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Properties of an 'LNA'-modified ricin RNA aptamer

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ABSTRACT

'Locked nucleic acids' (LNAs) are sugar modified nucleic acids containing the 2′-*O*-4′*C*-methylene-β-D-ribofuranoses. The substitution of RNAs with LNAs leads to an enhanced thermostability. Aptamers are nucleic acids, which are selected for specific target binding from a large library pool by the 'SELEX' method. Introduction of modified nucleic acids into aptamers can improve their stability. The stem region of a ricin A chain RNA aptamer was substituted by locked nucleic acids. Different constructs of the LNA-substituted aptamers were examined for their thermostability, binding activity, folding and RNase sensitivity as compared to the natural RNA counterpart. The LNA-modified aptamers were active in target binding, while the loop regions and the adjacent stem nucleotides remained unsubstituted. The thermostability and RNase resistance of LNA substituted aptamers were enhanced as compared to the native RNA aptamer. This study supports the approach to substitute the aptamer stem region by LNAs and to leave the loop region unmodified, which is responsible for ligand binding. Thus, LNAs possess an encouraging potential for the development of new stabilized nucleic acids and will promote future diagnostic and therapeutic applications.

1. Introduction

Aptamers are nucleic acids that are selected for specific target binding from a large library pool by the 'SELEX' method ('systematic evolution of ligands by exponential enrichment') [1,2]. In principle, aptamers synthesized *in vitro* can be designed against any molecular target, serving thus as a valuable tool in medical diagnostics and therapy. Aptamers are much smaller in molecular mass than antibodies and show low immunogenicity. The therapeutic and diagnostic potential of aptamers has been investigated thoroughly and reviewed extensively [3,4]. An excellent example of a successful clinical application of an aptamer is the drug Macugen, which acts as an endothelial growth factor antagonist and is already approved for the therapy of neurovascular age-dependent macular degeneration [5].

As natural nucleic acids are sensitive against nuclease degradation and often possess low thermal stability, a challenging approach is to stabilize them by introducing modified nucleic acid building blocks that are nuclease-insensitive and to induce higher melting temperatures. This is of special interest in oligonucleotide-based diagnostics and therapeutics, not only in aptamer-based drug design but also in gene silencing by RNA interference. Upcoming approaches are single or multiple substitutions of natural nucleic acids by so-called 'locked nucleic acids' (LNAs). These nucleic acids contain a variety of sugar modifications [6]. The most commonly used derivative is the 2′-*O*-4′*C*-methylene-β-D-ribofuranose moiety. Nucleotides containing this modification are known to introduce a substantial increase in the melting temperature of substituted RNAs [6]. A series of functional and structural investigations has already been reported for LNA-substituted nucleic acids. Structural investigations, using 2′-*O*-4′*C*-methylene-β-D-ribofuranose LNA–RNA mix-mers, could demonstrate that introduction of single LNA nucleotides into one strand of a RNA duplex did not interfere with the RNA A-type nucleic acid conformation. Likewise, the use of 2′-*O*-4′*C*-methylene-α-L ribofuranose LNA–DNA mix-mers in one strand of a DNA duplex is compatible to the B-type geometry [7,8].

Extensive investigations were performed towards understanding the enhanced thermostability of LNAs. Studies with RNA–LNA mix-mers and with RNA–LNA heteroduplexes show that the 2′-*O*-4′*C*-β-D-methylene ribofuranose 'locks' the LNA in the C2′-exo conformation. This influences the geometry of the sugar–phosphate backbone and orients the duplex in a way to facilitate a more efficient base stacking [7–10]. For our investigations, we have chosen a well-established aptamer, RA80.1.d1, which has been developed against the ricin A chain, as a model substance to introduce LNA modifications into the stem region [11]. We substituted different

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regions of the aptamer stem with continuous LNAs, in most cases forming a duplex consisting of 7 base pairs. As the loop region of the aptamer is supposed to be responsible for ricin recognition [11], we left this part unmodified to maintain the binding activity. We examined the LNA substituted aptamers in comparison to the natural RNA aptamer with respect to thermostability, ricin binding capacity and RNase sensitivity.

2. Materials and methods

2.1. Reagents

Ricin extracted from the castor plant was a kind gift of Brigitte Dorner, Robert Koch Institute, Berlin, Germany. RNases were obtained from Applied Biosystems (Darmstadt, Germany). All other reagents have been purchased from Sigma–Aldrich (Taufkirchen, Germany) unless otherwise stated.

2.2. Construction and preparation of the RNA aptamer and the LNA–RNA aptamers

The sequence of the LNA helix has been adapted from the terminal domain of the ricin RNA aptamer [11] and was designed as an 'all LNA' for this study. The helix consists exclusively of nucleotide building blocks which contain the β-D-2′-*O*-4′-*C*-methylene modified ribofuranose. The chemically synthesized oligonucleotides with the sequences 5′-Amino-C6-(UGG AUC C)^{L}-GGC GAA UUC AGG GGA CGU AGC AAU GAC UGC C-(GGG UUC A)^{L}-3′ (aptamer-1, 45mer), 5′-Amino-C6-(UGG AUC C)^{L}-GAA UUC AGG GGA CGU AGC AAU GAC U-(GGG UUC A)^{L}-3′ (aptamer-2, 39mer), 5′-Amino-C6-(GGC GAA UUC A)^{L}-GGG GAC GUA GCA A-(UGA CUG CC)^{L}-3′ (Aptamer-3, 27mer) and the RNA aptamer 5′-Amino-C6-GGC GAA UUC AGG GGA CGU AGG AAU GAC UGC C-3′ (native aptamer) were purchased from IBA (Göttingen, Germany). The LNA modifications possess the standard U to T and C to m^{5}C exchange in LNAs as compared to RNAs. Sample preparation and annealing of the aptamers was done by heating the samples for 5 min to 95 °C at a concentration of 0.1 mM in water or PBS containing 5 mM $MgCl_2$. Subsequent cooling to room temperature occurred either within several hours or by 'snap cooling' directly on ice, depending on the experiment.

2.3. Measurement of melting temperatures

As reported previously, we measured the melting curves of the RNA and LNA 7mer duplex to examine their thermostability [12]. Melting curves of the aptamers were recorded on a HP Agilent 8453 UV/visible diode array spectrophotometer (Agilent ChemStation software) in PBS using 2.5 μM aptamer. Absorbance A_{260} was measured as a function of temperature within a range of 10–90 °C upon increasing the temperature by 1 °C increase per min. The T_m values were obtained from the maxima of the first derivatives of the melting curves.

2.4. Polyacrylamide gel electrophoresis

Native polyacrylamide (PAA) gel electrophoresis was performed using 15% or 20% PAA gels in TBE-buffer: 89 mM Tris/HCl, 89 mM boric acid, 2.0 mM EDTA, pH 8.4. Samples were combined with loading buffer that consisted of TBE buffer, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol and 20% (v/v) glycerol. Roughly 5 μg of aptamer was used per lane. Gels were run at 300–400 V for 2–3 h. Staining of the gels was performed with 0.1% (w/v) 'Stains all' (Sigma–Aldrich, Hamburg) in 50% aqueous formamide for 1–2 h at room temperature. Destaining was done in water for several hours.

2.5. RNase digestions of aptamers

RNase R (20 U/μl) was supplied in 50% glycerol solution containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton® X-100. Digestion of 5 μg RNA each was performed in 20 μl 20 mM Tris–HCl, pH 8.0, 0.1 M KCl, and 0.1 mM $MgCl_2$ at a temperature of 37 °C for 30 min. RNase T1 (1000 U/μl) was purchased in a 50% glycerol solution containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA. Cleavage of 5 μg RNA with 1 μl RNase T1 was carried out in 50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.1 mM EDTA for 30 min at 37 °C. RNase A (70 U/μl) was supplied in 50 mM Tris–HCl, pH 7.4 and 50% (v/v) glycerol. Identical reaction conditions were used as described for RNase T1 cleavage, but with addition of 5 mM $MgCl_2$ instead of 0.1 mM EDTA. For cleavage with RNase V1 (Applied Biosystems, Darmstadt, Germany), 5 μg RNA each were treated with 10 μl RNase V1 (0.1 U/μl) in the provided reaction buffer at a temperature of 37 °C for 30 min.

Each reaction was stopped by the addition of 20 μl phenol/chloroform. The RNA in the aqueous solution was precipitated from the aqueous phase by adjusting the solution to 0.3 mM sodium acetate, pH 4.8, and adding 120 μl ethanol at a temperature of −20 °C. Following to an overnight incubation at −20 °C, the RNA was recovered by centrifugation at 13,000 g for 10 min. The RNA pellet was washed with 70% ethanol/water at −20 °C and dried at room temperature. For the RNase digestion and gel electrophoresis, the RNA was resuspended in 5 μl water and treated as described above.

2.6. Surface plasmon resonance measurements

Binding between aptamers and ricin was investigated with a Biacore T100 instrument (GE Healthcare, Freiburg, Germany) at a temperature of 25 °C in PBS containing 5 mM $MgCl_2$ as running buffer. Gold sensor chips (SIA Kit, GE Healthcare) were functionalized with mercaptoundecanoic acid (MUA, 7 mM solution in ethanol, 72 h, RT) and thoroughly washed with ethanol and water prior to use. Immobilization of 800 RU ricin was performed using EDC (1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride) and NHS (*N*-hydroxysuccinimide) amine coupling chemistry at a flow rate of 10 μl/min. Briefly, after activation of the chip surface with a mixture of EDC and NHS (200 mM/50 mM in water) for 7 min, a solution of 10 μg/ml ricin in 10 mM sodium acetate buffer, pH 5.0, was injected until reaching the designated immobilization level. Remaining activated carboxylic groups were saturated with 1 M glycine (in PBS, pH 7.4) for 7 min.

For binding analysis, nucleic acids were diluted with running buffer to a concentration of 1 μM and pumped over the chip surface for 3 min at a flow rate of 30 μl/min. Pure running buffer was continued for another 10 min. A 30 nucleotide DNA (5′-GTA GCG TCC CTG ATA GAG CTG ACA TGA ACG-3′) and a mutant of the native RNA aptamer (C9 to A transition, 5′-Amino-C6-GGC GAA UUA AGG GGA CGU AGC AAU GAC UGC C-3′) were used as negative controls. Regeneration was achieved by a short injection of 5 mM NaOH (30 s, 30 μl/min) followed by a stabilization period of 2 min. Data were evaluated using Biacore T100 Evaluation Software version 1.1.1.

3. Results and discussion

We investigated the stabilization of aptamers by introducing modified nucleic acids into the stem regions, as these parts are

Table 1
Thermostability of native and chemically modified nucleic acid duplexes. The melting temperature T_m of the duplexes increases continuously from top to bottom [13].

Abbreviation	Type of nucleic acid
2′-F/2′-OMe	2′-Fluoro-RNA/2′-*O*-Methyl-RNA
RNA/RNA	Native RNA/RNA
2′-OMe/2′-OMe	2′-*O*-Methyl-RNA/2′-*O*-Methyl-RNA
2′-F/LNA	2′-Fluoro-RNA/2′-*O*-4′*C*-methylene-*β*-D-ribofuranose nucleic acid
RNA/LNA	Native RNA/2′-*O*-4′*C*-methylene-*β*-D-ribofuranose nucleic acid
LNA/RNA	2′-*O*-4′*C*-methylene-*β*-D-ribofuranose nucleic acid/native RNA
2′-OMe/LNA	2′-*O*-Methyl-RNA/2′-*O*-4′*C*-methylene-*β*-D-ribofuranose nucleic acid
LNA/LNA	2′-*O*-4′*C*-methylene-*β*-D-ribofuranose nucleic acid homoduplex

usually not involved in target binding. As the order of thermostability for modified nucleic acids is described to be: 2′-F/2′-OMe < RNA/RNA ⩽ 2′-OMe/2′-OMe < 2′-F/LNA < RNA/LNA = LNA/RNA < 2′-OMe/LNA < LNA/LNA [13] (Table 1), we focussed our interest on introducing 2′-*O*-4′*C*-methylene-*β*-D-ribofuranose modifications into the aptamer stem structures. In this study, we chose a ricin-specific RNA aptamer as a model [11] and modified the stem region with LNAs (Fig. 1). We designed three different aptamers (labeled 1, 2 and 3) that resemble the original aptamer (RA80.1.d1) but carry shortened stem regions containing exactly 7 continuous LNA basepairs. For aptamers 1 and 2, the terminal 7 basepairs of the original aptamer (RA80.1.d1) were converted into LNAs and attached to the shortened published version (RA80.1.d2) to form aptamer 1 or attached directly the G–U basepair that closes the AAU bulge on its terminal end (aptamer 2, Fig. 2). Finally, a third aptamer was constructed resembling the short published version (RA80.1.d2) but with the complete stem, including the bulge, synthesized as LNA (aptamer 3, Fig. 2). All three LNA variants as well as the two published RNA versions of the ricin aptamer were analyzed with respect to their binding affinity, thermostability, folding behavior and nuclease sensitivity.

The binding between aptamers and the target ricin was examined by surface plasmon resonance spectroscopy. This allows label-free on-line-detection as used not only for the study of artificial aptamer–protein interactions [14,15] but also for protein binding to natural nucleic acid structures [16]. As expected, the unmodified RNA aptamer (RA80.1.d1) binds to the immobilized ricin. The LNA-modified aptamers show the following behavior (Figs. 2 and 3): aptamer 1, which possesses the LNA-duplex with the largest distance away from the loop region, also showed binding to the target ricin and reached a binding level of approximately 50% as compared to aptamer RA80.1.d1. Aptamer 2, with the LNA cassette in shorter distance to the aptamer loop, showed markedly reduced binding affinity. We were unable to detect binding to ricin for aptamer 3, in which the small extra bulge was included into the LNA modification scheme. In a control experiment, we measured the affinity to ricin of a 30 nucleotide DNA with random sequence and of a mutant of the native RNA aptamer (C9 to A transition). As expected, both constructs exhibited no detectable binding affinity. In accordance to [11], the large single stranded loop as well as the single stranded bulge region are essential for the interaction with the ligand. LNA modification of the bulge completely abrogates binding affinity for the aptamer ligand ricin. We conclude that binding of the LNA-modified aptamers to ricin is maintained, as long as the LNA-modifications are not in structural coherence with the loop or bulge regions of the aptamer.

The thermostability and the structural properties of LNAs provide new perspectives for LNA modifications in nucleic acid drug application, which is an encouraging outlook. As has been reviewed, an increase of +2 to +10 °C can be observed per LNA building block added in strands hybridized to RNA [6]. As reported previously [12], we have investigated the melting temperature of the aptamer's 7mer RNA helix and compared the data to the LNA's 7mer counterpart. Whereas the RNA duplex possesses a T_m value of 22 °C, the corresponding 'all LNA' duplex shows a T_m value of 84 °C. This dramatic shift in thermostability corresponds to an average of 4.4 °C per monomeric nucleotide. This is consistent with reviewed observations [6]. The unmodified short RNA aptamer (RA80.1.d2, Fig. 2) has a T_m value of 40–42 °C, whereas the LNA-modified aptamer (aptamer 1, Fig. 2) shows a T_m of 50–52 °C. As expected, the melting temperature of the LNA-modified aptamer is increased as compared to the native RNA molecule. Surprisingly, the shift in thermostability is far lower than expected, since the T_m value of the LNA–RNA aptamer is lower than the T_m of the 'all LNA' 7mer duplex. It is conceivable that this is due to the special structural arrangement in a mixed type RNA/LNA helix, as the RNA part resembles the structure of an A-type nucleic acid helix, whereas the LNA duplex has a totally different structural arrangement with a stronger hydrophobic interaction between the base pairs, resembling rather the structure of a 'ribbon' than of that of a helix [17]. There should be a transition of these different structural types in a mixed RNA/LNA helix. We conclude that the initial melting of the RNA part induces a faster melting of the adjacent LNA helix, leading to a T_m, which is lower as compared to that of the very stable 'all LNA' 7mer helix. Nevertheless, the introduction of the LNA helix into the RNA ricin aptamer enhances the melting temperature to a considerable degree.

We further examined the folding behavior and stability of the native *vs.* the LNA-modified aptamers by polyacrylamide gel electrophoresis (PAGE) and RNase treatments. Hairpin RNAs are prone

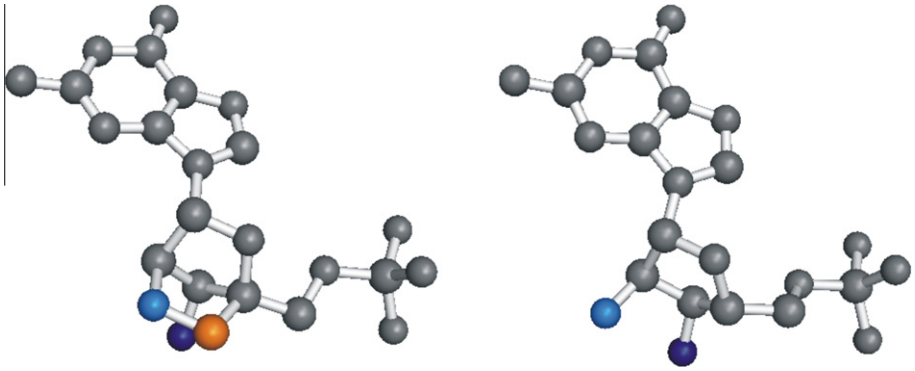

Fig. 1. Guanosine nucleotides are shown as LNA with the 2′-*O*-4′*C*-*β*-D-methylene ribofuranose modification (left) and as RNA with the naturally occurring ribose (right). The 2′- and 3′-oxygen atoms are coloured in cyan and blue. The additional methylene group in the LNA is shown in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

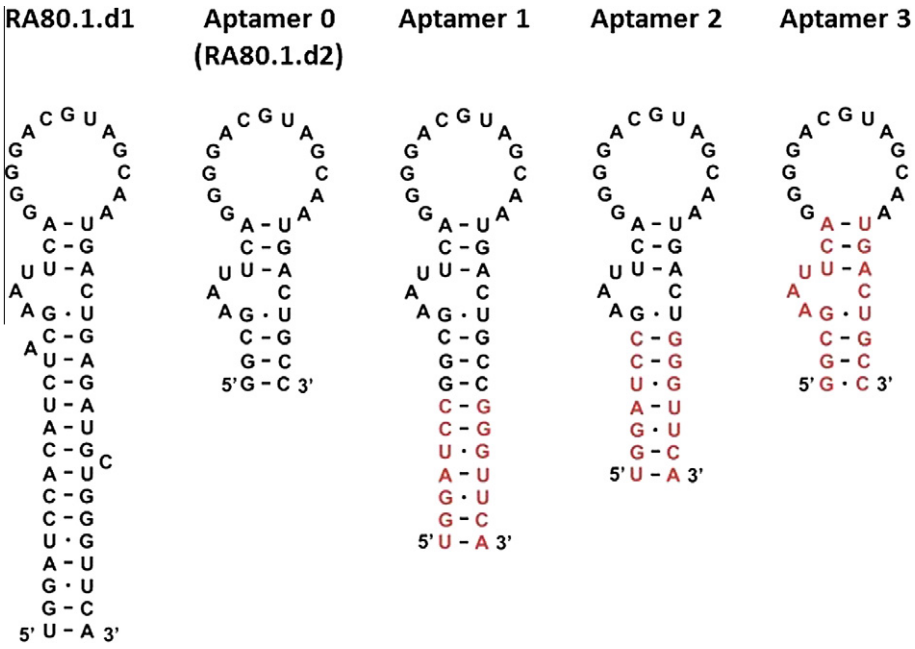

Fig. 2. Loop–stem structure of the analyzed ricin aptamers. The 54 nucleotide long RNA sequence of the ricin-specific aptamer RA80.1.d1 and the 31 nucleotide long sequence of the shortened aptamer RA80.1.d2 ('aptamer 0') were adapted from [11]. The terminal 7 base pair helix was constructed as 'LNA modified stem' and introduced into the following shortened LNA-modified aptamers: aptamer 1, (45 nucleotides), aptamer 2, (39 nucleotides) and aptamer 3 (31 nucleotides). LNA substitutions are pointed out in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

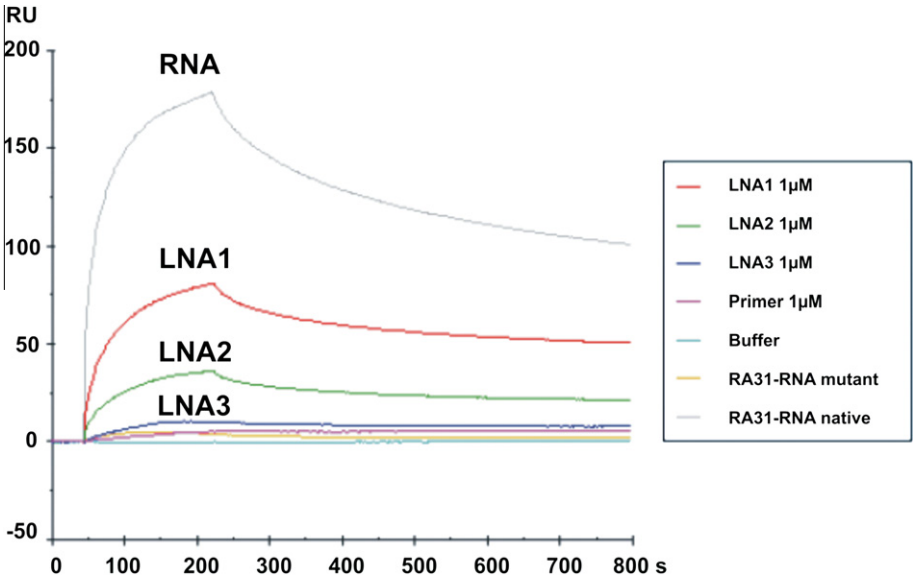

Fig. 3. Analysis of the binding properties of unmodified and LNA-modified aptamers to immobilized ricin by surface plasmon resonance spectroscopy. Aptamers (1 µM in PBS including 5 mM $MgCl_2$) were applied to the chip surface (binding) and subsequently washed off with running buffer (unbinding). Association and dissociation were monitored in real time.

to form stable dimers besides monomeric structures [18]. Therefore, we investigated the melting of the native aptamer RA80.1.d1 and an LNA modified construct with the highest binding affinity for ricin, aptamer 1 (Fig. 2) by heating them to a defined temperature and cooling them quickly on ice before separating the different conformers by a native gel electrophoresis. In Fig. 4A, we show a native PAGE with the RNA aptamer and LNA-modified aptamer 1 after heating to 30°, 50°, 70° and 90° in water and subsequent 'snap-cooling'. The aptamer dimers appear as upper bands on the gel, whereas the monomers can be seen as lower bands. The dimeric form of the unmodified aptamer disappears after heating to 70 °C, whereas traces of the dimeric form of the LNA-modified aptamer were even detectable after treatment at 90 °C. To verify the molecular integrity of the aptamers, all samples were in addition analyzed on a denaturing PAGE with 7 M urea. This experiment revealed that all aptamers withstood even the highest temperature without any sign of degradation (Fig. 4B). The observed thermostability was a prerequisite for measuring the RNase sensitivity of the aptamers.

Both aptamers, the unmodified RNA aptamer RA80.1.d1 and the LNA-modified aptamer 1 were examined with respect to their nuclease stability using four different ribonucleases: RNase V1, A, T1 and R. The resulting digestion profile is depicted in Fig. 4C. Undigested aptamers served as a control and were separated on

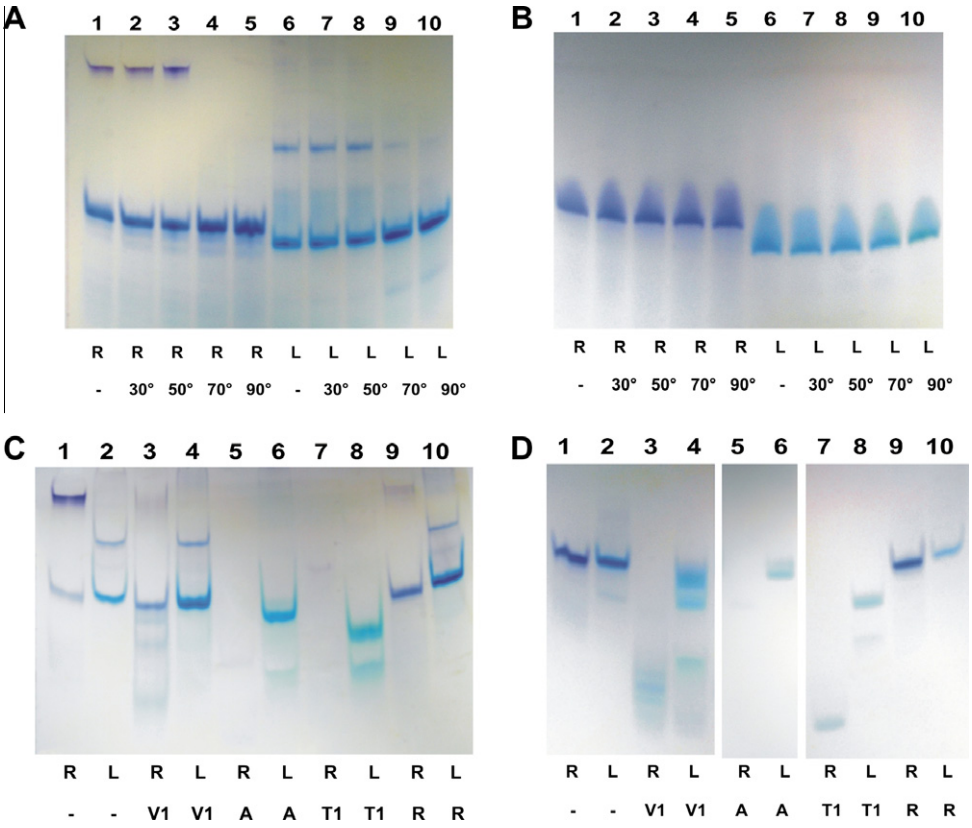

Fig. 4. RNase resistance of the native ricin RNA aptamer and the LNA-modified variant 1. (A) Native PAA electrophoresis of both aptamers subsequent to heat treatment and 'snap cooling' on ice. R (RNA) and L (LNA-modified aptamer); temperatures are indicated. The lower bands correspond to the correctly folder monomer, whereas the upper bands reflect the inactive dimer of the hairpin aptamer. (B) Denaturing PAGE of both aptamers after heat treatment according to (A). R (RNA) and L (LNA-modified aptamer 1), temperatures are indicated. The bands show the unfolded aptamer monomers without any sign of degradation. (C) Treatment of RNA aptamer and LNA-modified aptamer 1 with RNase V1, A, T1 and R. The aptamers were hybridized in PBS containing 5 mM $MgCl_2$ and slowly cooled to room temperature prior to RNase cleavage. The hybridization condition induced the aptamers to fold into monomers (lower bands) and into dimers (upper bands). Details are described in the text. (D) RNase treatment of aptamers that were quantitatively folded into monomers by 'snap cooling' on ice prior to RNase cleavage. Details are described in the text.

lanes 1 and 2 of the native PAA gel. After RNase V1 digestion, we observed a significant difference in the cleavage pattern of the two RNA aptamers: Whereas the dimeric form of the native aptamer was almost completely degraded, the monomeric form remained intact (lane 3). In contrast, the LNA-modified aptamer was not affected at all (lane 4). A higher stability of the modified aptamer was expected, since LNA is less susceptible towards cleavage of the double strand specific RNase V1 as compared to RNA. Surprisingly, even the double stranded RNA part of the stem remained uncleaved. This result indicates that the LNA part of the stem in aptamer 1 alters the helical conformation of the RNA double stranded part into a stabilized form, which is inaccessible for RNase V1.

Next, the aptamers were digested with the single-strand specific RNases A and T1. Surprisingly, we observed a complete degradation of the native RNA aptamer (lanes 5 and 7). This can be explained by the low melting temperature of the RNA aptamers, rendering even the former helical part of the RNA aptamer accessible to RNase A and T1 digestion. In contrast, the LNA-modified aptamer showed only a partial cleavage of the monomer, resulting in a faster migration in the gel. This indicates that only the single-stranded, unmodified fraction of the aptamer was degraded, whereas the LNA stem resisted the RNases (lanes 6 and 8). The dimeric form of the LNA-modified aptamer completely disappeared due to cleavage in the loop regions, leading to monomeric products in their digested form.

Finally, cleavage by RNase R, which is a 3′-exonuclease specific for single stranded RNA, is shown in lanes 9 and 10. The dimeric form of the native RNA aptamer is significantly reduced, possibly due to a temporarily unfolding and the subsequent 3′-5′-exonucleatic activity of RNase R. In contrast, the monomeric form of the native RNA aptamer remained unaffected by the RNase R treatment. In Fig. 4D, we show the same RNase digestions as in Fig. 3C, but the aptamers were hybridized by a different technique: The nucleic acids were heated to 95 °C in water for 5 min and directly 'snap cooled' on ice, which resulted in the exclusive formation of the monomeric aptamers. The results of the RNase V1, A, T1 and R cleavages correspond well to the findings shown in Fig. 3C.

The nuclease cleavage reactions reveal an increased nuclease resistance within the LNA-modified aptamer, which is due not only to the introduction of RNase-insensitive LNA modifications, but also to the stabilizing effects of the LNA-helix on the overall conformation of the aptamer. The structure of an LNA helix visualizes a picture of a stretched helical ladder with altered local and overall geometric parameters [17]. This geometry can rather be placed in vicinity to the structure of glycol nucleic acids (GNAs) [19], peptide nucleic acids (PNAs) [20,21] or homo DNAs [22]. We detected a notable decrease in several local and overall helical parameters like twist, roll and propeller twist as compared to standard RNA molecules. This induces a widening of the major groove and a decrease in helical winding and an increased helical pitch. The enhanced base stacking within LNAs is likely associated with stronger Π–Π interactions of the base pairs, which could explain the high thermostability of LNA duplexes. Consequently, the substitution of double helical regions in RNA aptamer stems by LNA duplexes will induce an improved thermostability and enlarged RNase resistance, by

simultaneously maintaining the biological activity whilst leaving the ligand interacting loop regions largely unaltered.

Nevertheless, we observed a reduced binding affinity of LNA-modified aptamers to the ligand ricin. This finding, however, is in accordance with the data obtained by melting curves and nuclease cleavage reactions. Our findings support a model of a special structural arrangement in the mixed type RNA/LNA helices, as the RNA part resembles the structure of an A-type nucleic acid duplex, whereas the LNA possesses a totally different helical arrangement. In consequence, the LNA modifications might have a significant effect on the tertiary folding of the bulge loop (Fig. 2). The reduced binding affinity of LNA-modified aptamers indicates that this bulge might be part of the binding motif. Thus, the influence of LNAs introduced near bulge regions needs to be considered for future studies. Regarding the ricin aptamer, substitution of the stem region by using thermostable and nuclease-insensitive modified nucleic acid derivatives improve the aptamer stability and could facilitate its application in future ricin and ricin A chain detection assays.

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