a structure commonly present in most structured nucleic acids and is known to exhibit an array of biological functions. The loop of the hairpin is complementary to the probe whereas its stem is dual end-labeled with a fluorophore (fluorescein) at one end and a quencher at the other end. By observing fluorescence intensity changes of the fluorophore (DABCYL), the relative opening of the hairpin upon hybridization to the unmodified and LNA-modified oligonucleotide has been studied. Our findings clearly demonstrate that LNA-modified oligonucleotides readily outperform the conventional DNA oligonucleotides in targeting highly structured-nucleic acids and thus, may prove as potential therapeutic candidates to manipulate gene expression by competing with biologically relevant nucleic acid secondary structure.

Materials and methods

Oligonucleotides. To study the potential of LNA-modified oligonucleotides to target highly stable, 13 mer stem–loop hairpin oligonucleotide, a set of four oligonucleotides (8mer) were considered that were modified to various extent and at various positions with adenine locked nucleic acid bases. The extent of LNA substitution varied from zero to three (Table 1). The end-labeled stem of the target hairpin consisted of three base pairs whereas its loop region, consisting of eight bases, exhibited perfect complementarity to the 8mer oligonucleotide.

The LNA containing octameric oligonucleotides were synthesized and purified as described in [5] whereas the unmodified DNA oligonucleotide, along with 13 mer oligonucleotide d(CGCTGGTGCTGCG) (HPLC purified) labeled with 5′-fluorescein and 3′-quencher (DABCYL) were obtained from Sigma Genosys. The solution concentrations of the unmodified oligonucleotide were determined optically at 260 nm and at 25 °C using the following molar extinction coefficients [per $(\text{mM cm})^{-1}$ of strands]: 81.3 for d(AGCACCAG). This value was calculated by extrapolation of the tabulated values of the dimers and monomers bases at 25 °C to high temperature using protocols reported earlier [16]. For the modified oligonucleotides, the molar absorptivities were assumed to be identical to the DNA monomeric oligonucleotide. Concentration of the labeled oligonucleotide was determined by measuring the absorbance of the attached fluorescein moiety at 496 nm using a molar extinction coefficient of $4.1 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ [17]. Milli Q water was used in all the experiments. All experiments except Native-PAGE were performed in 10 mM sodium cacodylate buffer (pH 7.0) with 100 mM NaCl.

Table 1
Sequence of the target hairpin (13mer) and differentially modified LNA-probes (8mer)[a]

Nomenclature	Sequence
Target hairpin	5′-CGCTGGTGCTGCG-3′
Unmodified probe	5′-AGCACCAG-3′
LNA 1	5′-**A^{L}**GCACCAG-3′
LNA 2	5′-**A^{L}**GC**A^{L}**CCAG-3′
LNA 3	5′-**A^{L}**GC**A^{L}**CC**A^{L}**G-3′

[a] LNA-modified bases marked bold with 'L' superscript.

FRET-based analysis. Temperature-dependent fluorescence studies for preformed hairpin (250 nM) in the absence and presence of unmodified and LNA-modified oligonucleotides were carried out using a Fluoromax 4 (Spex) spectrofluorimeter with an excitation and emission bandwidth of 5 nm and a 1 cm × 1 cm quartz cuvette. Melting profile was constructed at the hairpin to probe concentration of 1:1, 1:5 and 1:10. Excitation wavelength was set at 480 nm and emission spectra were recorded from 500 to 750 nm. Melting profile with a heating rate of 0.3 °C/min was constructed by plotting fluorescein intensity as a function of temperature (10–90 °C).

To determine the binding affinity of modified and unmodified oligonucleotides towards the target hairpin, fluorescence experiments were carried out at 20 °C using fixed concentration (250 nM) of dual-labeled hairpin and varying the oligonucleotide concentration (0–500 nM). For analysis of data, the observed fluorescence intensity was considered as the sum of the weighted contributions from folded hairpin strand and converted hairpin to duplex:

$$F = (1 - \alpha_b)F_0 + \alpha_b F_b, \tag{1}$$

where F is the observed fluorescence intensity at each titrant concentration, F_0 and F_b are the respective fluorescence intensities of initial and final states of titration, and α_b is the mole fraction of duplex in duplex form. Assuming 1:1 stoichiometry for the interaction in case of oligonucleotide binding, it can be shown that:

$$[\text{HP}_0]\alpha_b^2 - ([\text{HP}]_0 + [\text{C}] + 1/K_A)\alpha_b + [\text{C}] = 0, \tag{2}$$

where K_A is the binding constant, $[\text{HP}]_0$ is the total hairpin concentration, and [C] is the added complementary oligonucleotide concentration. From Eqs. (1) and (2), it can be shown that:

$$\Delta F = (\Delta F_{\max}/2[\text{HP}_0]) \times \left\{([\text{HP}]_0 + [\text{C}] + 1/K_A) - \sqrt{([\text{HP}]_0 + [\text{C}] + 1/K_A)^2 - 4[\text{HP}]_0[\text{C}]}\right\}, \tag{3}$$

where $\Delta F = F - F_0$ and $\Delta F_{\max} = F_{\max} - F_0$ and F and F_0 are the initial and subsequent fluorescence intensities of the fluorophore at 520 nm, upon addition of complementary strand. The binding affinity (K_A) derived from Eq. (3) was used to determine the fraction of duplex (D_{eq}) formed at equimolar hairpin (HP_0) and complementary strand concentration (C_0) (250 nM), using the equation

$$K_A = D_{eq}/(\text{HP}_0 - D_{eq}) \times (C_0 - D_{eq}). \tag{4}$$

Polyacrylamide gel electrophoresis (PAGE). Nondenaturing PAGE 20% was carried out at 4 °C. Both the gel and the running buffer contained 90 mM Tris borate buffer (TBE), pH 8.0. Experiments were performed with hairpin to oligonucleotide concentration of 1:1 (0.5 μM). Glycerol was added [5% (v/v)], and a final sample volume of 30 μl was loaded onto the gel and electrophoresed at 4 V/cm. Bands were visualized using phosphorimager (Fujifilm FLA 2000).

Results

To demonstrate the superiority of LNA-modified oligonucleotides over conventionally used DNA probes in competing with highly structured state of nucleic acids; we carried fluorescence-based study. In the absence of oligonucleotide, the two ends of target base-pair to form a highly stable stem–loop hairpin structure that brings the fluorophore (fluorescein) in close proximity of the quencher (DABCYL), as a result of which the relative fluorescence intensity of the fluorophore is quenched. Upon hybridization to the complementary oligonucleotide, the stem–loop hairpin structure of the target opens, which separates the donor dye (fluorescein) from the quencher, resulting in an increase in fluorescence intensity of the fluorophore. Fluorescence spectra of the hairpin in the absence and presence of equimolar concentration (250 nm) of unmodified and LNA-modified oligonucleotides at 20 °C showed an increase in the fluorescence intensity as the number of LNA substitutions in the oligonucleotides was increased from LNA 0 (unmodified probe) to LNA 3 (Fig. 1).

Further, temperature-dependent fluorescence melting curves were obtained for dual-labeled, stem–loop target hairpin at the hairpin to oligonucleotide concentration of 1:1, 1:5, and 1:10. Melting profile of hairpin in the absence of complementary oligonucleotide was constructed by plotting fluorescein intensity as a function of temperature. The plot exhibited a sigmoid curve with a T_M of 65 °C, reflecting the high stability of the hairpin (Fig. 2). Typical rise in the fluorescein intensity observed in the temperature range of 40–100 °C represented region of hairpin melting. The presence of oligonucleotide (unmodified or LNA-modified)

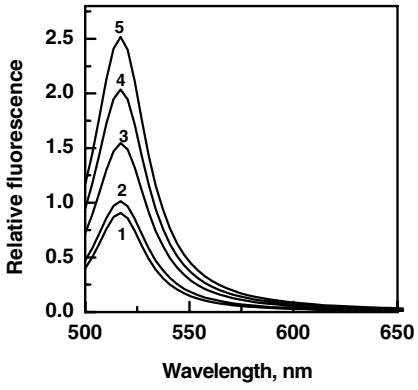

Fig. 1. Fluorescence intensity changes of the labeled hairpin (trace 1) at 250 nM, 20 °C, in the presence of equimolar concentration of unmodified (trace 2), single modified (trace 3), double modified (trace 4), and triple modified (trace 5) complementary oligonucleotide in 10 mM sodium cacodylate buffer (pH 7) containing 100 mM NaCl.

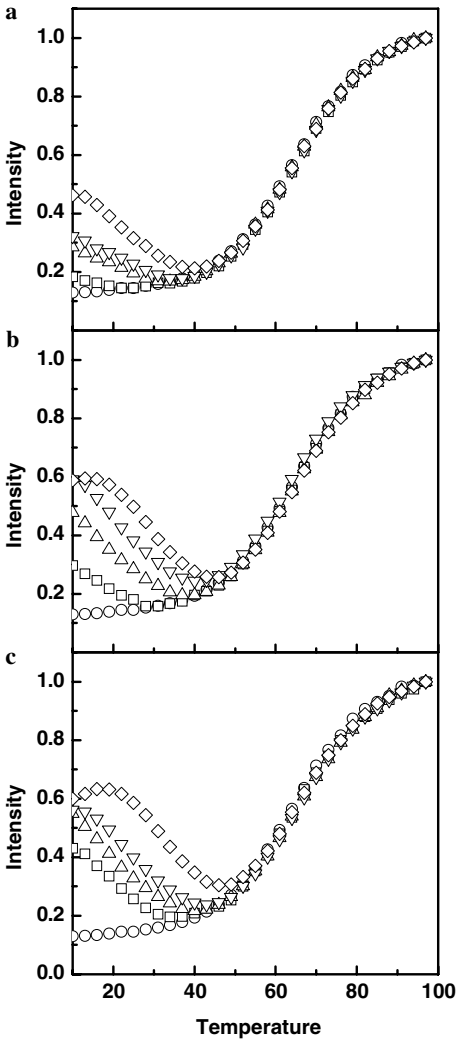

Fig. 2. Fluorescence emission intensity of the labeled hairpin (circle) at 250 nM, λ_{520nm}, in the presence of unmodified (square), single modified (upright triangle), double modified (inverted triangle) and triple modified (diamond) complementary oligonucleotides, as a function of temperature (10–100 °C) at hairpin to probe concentration of 1:1 (a), 1:5 (b), and 1:10 (c).

made no difference to this hairpin-melting region, however, led to an initial dip in the fluorescein intensity in the range of 10–45 °C (Fig. 2). The decline in the signal became pronounced as the number of LNA substitutions in the complementary oligonucleotide was increased. The dip became further significant as the hairpin to oligonucleotide concentration was increased from 1:1 to 1:5 and 1:10 (Fig. 2).

To further analyze the binding of LNA-modified oligonucleotides, we carried out fluorescence-based binding experiments. By monitoring increase in fluorescein intensity as a function of complementary oligonucleotide concentration, the relative extent of duplex formation indicative of the binding of modified versus unmodified oligonucleotide was estimated. The binding affinities were analyzed by fitting the plot of change in fluorescence intensity of the fluorophore, at 520 nm against complementary strand concentration using Eq. (3) (Fig. 3). The obtained binding

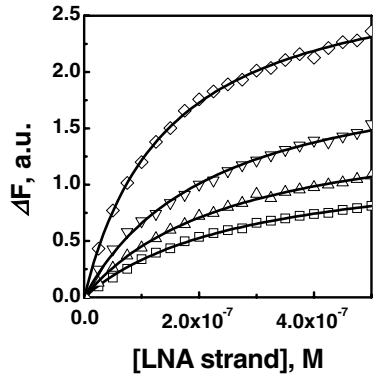

Fig. 3. Opening up of the hairpin (250 nM) at 20 °C upon addition of different concentrations of (0–500 nM) complementary oligonucleotide, measured in 10 mM sodium cacodylate buffer, pH 7 containing 100 mM NaCl. Complementary oligonucleotides used were unmodified (square), single modified (upright triangle), double modified (inverted triangle), and triple modified (diamond).

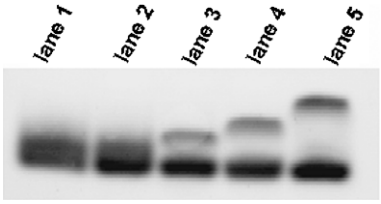

Fig. 4. Gel mobility profile of the hairpin (control, lane 1) in the presence of unmodified (lane 2), single modified (lane 2), double modified (lane 3), and triple modified (lane 4) complementary oligonucleotides.

affinities were 5.0×10^6, 7.7×10^6, 1.7×10^7, and $5.6\times10^7\ M^{-1}$ for LNA 0, LNA 1, LNA 2, and LNA 3, respectively. Further, using Eq. (4) the ratio (D_{eq}/HP_0) of duplex formed versus free hairpin was determined that corresponded to 0.72, 0.97, 1.62, and 3.27 for LNA 0, LNA 1, LNA 2, and LNA 3, respectively. The binding data thus, revealed that the binding affinity and the amount of duplex formed increased upon increasing the number of LNA substitutions, in the oligonucleotide.

An electrophoretic mobility shift assay for the estimation of relative hairpin to duplex conversion was carried out to further validate our findings. Hairpin to complementary strand concentration was adjusted to 1:1 (0.5 μM). The hairpin structure being compact enough migrates at a faster rate in the gel whereas, the corresponding duplex with high molecular weight lags behind [18]. The shift in the hairpin band indicated (Fig. 4) that at a given concentration the relative opening of hairpin, as reflected by conversion to the duplex form, increased as the extent of LNA substitutions in the complementary oligonucleotide was increased.

Discussion

The concept of gene inhibition by employing complementary oligomers (antisense or antigene approaches) is not new and theoretically, possesses enormous potential to modulate gene expression particularly, at the level of RNA. In practice, however, these methods have not proven to be robust or reliable, because all available antisense agents used till date, suffer from one or more inherent weaknesses [19]. This has led to the search for new reliable therapeutic oligonucleotides with desirable gene-silencing properties and among these LNA oligonucleotides are obvious candidates. The rapid development of locked nucleic acid (LNA)-modified oligonucleotide has significantly improved the sensitivity and specificity of nucleic acid targeting [3,20]. In order to demonstrate higher efficacy of LNA-modified oligonucleotides in competing with highly stable intramolecular state of nucleic acids, we undertook fluorescence-based study. Unlike CD or UV, fluorescence allowed us to work at nanomolar range thereby, enabling monitoring of oligonucleotide–target interaction at a concentration range in which biomolecules actually interact in the cellular milieu.

The presence of complementary oligonucleotide ensues hairpin opening leading to an increase in fluorescence intensity of the fluorophore. Compared to an unmodified oligonucleotide, an LNA-modified oligonucleotide exhibits increased hybridization affinity towards the target hairpin, as a result of which the relative rise in fluorescence intensity increases as the extent of LNA substitution is increased from LNA 0 to LNA 3 (Fig. 1). This increase in intensity indicates that even with the same oligonucleotide concentration, a higher 'hairpin to duplex' conversion can be achieved with LNA-modified oligonucleotides, as compared to the conventionally used DNA oligonucleotides. The relative stability of the so-formed duplex was examined using fluorescence melting study (Fig. 2). Hairpin melting in the absence of complementary oligonucleotide exhibited a T_M of 65 °C, reflecting the high stability of the hairpin. The intention of considering this highly stable target for our study was to assess if LNA-modified probes could capture such highly stable, structured targets, which are most commonly encountered in *in vivo* settings. Since, these structures are difficult to overcome they serve as rigorous test for any therapeutic oligonucleotide that relies on hybridization-based gene-silencing approach to modulate gene expression. Melting in presence of complementary oligonucleotide exhibited no change to the hairpin-melting region; however, it disrupted the profile in the temperature range of 10–45 °C, where a decline in the fluorescein intensity was observed. This decline in signal results from the melting of the duplex formed by oligonucleotide–target hybridization. As the duplex melts, the denatured hairpin strand again tends to resume the stable stem–loop conformation, thereby, bringing the donor fluorophore (fluorescein) in close proximity of the quencher (DABCYL). The relative decrease in the intensity was more for LNA-modified oligonucleotides, which reflects their tendency to form increasingly stable duplexes that melt at temperatures higher than the duplexes formed by unmodified oligonucleotides [21]. This higher stability of the LNA containing duplexes directly relates to their superior performance in targeting highly stably target hairpins. The decrease in fluorescein signal became further pronounced when the relative concen-

tration of the complementary oligonucleotide with respect to hairpin was increased, ascribed to the fact that increasing the concentration of the complementary strand leads to increased hairpin to duplex conversion [22]. A further rise in temperature melts the hairpin, as reflected by the increase in fluorescein intensity. In the light of above facts it can be concluded that LNA-modified oligonucleotides form far more stable duplexes as compared to the unmodified DNA oligonucleotides and are thus, more efficient in competing with the highly stable intramolecular state of nucleic acids. Further, our FRET-based binding experiments allowed estimation of relative binding affinity of the unmodified or LNA-modified oligonucleotide towards the target, along with the relative duplex formation, when equimolar concentration of complementary strand is used. Our results showed an increased association of LNA-modified oligonucleotides to the target hairpin leading to an increased duplex formation, which indicates that compared to the unmodified oligonucleotides, lower concentration of LNA-modified oligonucleotides is required to mediate transition of intramolecular-hairpin state to intermolecular duplex state. Our Native-PAGE experiment further strengthened our results, where at a given concentration we observed increased conversion of hairpin to duplex form by LNA-modified oligonucleotide as compared to the unmodified oligonucleotide. The fraction of duplex formed increased with the increase in the number of LNA substitutions in the oligonucleotide. Taken together, our results indicate that LNA-modified oligonucleotides are far more superior in targeting highly stable hairpin structures, even at low concentrations. Their higher binding affinity and high duplex stability indicate that they can be successfully employed in cases where the target is present in either lower amounts or where the high stability of the hairpin renders the complementary oligonucleotide inaccessible for hybridization. The use of LNA oligonucleotides in such a scenario will significantly improve signal to background ratios by enhancing the effectiveness of the therapeutic oligonucleotide to compete and disrupt highly stable, structured targets, even at low oligonucleotide concentration. These findings thus, have significant implications for the application of LNA-modified oligonucleotides for efficient targeting of highly structured nucleic acids.

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