ACCOUNTS of chemical research

Article

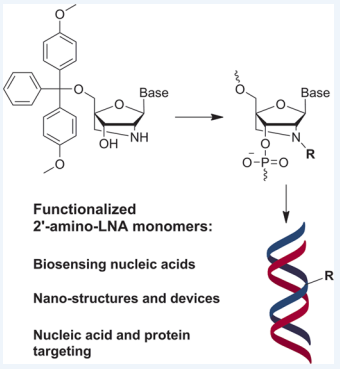
pubs.acs.org/accounts

Scaffolding along Nucleic Acid Duplexes Using 2′-Amino-Locked Nucleic Acids

I. Kira Astakhova* and Jesper Wengel

Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

CONSPECTUS: Incorporation of chemically modified nucleotide scaffolds into nucleic acids to form assemblies rich in function is an innovative area with great promise for nanotechnology and biomedical and material science applications. The intrinsic biorecognition potential of nucleic acids combined with advanced properties of the locked nucleic acids (LNAs) provide opportunities to develop new nanomaterials and devices like sensors, aptamers, and machines. In this Account, we describe recent research on preparation and investigation of the properties of LNA/DNA hybrids containing functionalized 2′-amino-LNA nucleotides.



By application of different chemical reactions, modification of 2′-amino-LNA scaffolds can be efficiently performed in high yields and with various tags, postsynthetically or during the automated oligonucleotide synthesis. The choice of a synthetic method for scaffolding along 2′-amino-LNA mainly depends on the chemical nature of the modification, its price, its availability, and applications of the product. One of the most useful applications of the product LNA/DNA scaffolds containing 2′-amino-LNA is to detect complementary DNA and RNA targets. Examples of these applications include sensing of clinically important single-nucleotide polymorphisms (SNPs) and imaging of nucleic acids *in vitro*, in cell culture, and *in vivo*. According to our studies, 2′-amino-LNA scaffolds are efficient within diagnostic probes for DNA and RNA targets and as therapeutics, whereas both 2′-amino- and isomeric 2′-α-L-amino-LNA scaffolds have promising properties for stabilization and detection of DNA nanostructures. Attachment of fluorescent groups to the 2′-amino group results in very high fluorescent quantum yields of the duplexes and remarkable sensitivity of the fluorescence signal to target binding. Notably, fluorescent LNA/DNA probes bind nucleic acid targets with advantages of high affinity and specificity. Thus, molecular motion of nanodevices and programmable self-assembly of chemically modified LNA/DNA nanomaterials can be followed by bright fluorescence signaling from the functionalized LNA units. Another appealing aspect of the amino-LNA scaffolds is specific targeting of nucleic acids and proteins for therapeutic applications. 2′-Amino-LNA/DNA conjugates containing peptide and polyaromatic hydrocarbon (PAH) groups are promising in this context as well as for advanced imaging and diagnostic purposes *in vivo*. For imaging applications, photostability of fluorescence dyes is of crucial importance. Chemically stable and photostable fluorescent PAH molecules attached to 2′-amino functionality of the 2′-amino-LNA are potent for *in vitro* and *in vivo* imaging of DNA and RNA targets.

We believe that rational evolution of the biopolymers of Nature may solve the major challenges of the future material science and biomedicine. However, this requires strong scientific progress and efficient interdisciplinary research. Examples of this Account demonstrate that among other synthetic biopolymers, synthetic nucleic acids containing functionalized 2′-amino-LNA scaffolds offer great opportunities for material science, diagnostics, and medicine of the future.

1. INTRODUCTION

Nucleic acids are key biopolymers in all living organisms, which provide genetic inheritance, evolution, and adaptation of species to the environment. Even small alterations in nucleic acid sequences, such as single nucleotide polymorphisms (SNPs) or deletion of one or two nucleotides, might induce genetic predispositions, frank disorders, and diverse drug responses.[1] As discovered by Watson and Crick in 1953, the principle of complementarity plays a central role in nucleic acid structure and function.[2] Presently, multiple approaches have been developed for closer insight into structure and function of natural nucleic acids and to create materials and tools inspired by and resembling natural nucleic acids. Automated chemical synthesis of short nucleic acids, that is, oligonucleotides, complementary to a certain genomic sequence, together with design and preparation of self-assembling structures and devices from nucleic acid building blocks, are among these approaches.[3,4]

Chemical modification of nucleic acids is a rapidly developing research area, which provides the possibility to create advanced tools for diagnostics,[3,5] therapy,[6] and material science.[4,7] By incorporation of synthetic analogues, the intrinsically high biorecognition potential of nucleic acids can be improved with respect to both affinity and specificity of target bindng.[5] Furthermore, stability to degradation by enzymes and cellular uptake of therapeutic and bioanalytical oligonucleotides can be

Special Issue: Nucleic Acid Nanotechnology

Received: January 10, 2014
Published: April 21, 2014

   dx.doi.org/10.1021/ar500014g | *Acc. Chem. Res.* 2014, 47, 1768−1777

increased using modified nucleic acid scaffolds.[6] Finally, additional function can be brought into nucleic acids, for example, by incorporation of sensor molecules such as fluorescent dyes,[8] spin labels,[9] and radioisotopes.[10]

Nucleic acid scaffolding can be performed by introducing modifications into the sugar or nucleobase parts of nucleotides or the phosphate backbone.[11] Multiple studies have shown that the conformation of the furanose ring plays a vital role in overall structure and in biophysical properties of the natural ribose-based nucleic acids.[12] A plethora of synthetic DNA and RNA scaffolds containing modifications in the sugar part have been prepared and investigated.[11,13] Among other modifications, locked nucleic acids (LNAs) have shown appealing properties such as improved affinity and specificity of binding to DNA/RNA targets[14] together with high enzymatic stability (Figure 1).[15,16] The reason for these properties is the bicyclic skeleton, which "locks" the furanose ring of LNA nucleotides into an RNA-mimicking N-type conformation.[17]

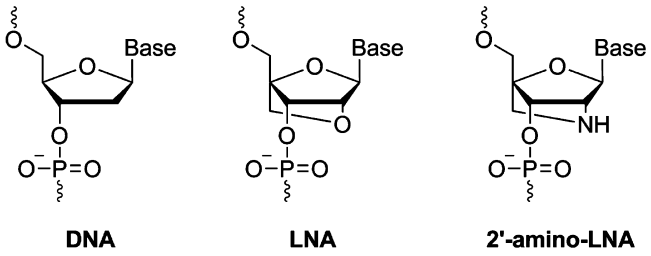


Figure 1. Chemical structures of DNA, LNA, and 2′-amino-LNA nucleotides.

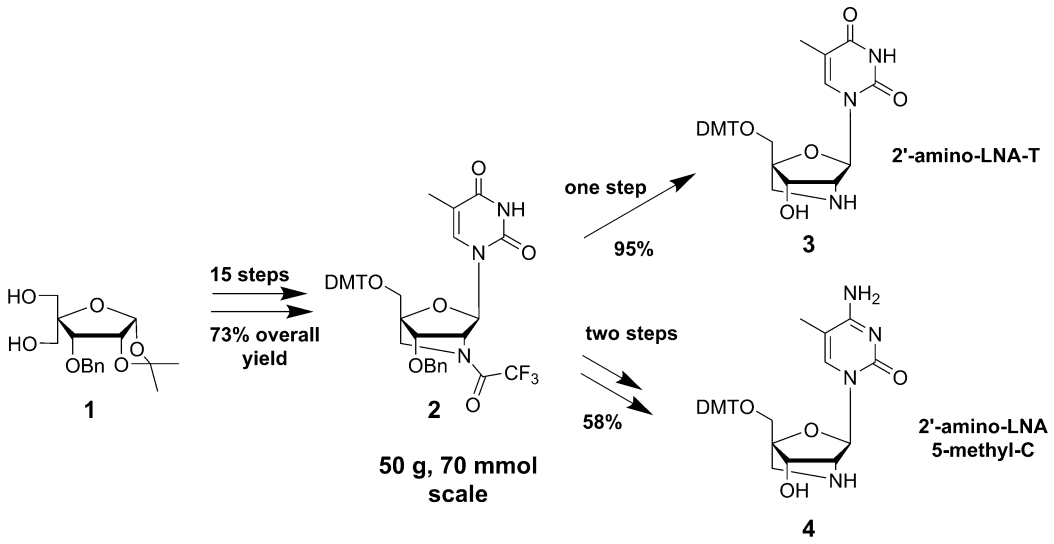
Shortly after the synthesis of LNA, 2′-amino-LNA analogues were developed in order to further incorporate functional tags into oligonucleotides.[18] As demonstrated below, the amino group of 2′-amino-LNA is a good choice for advanced modification by various chemical reactions at different stages of nucleic acids synthesis.[16,19−21]

In this Account, we present an overview of synthetic routes leading to modified 2′-amino-LNA scaffolds, as well as examples of their application in modern diagnostics, nanobiotechnology, and development of personalized therapy of human diseases.

2. SYNTHESIS OF 2′-AMINO-LNA AND ITS DERIVATIVES

A robust and straightforward synthetic route to a nucleic acid analogue and its derivatives makes the modified scaffold easily available and enables its exploration and use. Therefore, optimization of a quite long synthetic route would increase application of 2′-amino- and isomeric 2′-α-L-amino-LNA. The first synthesis of 2′-amino- and 2′-methylamino-locked nucleic acids (2′-amino-LNAs) was reported by Singh et al. in 1998.[18] The initial synthetic route included more than 20 steps resulting in an overall yield of ∼15% starting from 4-*C*-acetoxymethyl-1,2-di-*O*-acetyl furanose.[18,22] Nevertheless, thermal stability of duplexes with complementary RNA and DNA strands containing 2′-amino- and 2′-methylamino-LNA nucleotides were remarkably high (ΔT_m + 3.0 °C toward DNA and +6.6 °C toward RNA per one 2′-amino-LNA modification).[18] Therefore, despite a rather complicated synthesis, the attractive biophysical properties inspired further functionalization of 2′-amino-LNA and a broad range of studies of their derivatives as described below. Stimulated by the first exciting data (vide infra) on modified 2′-amino-LNA scaffolds, an improved synthetic route to key 2′-amino-LNA intermediates was developed by Rosenbohm et al. in 2003.[22] The authors reported that the optimal route is convergent with the synthesis of LNA monomers (Figure 1)[23] via a common intermediate obtained by a mild intramolecular cyclocondensation reaction. This new synthetic strategy included 13 steps starting from 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-*erythro*-pentofuranose with an improved overall yield of 2′-amino-LNA nucleosides up to 37%.[22] Next, in 2006, Ravn et al. developed a synthetic route via a transnucleosidation that enabled the preparation of oligonucleotides incorporating 2′-amino-LNA with all four natural bases,[24] whereas a large scale synthesis of 2′-amino-LNA thymine and 5-methylcytosine nucleosides (up to 50 g) was reported by Madsen et al. in 2012 (Scheme 1).[25] In the latter work, thymidine

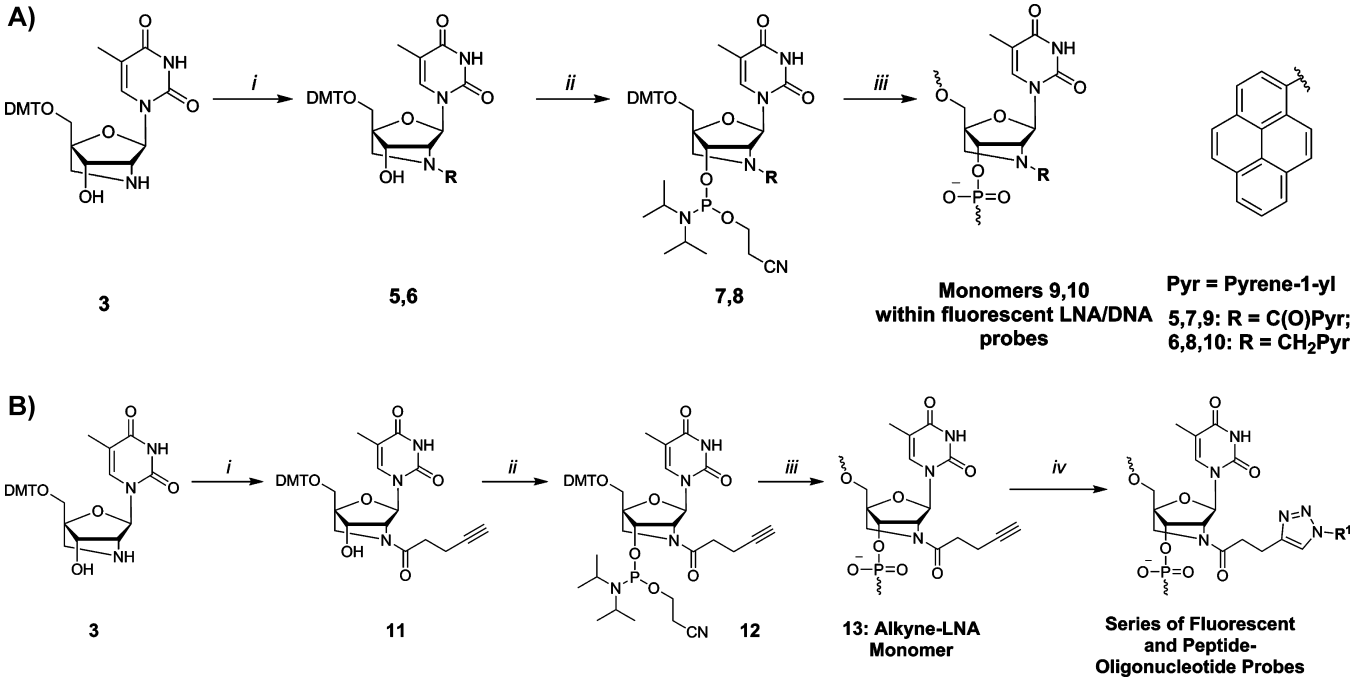
Scheme 1. General Strategy of Large-Scale Synthesis of 2′-Amino-LNA Thymine and 5-Methylcytosine Nucleosides Reported by Madsen et al.[25a]



[a]DMT = 4,4′-dimethoxytrityl.

dx.doi.org/10.1021/ar500014g | *Acc. Chem. Res.* 2014, 47, 1768−1777

Scheme 2. Representative Strategies for Functionalization of a 2′-Amino-LNA Scaffold[a]



[a]Reagents and conditions: (A) (i) (giving product **5**) (1) ethyl trifluoroacetate, DMAP, CH_3OH; (2) sat. NH_3 in MeOH, (3) pyren-1-ylcarbonyl chloride, Na_2CO_3, MeOH, 0 °C, 50% for three steps; (giving product 6) pyrene-1-carbaldehyde, AcOH, $NaCNBH_3$, MeOH, 94%; (ii) (giving products 7 and **8**) $NC(CH_2)_2OP(Cl)N(i\text{-}Pr)_2$, $(i\text{-}Pr)_2NEt$, CH_2Cl_2 (7, 76%; **8**, 57%); (ii) (giving product **12**) $NC(CH_2)_2OP(N(i\text{-}Pr)_2)_2$, diisopropylammonium tetrazolide, DCM, 73%; (iii) automated DNA synthesis, 95-99%; (B) (i) pent-4-ynoic acid, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIPEA), DMF, 71%; (iv) R^1N_3, CuAAC click chemistry, 60−89%. DMT = 4,4′-dimethoxytrityl, R^1 = peptide, fluorescent dye.[31,32].

intermediate **2** was synthesized on a multigram scale (50 g, 70 nmol) from starting sugar **1** in 15 steps and overall yield of 73%, with only five purification steps. Next, the key thymine nucleoside **3** was obtained from **2** in a single step in 96% yield, whereas the key 5-methylcytosine compound **4** was obtained from **2** in two steps in 58% yield (Scheme 1). This highly efficient large scale route allows convenient and high-yielding syntheses of thymine and 5-methylcytosine LNA derivatives, which can be further functionalized with a functional group at the N2′-position, converted into phosphoramidite reagents (in two divergent steps following standard methods), and incorporated into synthetic oligonucleotide analogues.[25]

Functionalization of 2′-amino-LNA can be performed either prior to automated oligonucleotide synthesis[26,27] or after completion of the desired oligonucleotide sequence (post-synthetically)[28,29] and by a wide variety of chemical reactions. In general, the choice of a synthetic method for scaffolding along 2′-amino-LNA mainly depends on the chemical nature of the modification, its price, its availability, its sensitivity to solid-phase synthesis or deprotection conditions, applications of the product, and amount of the product needed. Diverse N-acylated[26] and N-alkylated[26,27] derivatives of 2′-amino-LNA can be obtained by amide coupling[28] and reductive ammination[29] reactions, respectively (Scheme 2A). These methods give modified 2′-amino-LNA nucleosides such as **5** and **6** in high yields, which afterward can be converted into phosphoramidite reagents **7** and **8** for solid-phase DNA synthesis of the desired modified oligonucleotides (Scheme 2A). Incorporation of bulky 2′-amino-LNA derivatives result in lowered coupling yields for the corresponding phosphoramidites, which can be improved up to 85−92% by applying microwave conditions and hand coupling procedures.[26,27] Alternatively, 2′-amino-LNAs can be functionalized after their incorporation into oligonucleotides by a coupling reaction or by click chemistry, for example, by the copper catalyzed azide−alkyne cycloaddition (CuAAC) reaction (Scheme 2B).[30,31] In this case, a much lower amount of the tag is needed for the reaction, which is economically a more convenient approach, for example, for small scale conjugation of rather expensive peptides and fluorophores.[30−32] An efficient demonstration of a postsynthetic amide coupling approach is the stepwise attachment of diverse amino acids to the 2′-nitrogen of 2′-amino-LNA monomers.[30] In order to perform CuAAC click chemistry on 2′-amino-LNA/DNA scaffolds, 2′-*N*-alkyne monomer **13** was prepared by coupling of a protected 2′-amino-LNA nucleoside with pent-4-ynoic acid.[31] The resulting nucleoside was converted into phosphoramidite reagent **12**, which was used in solid-phase synthesis of 21mer oligonucleotides with single to triple incorporations of modified 2′-amino-LNA scaffolds. Remarkably, click chemistry of the product oligonucleotides rapidly provided a library of fluorescent and peptide-labeled LNA/DNA conjugates in 62−88% yield without the need for additional purification steps (except for rapid gel filtration).[31,32]

A novel challenging approach to synthesis of modified nucleic acid scaffolds is the use of enzymes, for example, polymerases. Recently, the triphosphate derivative of 2′-amino-LNA thymidine (2′-amino-LNA-TTP) was synthesized and found to be a good substrate for Phusion HF DNA polymerase, allowing enzymatic synthesis of modified DNA strands encoded by unmodified template strands.[33] To complement this, 2′-amino-LNA-T phosphoramidites were incorporated into oligodeoxyribonucleotides, which were used as templates for enzymatic

dx.doi.org/10.1021/ar500014g | Acc. Chem. Res. 2014, 47, 1768−1777

synthesis of unmodified DNA using different polymerases including KOD, KOD XL, or Phusion polymerase. It was reported that 2′-amino-LNA-T in the template and 2′-amino-LNA-TTP as a substrate both decreased reaction rate and yield compared with unmodified DNA, especially for sequences with multiple 2′-amino-LNA-T nucleotides.[33] We speculate that in the future the problem of low yield for incorporation of modified LNA nucleotides in such reactions can be solved using novel synthetic polymerases.[34]

3. DETECTION OF NUCLEIC ACIDS USING 2′-AMINO-LNA SCAFFOLDS

Generally, a suitable method for nucleic acid diagnostics is accurate, is adaptable for both homogeneous and solid-phase reaction formats, and provides a robust and easily interpretable signal. To create such a method, one must develop oligonucleotide probes that provide an intense signal (output) while efficiently discriminating between complementary and mismatched targets. These properties have recently been reported for fluorescent derivatives of 2′-amino-LNAs containing polyaromatic hydrocarbon (PAH) dyes.[31,35,36] Because of the expanded π-electronic structure of these dyes, fluorescence of these molecules is sensitive to even minor changes in their microenvironment by shifts of absorption or emission bands or by the appearance of additional signals resulting in the formation of excimers and exciplexes.[37] However, a relatively long excited state lifetime (>10 ns) is needed in order to monitor dynamic fluorescence effects within biopolymers. Therefore, pyrene and its derivatives display highest sensitivity of fluorescence to molecular environment and polarity.

In our research, we have shown that short fluorescent LNA/DNA probes containing PAHs such as pyrene,[38] (phenylethynyl)pyrenes,[35] perylene,[36] and coronene[39] represent a class of biosensors with advantages of high specificity and sensitivity in the context of sensing SNPs. Umemoto et al. thus applied pyrene excimer-forming short LNA/DNA probes for SNP sensing in a model system.[38] We further developed this system and applied excimer based design for genotyping of the HIV-1 protease encoding gene fragment (HIV-pol; Figure 2).[35] With novel (phenylethynyl)pyrene dyes with improved photophysical characterisctics compared with the parent pyrene (i.e., up to 20-fold higher fluorescence quantum yields and 50−120 nm longer excitation and emission wavelengths), the detection limit was below 5 nM, even in the presence of one or two polymorphic mutations located five to seven nucleotides away from the drug-resistance causing SNP. Furthermore, this system was efficiently applied for genotyping of clinical samples amplified by PCR, and the results were similar to those obtained by sequencing experiments performed as controls. Finally, FRET between pyrene and perylene was also useful for nucleotide-specific genotyping of the HIV PR gene using 2′-amino-LNA scaffolds within short LNA/DNA probes.[36] We believe that the extraordinarily high specificity of LNA/DNA probes and bright fluorescence response to a particular mismatched nucleotide when applying 2′-amino-LNA scaffolds can lead to a unique platform for development of next-generation enzyme-free diagnostics of SNPs.

The hybridization-induced modulation of fluorescence intensity is an exciting property of multiply labeled PAH−LNA/DNA probes making them useful for nucleic acid detection.[40,41] This is accompanied by high binding affinity and selectivity to both DNA and RNA targets and by efficient fluorescence sensing of single-nucleotide mismatches, also

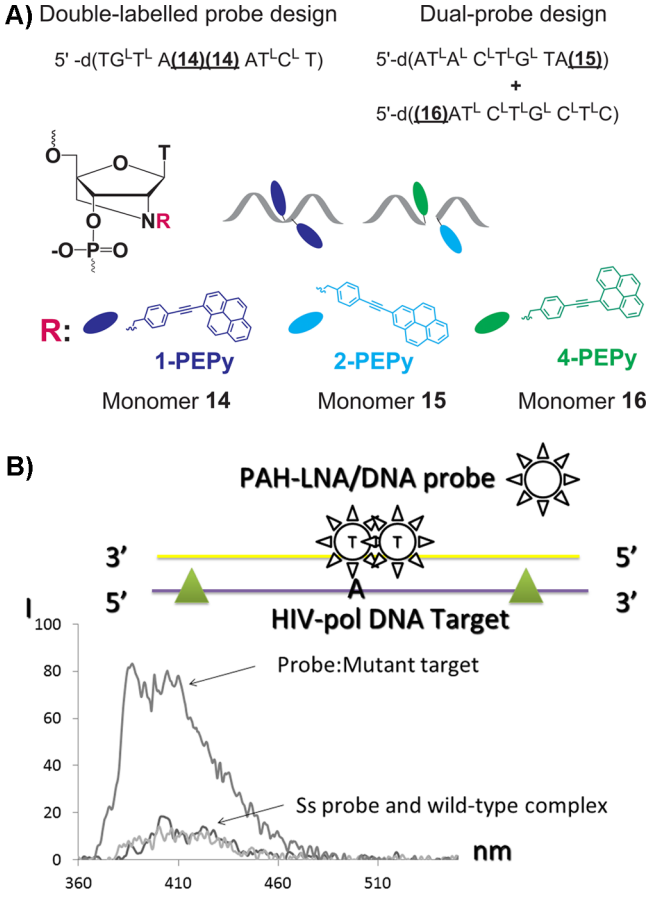


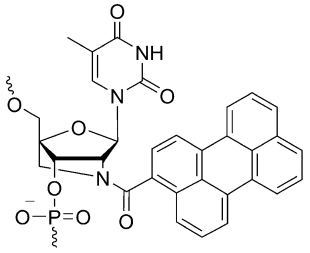
Figure 2. Example design of PAH−LNA/DNA probes for fluorimetric genotyping assays.[35] HIV-pol = HIV-1 gene fragment encoding protease. (A) Chemical structures and design rules for the PAH−LNA/DNA probes. LNAs are marked with uppercase L. (B) Fluorescence spectra of single-stranded dual-probe mix and their complexes with complementary (mutant) and mismatched (wild-type) DNA targets; λ_{ex} = 325 nm, 0.5 μM solutions in a medium salt buffer ([Na]$^+$ 110 mM, pH 7.2).

observed for derivatives of isomeric 2′-amino-α-L-LNA.[42,43] However, in case of the latter, the better results were obtained for targeting DNA rather than RNA, due to efficient intercalation of the tags attached to the 2′-amino functionality of the 2′-amino-α-L-LNA into the double-stranded DNA. In case of 2′-amino-LNA, efficient biosensing properties are achieved for both DNA and RNA targets. For example, upon duplex formation of appropriately designed 2′-*N*-(pyren-1-yl)- and 2′-*N*-(perylene-3-yl)carbonyl-2′-amino-LNA probes and complementary DNA or RNA, intensive fluorescence emission with quantum yields between 0.11 and 0.99 is obtained.[40,41] Molecular modeling studies suggest that the constrained bicyclic ribose (dioxabicyclo[2.2.1]heptane) skeleton and the amide linkage of PAH-modified 2′-amino-LNA monomers fix the orientation of the dye in the minor groove of a nucleic acid duplex. Interactions between the dye and nucleobases or media, which typically lead to quenching of fluorescence, are thereby reduced or even prevented. Duplexes between multiply modified probes and DNA or RNA complements exhibit additive increases in fluorescence intensity, while the fluorescence of single stranded probes becomes increasingly quenched. Thus, up to a 69-fold increase in fluorescence intensity (measured at λ_{em} 383 nm) was observed for pyrene-2′-amino-LNA monomer **9** upon hybridization to DNA/RNA. The emission from duplexes of multiply

dx.doi.org/10.1021/ar500014g | *Acc. Chem. Res.* 2014, 47, 1768−1777

modified probes containing the bright perylene dye (monomer **17**, Chart 1) and DNA or RNA complements at concentrations

Chart 1. Chemical Structure of Monomer 17



2'-*N*-(perylene-3-yl)carbonyl-2'-amino-LNA

down to less than 500 nM can easily be seen by the naked eye using standard illumination intensities, although the fluorescence increase for perylene upon hybridization is less than that for pyrene (~8-fold vs ~69-fold, respectively).[40,41] Less efficient quenching of fluorescence by nucleobases for perylene compared with pyrene is caused by shorter excited state lifetime of the former (~2−3 ns vs >10 ns, respectively).[37] Monomer **17** furthermore provides excellent binding affinity and mismatch discrimination of the probes when binding to complementary DNA or RNA targets. Finally, using perylene−LNA/DNA probes, we were able to detect the target mRNA in cell culture with advantages of high specificity and an excitation wavelength of perylene that completely eliminated cell autofluorescence (λ_{ex} 425 nm).[41]

4. 2′-AMINO-LNA SCAFFOLDS IN DRUG DISCOVERY

Synthetic oligonucleotide analogues have enormous therapeutic potential.[44] To date, modulation of gene expression has mainly been performed by three main approaches: (1) antisense oligonucleotides, which by complementarity to a given mRNA can inhibit translation through binding the target mRNA, (2) small interfering RNAs (siRNAs), which are double-stranded RNA molecules of which one strand bind RNA molecules thereby modulating gene expression, and (3) triplex-forming oligonucleotides (TFOs), which can bind specific genomic sequences of double-stranded DNA and thereby interfere with transcription.

Unlike LNA, 2′-amino-LNA scaffolds have not been extensively studied in antisense and siRNA technologies so far,[45−51] but 2′-amino-LNAs were found to be useful in TFOs[52] and as a constituent of nucleic acid aptamers (*vide infra*).[53] Interestingly, nonmodified 2′-amino-LNA monomers proved to be less stabilizing to triplexes than LNA monomers when incorporated into a triplex-forming third strand.[52] However, N2′-functionalization of 2′-amino-LNA monomers with a glycyl unit induced the formation of exceptionally stable triplexes (ΔT_m up to +14.0 °C compared with nonmodified TFOs), although without any data on the mismatch discrimination ability of these TFOs.

Developing aptamers toward a specific target molecule is another appealing approach for therapeutic nucleic acids. Hernandez et al. reported significantly improved affinity against the avidin protein upon incorporation of LNA and 2′-amino-LNA monomers into an avidin-binding DNA aptamer.[53] In this work, the kinetic profile of a selected modified aptamer was obtained by surface plasmon resonance experiments and compared with the profile of the parent unmodified DNA aptamer. This report established 2′-amino LNAs as novel monomers in aptamers which in addition to direct therapeutic action may find applications as a carrier unit, for example, for small-molecule drug entities.[53] Moreover, "clickable" 2′-amino-LNA scaffolds efficiently bind and sense nucleic acids and antibodies against double-stranded DNA (dsDNA-mAb32 and dsDNA-mAb33),[31] while simultaneously improving stability of the probes in nondiluted human serum.[32] We believe that our method of using affinity-enhancing 2′-amino-LNA will contribute to further development of advanced enzymatically stable peptide−oligonucleotide conjugates as useful tools addressing diverse biological and biomedical goals *in vitro* and *in vivo*.

5. APPLICATIONS OF 2′-AMINO-LNA SCAFFOLDS IN NUCLEIC ACID NANOTECHNOLOGY

DNA and RNA nanotechnology is an exciting research field, which focuses on design, synthesis, and applications of novel functional devices and materials with nanometer precision. Successful examples of nanometer-scale DNA and RNA engineering include origami,[54−56] DNA walkers,[57−59] and large arrays for immobilization of proteins.[60] However, as outlined by Wengel in 2003, modified nucleic acid scaffolds such as LNA and 2′-amino-LNA allow one to increase resolution of the nucleic acid design down to ångström-scale.[61] Thus, current efforts within nucleic acid ångström-scale engineering focus on generating rigid and stable low nanometer-sized structures carrying functionalities with predictable spatial positioning. By encoded self-assembly of complementary nucleic acid strands, this allows building functional nucleic acid architectures aimed at applications within the biological and material sciences.[62]

The first examples of incorporating LNA into nano-objects include several model interstrand communication systems, which were called "zipper" complexes.[27] The main principle of the "zipper" approach is synthesis of complementary DNA strands containing modifications in certain positions with respect to each other upon forming the double-stranded complex (Figure 2A). In doing this, the aforementioned polyaromatic hydrocarbon (PAH) dyes were found to be very potent. In addition to stabilization of duplexes by $\pi-\pi$ interactions between PAHs, PAH−LNA scaffolds provided a hybridization-induced bright fluorescence signal with characteristic wavelengths of excitation and emission, for example, ~340/490 nm, ~345/515 nm, and ~325/500 nm for the pyrene,[27] (phenylethynyl)-pyrene[63,64] excimer, and pyrene−perylene Förster resonance energy transfer (FRET) systems,[65] respectively. Typically, attachment to nucleic acids results in the quenching of fluorescence of a dye in both single strands and duplexes due to the presence of multiple quenchers of emission. Such an effect was previously observed for pyrene, perylene, xanthene, and cyanine dyes attached to various nucleic acid scaffolds.[37] However, as mentioned above, attachment to a rigid LNA-type skeleton prevents structural fluctuations of the dye within a double stranded complex and thus reduces quenching of fluorescence. Owing to increased binding affinity and selectivity of oligonucleotides, isomeric 2′-amino-α-L-LNAs are also promising nucleotide analogues for scaffolding along double-stranded DNA nanostructures.[42,43] Resulting quantum yields and brightness values of the PAH−LNA/DNA scaffolds are very high compared with other nucleic acid analogues (like 2′-*O*-(1-pyrenylmethyl)uridine,[66] and "clickable" derivatives of 2′-propargylated uridine containing phenoxazinium,[67] coumarin,[67] styryl,[68] and commercially available xanthene and cyanine

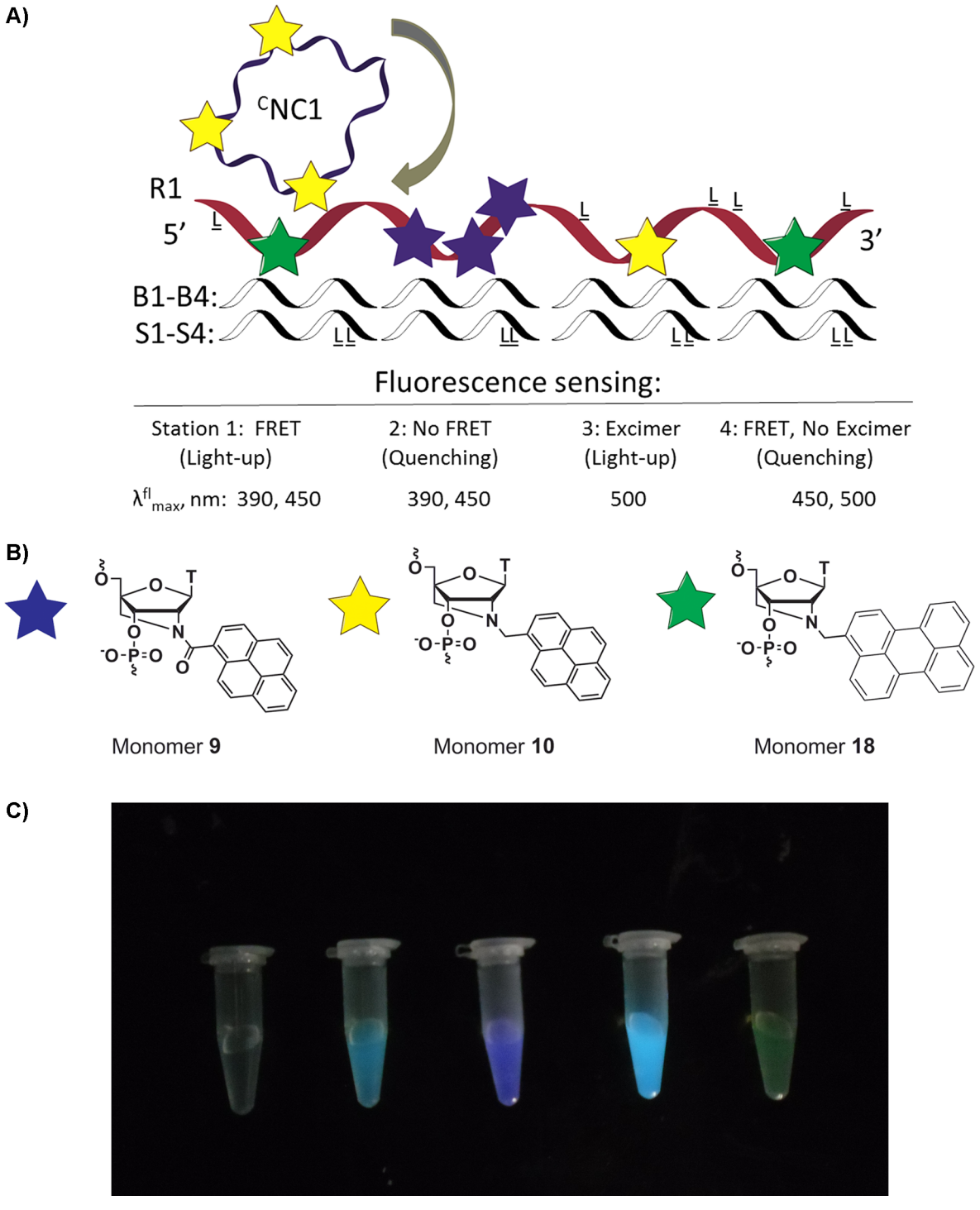


Figure 3. (A) Design of the labeled nanocrawler and complementary road, LNA/DNA brake and speed strands (^{C}**NC1**, **R1**, **B1**−**B4**, and **S1**−**S4**), and expected fluorescence sensing of binding ^{C}**NC**1 to **R1**.[77] L = LNA monomers. (B) Chemical structures of modified monomers. (C) Demonstration of following nanocrawler by color change (left to right): medium salt buffer (blank solution, 1), stations 1−4 (2−5). The picture was recorded in a medium salt phosphate buffer using 2.0 μM concentration of each strand and excitation wavelength of 365 nm (regular laboratory UV lamp). Reproduced from ref 77. Copyright 2013 American Chemical Society.

dyes[69]). Simultaneously, binding affinity and selectivity by the PAH−LNA containing probes are superior to higher compared with, for example, carbamates[70] or readily available C2′- and C5-modifications[68−72] As mentioned above, fluorescence of PAH−LNA units is also strongly affected in the presence of a single-nucleotide mismatch. Such a combination of biosensing and biophysical properties was not previously reported, for example, for base-modified[71−73] and intercalating non-nucleosidic scaffolds.[74−76]

Recently, we applied bright PAH−LNA/DNA scaffolds in construction of a novel fluorescent LNA/DNA machine called a nanocrawler, which reversibly moves along a directionally polar complementary road controlled by affinity-enhancing locked nucleic acid (LNA) monomers **9**, **10**, and **18** and by additional

dx.doi.org/10.1021/ar500014g | *Acc. Chem. Res.* 2014, 47, 1768−1777

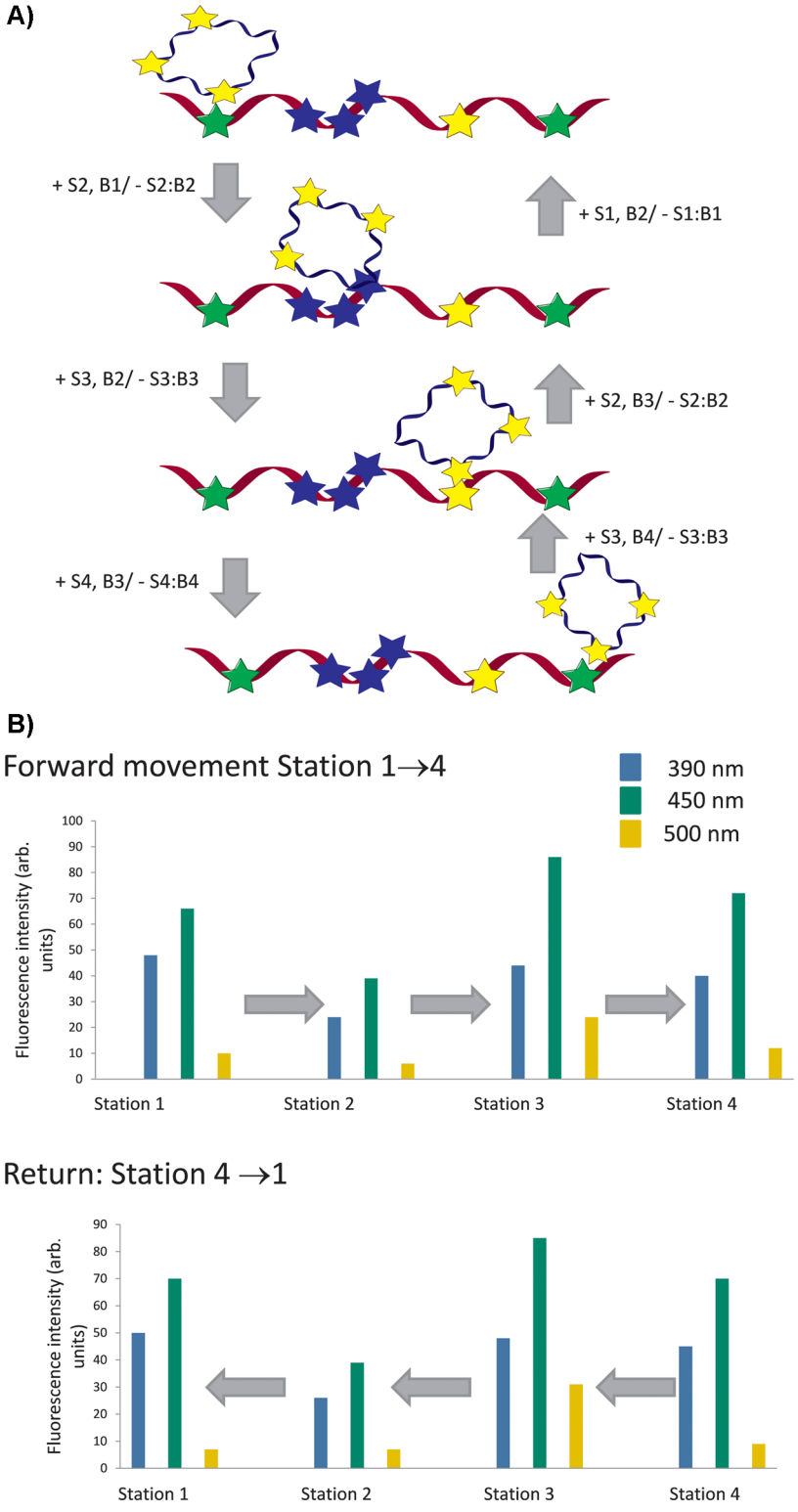


Figure 4. (A) Annealing scheme for the reversible movement of the nanocrawler (ᶜ**NC1**) along the road **R1**. (B) Fluorescent fingerprint of the movement of the nanocrawler from station 1 to station 4 at 390, 450, and 500 nm (blue, green, and yellow bars, respectively). Reproduced from ref 77. Copyright 2013 American Chemical Society.

regulatory strands (Scheme 2; Figures 3 and 4).[77] PAH dyes attached to 2′-amino-LNA monomers were incorporated at four stations of the system, enabling simple detection of the position of the nanocrawler via a step-specific color signal (Figure 3A). The sensing was provided by highly sensitive, chemically stable, and photostable PAH−LNA interstrand communication systems, including pyrene excimer formation and pyrene−perylene interstrand FRET (Figures 3 and 4). The nanocrawler selectively and reversibly moved along the complementary road, followed by a bright and consistent fluorescence fingerprint for up to 10 cycles without any loss of signal.

dx.doi.org/10.1021/ar500014g | *Acc. Chem. Res.* 2014, 47, 1768−1777

As a last aspect, the possibility to reversibly cross-link nucleic acid strands by a simple procedure is very desirable for nucleic acid nanotechnology. Using a 2′-amino-LNA scaffold, Pasternak et al. developed an efficient anthracene-functionalized cross-linking system.[78] Two DNA strands with the novel 2′-*N*-anthracenylmethyl-2′-amino-LNA monomers could be effectively cross-linked by photoligation at 366 nm in various types of DNA structures. Moreover, successful application of three differently functionalized 2′-amino-LNA monomers in self-assembled higher ordered structures for simultaneous cross-linking and monitoring of assembly formation was demonstrated.

6. CONCLUSIONS AND OUTLOOK

Chemical modification of nucleic acids is beginning to make a significant impact on development of novel functional tools for molecular diagnostics, therapy, and material science. High chemical and enzymatic stability, selectivity of target binding, and robust synthetic approaches are among the many challenges affiliated with use of nucleic acid analogues in living systems, biotechnology, and engineering. Incorporated into oligonucleotides, locked nucleic acids (LNAs) allow construction of efficient tools addressing these challenges. The additional advantage of 2′-amino-LNA scaffolds is a freedom to choose a molecular moiety for incorporation into nucleic acids while maintaining the potent biophysical properties of LNA. To realize such constructs, diverse chemical approaches can be used, including but not limited to amide coupling, reductive amination, and click chemistry. With regard to efficient bioconjugation methods, copper catalyzed azide−alkyne cycloaddition (CuAAC) has proven efficient for scaffolding along 2′-amino-LNA. The additional advantages of CuAAC click chemistry include high yields, simple purification techniques, and the possibility of performing modification in aqueous solution *in vitro* and even *in vivo*.[79] Recent advances in nucleic acid nanotechnology have enabled researchers to build large DNA and RNA structures and devices but with limited examples of utilizing modified nucleic acid scaffolds.[54−59] We believe that 2′-amino-LNAs and their diverse derivatives will bring additional functional dimensions into the field of nucleic acid nano-objects and nanodevices and will allow direct spectroscopic imaging of their actions and effects *in vitro* and *in vivo*.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ias@sdu.dk.

Funding

The authors acknowledge financial support from The Sapere Aude programme of The Danish Council for Independent Research, THE VILLUM FOUNDATION, and The European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC Grant Agreement No. 268776.

Notes

The authors declare no competing financial interest.

Biographies

Irina Kira Astakhova was born in Dubno, Ukraine, in 1985. She obtained her M.S. degree in chemistry from the Mendeleev Institute of Chemical Technology in Moscow, Russia, in 2006. In 2009, she completed her Ph.D. in bioorganic chemistry at Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry in Moscow, Russia, with Professor Vladimir Korshun. She then worked with Professor Jesper Wengel at Nucleic Acid Center, University of Southern Denmark, from 2009 through 2012, as a Postdoctoral Fellow. Since 2012, she joined the Nucleic Acid Center at the University of Southern Denmark as an Associate Professor.

Jesper Wengel was born in Odense, Denmark, in 1963. He obtained his Ph.D in nucleoside chemistry from Odense University in 1991 and became full professor in organic chemistry at University of Copenhagen in 1996. Since 2000, he has been full professor of bioorganic chemistry at University of Southern Denmark where he has been director of the Nucleic Acid Center since 2001, director of the Biomolecular Nanoscale Engineering Center since 2012, and an ERC advanced grant recipient since 2011. He is the coinventor of LNA (locked nucleic acid) and UNA (unlocked nucleic acid) technologies, and he is cofounder of RiboTask, a biotech company focused on developing and marketing novel RNA technologies for gene silencing. He has published more than 330 research papers in international journals.

REFERENCES

(1) Griffiths, A. J. F.; Wessler, S. R.; Carroll, S. B.; Doebley, J. *Introduction to Genetic Analysis*, 10th ed.; W.H. Freeman and Company: New York, 2012.
(2) Watson, J. D.; Crick, F. H. C. A Structure for Deoxyribose Nucleic Acid. *Nature* **1953**, *171* (4356), 737−738.
(3) Kirk, B. W.; Feinsod, M.; Favis, R.; Kliman, R. M.; Barany, F. Single Nucleotide Polymorphism Seeking Long Term Association with Complex Disease. *Nucleic Acid Res.* **2002**, *30* (15), 3295−3311.
(4) Douglas, S. M.; Dietz, H.; Liedl, T.; Högberg, B.; Graf, F.; Shih, W. M. Self-Assembly of DNA into Nanoscale Three-Dimensional Shapes. *Nature* **2009**, *459* (7245), 414−418.
(5) Thomas, D. C.; Nardone, G. A.; Randall, S. K. Amplification of Padlock Probes for DNA Diagnostics by Cascade Rolling Circle Amplification or the Polymerase Chain Reaction. *Arch. Pathol. Lab. Med.* **1999**, *123* (12), 1170−1176.
(6) Waters, J. S.; Webb, A.; Cunningham, D.; Clarke, P. A.; Raynaud, F.; di Stefano, F.; Cotter, F. E.; Phase, I. Clinical and Pharmacokinetic Study of Bcl-2 Antisense Oligonucleotide Therapy in Patients with Non-Hodgkin's Lymphoma. *J. Clin. Oncol.* **2000**, *18* (9), 1812−1823.
(7) Mohammed, A. M.; Schulman, R. Directing Self-Assembly of DNA Nanotubes Using Programmable Seeds. *Nano Lett.* **2013**, *13* (9), 4006−4013.
(8) Randolph, J. B.; Waggoner, A. S. Stability, Specificity and Fluorescence Brightness of Multiply-Labeled Fluorescent DNA Probes. *Nucleic Acid Res.* **1997**, *25* (14), 2923−2929.
(9) Azarkh, M.; Singh, V.; Okle, O.; Seemann, I. T.; Dietrich, D. R.; Hartig, J. S.; Drescher, M. Site-Directed Spin-Labeling of Nucleotides and the Use of in-Cell EPR to Determine Long-Range Distances in a Biologically Relevant Environment. *Nat. Protoc.* **2013**, *8* (1), 131−147.
(10) Zhao, X.; Yu, Y.-T. Detection and Quantitation of RNA Base Modifications. *RNA* **2004**, *10* (6), 996−1002.
(11) Blackburn, G. M.; Gait, M. J.; Loakes, D.; Williams, D. M. *Nucleic Acids in Chemistry and Biology*, 3rd ed.; RCS Publishing: Cambridge, UK., 2006.
(12) Meints, G. A.; Karlsson, T.; Drobny, G. P. Modeling Furanose Ring Dynamics in DNA. *J. Am. Chem. Soc.* **2001**, *123* (41), 10030−100038.
(13) Plashkevych, O.; Chatterjee, S.; Honcharenko, D.; Pathmasiri, W.; Chattopadhyaya, J. Chemical and Structural Implications of 1′,2′- versus 2′,4′-Conformational Constraints in the Sugar Moiety of Modified Thymine Nucleosides. *J. Org. Chem.* **2007**, *72* (13), 4716−4726.
(14) Latorra, D.; Campbell, K.; Wolter, A.; Hurley, J. M. Enhanced Allele-Specific PCR Discrimination in SNP Genotyping Using 3′ Locked Nucleic Acid (LNA) Primers. *Hum. Mutat.* **2003**, *22* (1), 79−85.
(15) Schmidt, K. S.; Borkowski, S.; Kurreck, J.; Stephens, A. W.; Bald, R.; Hecht, M.; Friebe, M.; Dinkelborg, L.; Erdmann, V. A. Application of

Locked Nucleic Acids to Improve Aptamer *in Vivo* Stability and Targeting Function. *Nucleic Acid Res.* **2004**, *32* (19), 5757−5765.
(16) Kaur, H.; Babu, R. B.; Maiti, S. Perspectives on Chemistry and Therapeutic Applications of Locked Nucleic Acid. *Chem. Rev.* **2007**, *107*, 4672−4697.
(17) Nielsen, K. E.; Singh, S. K.; Wengel, J.; Jacobsen, J. P. Solution Structure of an LNA Hybridized to DNA: NMR Study of the d(CT^{L}GCT^{L}T^{L}CT^{L}GC):d(GCAGAAGCAG) Duplex Containing Four Locked Nucleotides. *Bioconjugate Chem.* **2000**, *11* (2), 228−238.
(18) Singh, S. K.; Kumar, R.; Wengel, J. Synthesis of 2′-Amino-LNA: A Novel Conformationally Restricted High-Affinity Oligonucleotide Analog with a Handle. *J. Org. Chem.* **1998**, *63* (26), 10035−10039.
(19) Lundin, K. E.; Højland, T.; Hansen, B. R.; Persson, R.; Bramsen, J. B.; Kjems, J.; Koch, T.; Wengel, J.; Smith, C. I. Biological Activity and Biotechnological Aspects of Locked Nucleic Acids. *Adv. Genet.* **2013**, *82*, 47−107.
(20) Obika, S.; Rahman, S. M. A.; Fujisaka, A.; Kawada, Y.; Baba, T.; Imanishi, T. Bridged Nucleic Acids: Development, Synthesis, and Properties. *Heterocycles* **2010**, *81*, 1347−1393.
(21) Zhou, C.; Chattopadhyaya, J. The Synthesis of Therapeutic Locked Nucleos(t)ides. *Curr. Opin. Drug Discovery Dev.* **2009**, *12* (6), 876−898.
(22) Rosenbohm, C.; Christensen, S. M.; Sørensen, M. D.; Pedersen, D. Sejer; Larsen, L.-E.; Wengel, J.; Koch, T. Synthesis of 2′-Amino-LNA: A New Strategy. *Org. Biomol. Chem.* **2003**, *1* (4), 655−663.
(23) Singh, S. K.; Koshkin, A. A.; Wengel, J.; Nielsen, P. LNA (Locked Nucleic Acids): Synthesis and High-Affinity Nucleic Acid Recognition. *Chem. Commun.* **1998**, *4*, 455−456.
(24) Ravn, J.; Rosenbohm, C.; Christensen, S. M.; Koch, T. Synthesis of 2′-Amino-LNA Purine Nucleosides. *Nucleosides Nucleotides Nucleic Acids* **2006**, *25* (8), 843−847.
(25) Madsen, A. S.; Jørgensen, A. S.; Jensen, T. B.; Wengel, J. Large Scale Synthesis of 2′-Amino-LNA Thymine and 5-Methylcytosine Nucleosides. *J. Org. Chem.* **2012**, *77* (23), 10718−10728.
(26) Sørensen, M. D.; Petersen, M.; Wengel, J. Functionalized LNA (Locked Nucleic Acid): High-Affinity Hybridization of Oligonucleotides Containing *N*-Acylated and *N*-Alkylated 2′-Amino-LNA Monomers. *Chem. Commun.* **2003**, *17*, 2130−2131.
(27) Hrdlicka, P. J.; Babu, B. R.; Sørensen, M. D.; Wengel, J. Interstrand Communication between 2′-*N*-(Pyren-1-yl)methyl-2′-amino-LNA Monomers in Nucleic Acid Duplexes: Directional Control and Signalling of Full Complementarity. *Chem. Commun.* **2004**, *13*, 1478−1479.
(28) Valeur, E.; Bradley, M. Amide Bond Formation: Beyond the Myth of Coupling Reagents. *Chem. Soc. Rev.* **2009**, *38* (2), 606−631.
(29) Baxter, E. W.; Reitz, A. B. Reductive Aminations of Carbonyl Compounds with Borohydride and Borane Reducing Agents. *Org. React.* **2002**, *59*, 1−714.
(30) Johannsen, M. W.; Crispino, L.; Wamberg, M. C.; Kalra, N.; Wengel, J. Amino Acids Attached to 2′-Amino-LNA: Synthesis and Excellent Duplex Stability. *Org. Biomol. Chem.* **2011**, *9* (1), 243−252.
(31) Jørgensen, A. S.; Gupta, P.; Wengel, J.; Astakhova, I. K. "Clickable" LNA/DNA Probes for Fluorescence Sensing of Nucleic Acids and Autoimmune Antibodies. *Chem. Commun.* **2013**, *49* (91), 10751−10753.
(32) Astakhova, I. K.; Hansen, L. H.; Vester, B.; Wengel, J. Peptide-LNA Oligonucleotide Conjugates. *Org. Biomol. Chem.* **2013**, *11* (25), 4240−4249.
(33) Johannsen, M. W.; Veedu, R. N.; Madsen, A. S.; Wengel, J. Enzymatic Polymerisation Involving 2′-Amino-LNA Nucleotides. *Bioorg. Med. Chem. Lett.* **2012**, *22* (10), 3522−3526.
(34) Pinheiro, V. B.; Taylor, A. I.; Cozens, C.; Abramov, M.; Renders, M.; Zhang, S.; Chaput, J. C.; Wengel, J.; Peak-Chew, S. Y.; McLaughlin, S. H.; Herdewijn, P.; Holliger, P. Synthetic Genetic Polymers Capable of Heredity and Evolution. *Science* **2012**, *336* (6079), 341−344.
(35) Astakhova, I. K.; Samokhina, E.; Babu, B. R.; Wengel, J. Novel (Phenylethynyl)pyrene−LNA Constructs for Fluorescence SNP Sensing in Polymorphic Nucleic Acid Targets. *ChemBioChem* **2012**, *13* (10), 1509−1519.
(36) Kumar, T. S.; Myznikova, A.; Samokhina, E.; Astakhova, I. K. Rapid Genotyping Using Pyrene-Perylene Locked Nucleic Acid Complexes. *Artif. DNA: PNA XNA* **2013**, *4* (2), 58−68.
(37) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: Singapore, 2006.
(38) Umemoto, T.; Hrdlicka, P. J.; Babu, B. R.; Wengel, J. Sensitive SNP Dual-Probe Assays Based on Pyrene-Functionalized 2′-Amino-LNA: Lessons to Be Learned. *ChemBioChem* **2007**, *8* (18), 2240−2248.
(39) Gupta, P.; Langkjaer, N.; Wengel, J. Synthesis and Biophysical Studies of Coronene Functionalized 2′-Amino-LNA: A Novel Class of Fluorescent Nucleic Acids. *Bioconjugate Chem.* **2010**, *21* (3), 513−520.
(40) Hrdlicka, P. J.; Babu, B. R.; Sorensen, M. D.; Harrit, N.; Wengel, J. Multilabeled Pyrene-Functionalized 2′-Amino-LNA Probes for Nucleic Acid Detection in Homogeneous Fluorescence Assays. *J. Am. Chem. Soc.* **2005**, *127* (38), 13293−13299.
(41) Astakhova, I. V.; Korshun, V. A.; Jahn, K.; Kjems, J.; Wengel, J. Perylene Attached to 2′-Amino-LNA: Synthesis, Incorporation into Oligonucleotides, and Remarkable Fluorescence Properties in Vitro and in Cell Culture. *Bioconjugate Chem.* **2008**, *19* (10), 1995−2007.
(42) Andersen, N. K.; Anderson, B. A.; Wengel, J.; Hrdlicka, P. Synthesis and Characterization of Oligodeoxyribonucleotides Modified with 2′-Amino-α-L-LNA Adenine Monomers: High-Affinity Targeting of Single-Stranded DNA. *J. Org. Chem.* **2013**, *78*, 12690−12702.
(43) Kumar, T. S.; Wengel, J.; Hrdlicka, P. 2′-N-(Pyren-1-yl)acetyl-2′-Amino-α-L-LNA: Synthesis and Detection of Single Nucleotide Mismatches in DNA and RNA Targets. *ChemBioChem* **2007**, *8*, 1122−1125.
(44) Goodchild, J. Therapeutic Oligonucleotides. *Methods Mol. Biol.* **2011**, *764*, 1−15.
(45) Fluiter, K.; Frieden, M.; Vreijling, J.; Rosenbohm, C.; De Wissel, M. B.; Christensen, S. M.; Koch, T.; Ørum, H.; Baas, F. On the in Vitro and in Vivo Properties of Four Locked Nucleic Acid Nucleotides Incorporated into an Anti-H-Ras Antisense Oligonucleotide. *ChemBioChem* **2005**, *6* (6), 1104−1109.
(46) Fluiter, K.; Frieden, M.; Vreijling, J.; Rosenbohm, C.; De Wissel, M. B.; Christensen, S. M.; Koch, T.; Orum, H.; Baas, F. On the in Vitro and in Vivo Properties of Four Locked Nucleic Acid Nucleotides Incorporated into an Anti-H-Ras Antisense Oligonucleotide. *ChemBioChem* **2005**, *6* (6), 1104−1109.
(47) Kjaerulff, L. S.; Asklund, M.; Westergaard, M.; Rosenbohm, C.; Wissenbach, M.; Hansen, B. Survivin nucleic acid-targeting antisense LNA oligonucleotides for treatment of cancer. PCT Int. Appl. WO 2006050732 A2, 2006.
(48) McCullagh, K.; Straarup, E. M.; Nielsen, N. F. Antisense oligonucleotides for modulation of mammalian PCSK9 and treatment of disease. PCT Int. Appl. WO 2009027527 A2, 2009.
(49) Dutkiewicz, M.; Grunert, H.-P.; Zeichhardt, H.; Wengel, S. L.; Wengel, J.; Kurreck, J. Design of LNA-Modified siRNAs against the Highly Structured 5′ UTR of Coxsackievirus B3. *FEBS Lett.* **2008**, *582* (20), 3061−3066.
(50) Brunet, E.; Alberti, P.; Perrouault, L.; Babu, R.; Wengel, J.; Giovannangeli, C. Exploring Cellular Activity of Locked Nucleic Acid-Modified Triplex-Forming Oligonucleotides and Defining Its Molecular Basis. *J. Biol. Chem.* **2005**, *280*, 20076−20085.
(51) Obika, S.; Hari, Y.; Sugimoto, T.; Sekiguchi, M.; Imanishi, T. Triplex-Forming Enhancement with High Sequence Selectivity by Single 2′-O,4′-C-Methylene Bridged Nucleic Acid (2′,4′-BNA) Modification. *Tetrahedron Lett.* **2000**, *41* (46), 8923−8927.
(52) Højland, T.; Kumar, S.; Babu, B. R.; Umemoto, T.; Albaek, N.; Sharma, P. K.; Nielsen, P.; Wengel, J. LNA (Locked Nucleic Acid) And Analogs As Triplex-Forming Oligonucleotides. *Org. Biomol. Chem.* **2007**, *5* (15), 2375−2379.
(53) Hernandez, F. J.; Kalra, N.; Wengel, J.; Vester, B. Aptamers as a Model for Functional Evaluation of LNA and 2′-Amino LNA. *Bioorg. Med. Chem. Lett.* **2009**, *19* (23), 6585−6587.
(54) Rothemund, P. W. K. Folding DNA to Create Nanoscale Shapes and Patterns. *Nature* **2006**, *440*, 297−302.

(55) Endo, M.; Yamamoto, S.; Tatsumi, K.; Emura, T.; Hidakac, K.; Sugiyama, H. RNA-Templated DNA Origami Structures. *Chem. Commun.* **2013**, *49*, 2879−2881.
(56) Andersen, E. S.; Dong, M.; Nielsen, M. M.; Jahn, K.; Subramani, R.; Mamdouh, W.; Golas, M. M.; Sander, B.; Stark, H.; Oliveira, C. L. P.; Pedersen, J. S.; Birkedal, V.; Besenbacher, F.; Gothelf, K. V.; Kjems, J. Self-Assembly of a Nanoscale DNA Box with a Controllable Lid. *Nature* **2008**, *459*, 73−76.
(57) Lund, K.; Manzo, A. J.; Dabby, N.; Michelotti, N.; Johnson-Buck, A.; Nangreave, J.; Taylor, S.; Pei, R.; Stojanovic, M. N.; Walter, N. G.; Winfree, E.; Yan, H. Molecular Robots Guided by Prescriptive Landscapes. *Nature* **2010**, *465* (7295), 206−210.
(58) Gu, H.; Chao, J.; Xiao, S. J.; Seeman, N. C. A Proximity-Based Programmable DNA Nanoscale Assembly Line. *Nature* **2010**, *465* (7295), 202−205.
(59) Yin, P.; Yan, H.; Daniell, X. G.; Turberfield, A. J.; Reif, J. H. A Unidirectional DNA Walker That Moves Autonomously along a Track. *Angew. Chem., Int. Ed.* **2004**, *43* (37), 4906−4911.
(60) Numajiri, K.; Kimura, M.; Kuzuya, A.; Komiyama, M. Stepwise and Reversible Nanopatterning of Proteins on a DNA Origami Scaffold. *Chem. Commun.* **2010**, *46*, 5127−5129.
(61) Wengel, J. Nucleic Acid Nanotechnology—towards Ångström-Scale Engineering. *Org. Biomol. Chem.* **2004**, *2* (3), 277−280.
(62) Malinovskii, V. L.; Wenger, D.; Häner, R. Nucleic Acid-Guided Assembly of Aromatic Chromophores. *Chem. Soc. Rev.* **2010**, *39* (2), 410−422.
(63) Astakhova, I. V.; Korshun, V. A.; Wengel, J. Highly Fluorescent Conjugated Pyrenes in Nucleic Acid Probes: (Phenylethynyl)-pyrenecarbonyl-Functionalized Locked Nucleic Acids. *Chem.—Eur. J.* **2008**, *14* (35), 11010−11026.
(64) Astakhova, I. V.; Lindegaard, D.; Korshun, V. A.; Wengel, J. Novel Interstrand Communication Systems within DNA Duplexes Based on 1-, 2- and 4-(Phenylethynyl)pyrenes Attached to 2′-Amino-LNA: High-Affinity Hybridization and Fluorescence Sensing. *Chem. Commun.* **2010**, *46* (44), 8362−8364.
(65) Lindegaard, D.; Madsen, A. S.; Astakhova, I. V.; Malakhov, A. D.; Babu, B. R.; Korshun, V. A.; Wengel, J. Pyrene-Perylene as a FRET Pair Coupled to the N2′-Functionality of 2′-Amino-LNA. *Bioorg. Med. Chem.* **2008**, *16* (1), 94−99.
(66) Nakamura, M.; Fukunaga, Y.; Sasa, K.; Ohtoshi, Y.; Kanaori, K.; Hayashi, H.; Nakano, H.; Yamana, K. Pyrene Is Highly Emissive When Attached to the RNA Duplex but Not to the DNA Duplex: The Structural Basis of This Difference. *Nucleic Acids Res.* **2005**, *33* (18), 5887−5895.
(67) Berndl, S.; Herzig, N.; Kele, P.; Lachmann, D.; Li, X.; Wolfbeis, O. S.; Wagenknecht, H.-A. Comparison of a Nucleosidic Vs Non-Nucleosidic Postsynthetic "Click" Modification of DNA with Base-Labile Fluorescent Probes. *Bioconjugate Chem.* **2009**, *20* (3), 558−564.
(68) Rubner, M. M.; Holzhauser, C.; Bohlnder, P. R.; Wagenknecht, H.-A. A "Clickable" Styryl Dye for Fluorescent DNA Labeling by Excitonic and Energy Transfer Interactions. *Chem.—Eur. J.* **2012**, *18* (5), 1299−1302.
(69) Astakhova, I. K.; Wengel, J. Interfacing Click Chemistry with Automated Oligonucleotide Synthesis for the Preparation of Fluorescent DNA Probes Containing Internal Xanthene and Cyanine Dyes. *Chem.—Eur. J.* **2013**, *19* (3), 1112−1122.
(70) Dioubankova, N. N.; Malakhov, A. D.; Stetsenko, D. A.; Gait, M. J.; Volynsky, P. E.; Efremov, R. G.; Korshun, V. A. Pyrenemethyl ara-Uridine-2′-carbamate: A Strong Interstrand Excimer in the Major Groove of a DNA Duplex. *ChemBioChem* **2003**, *4* (9), 841−847.
(71) Okamoto, A.; Tainaka, K.; Ochi, Y.; Kanatani, K.; Saito, I. Simple SNP Typing Assay Using a Base-Discriminating Fluorescent Probe. *Mol. BioSyst.* **2006**, *2*, 122−127.
(72) Seela, F.; Pujari, S. S. Azide−Alkyne "Click" Conjugation of 8-Aza-7-deazaadenine-DNA: Synthesis, Duplex Stability, and Fluorogenic Dye Labeling. *Bioconjugate Chem.* **2010**, *21*, 1629−1641.
(73) Skorobogatyi, M. V.; Malakhov, A. D.; Pchelintseva, A. A.; Turban, A. A.; Bondarev, S. L.; Korshun, V. A. Fluorescent 5-Alkynyl-2′-Deoxyuridines: High Emission Efficiency of a Conjugated Perylene Nucleoside in a DNA Duplex. *ChemBioChem* **2006**, *7* (5), 810−816.
(74) Moran, N.; Bassani, D. M.; Desvergne, J.-P.; Keiper, S.; Lowden, P. A. S.; Vyle, J. S.; Tucker, J. H. R. Detection of a Single DNA Base-Pair Mismatch Using an Anthracene Tagged Fluorescent Probe. *Chem. Commun.* **2006**, *48*, 5003−5005.
(75) Filichev, V. V.; Astakhova, I. V.; Malakhov, A. D.; Korshun, V. A.; Pedersen, E. B. 1-, 2-, and 4-Ethynylpyrenes in the Structure of Twisted Intercalating Nucleic Acids: Structure, Thermal Stability, and Fluorescence Relationship. *Chem.—Eur. J.* **2008**, *14* (32), 9968−9980.
(76) Berndl, S.; Herzig, N.; Kele, P.; Lachmann, D.; Li, X.; Wolfbeis, O. S.; Wagenknecht, H.-A. Comparison of a Nucleosidic vs Non-Nucleosidic Postsynthetic "Click" Modification of DNA with Base-Labile Fluorescent Probes. *Bioconjugate Chem.* **2009**, *20* (3), 558−564.
(77) Astakhova, I. K.; Pasternak, K.; Campbell, M. A.; Gupta, P.; Wengel, J. A Locked Nucleic Acid-Based Nanocrawler: Designed and Reversible Movement Detected by Multicolor Fluorescence. *J. Am. Chem. Soc.* **2013**, *135* (7), 2423−2426.
(78) Pasternak, K.; Pasternak, A.; Gupta, P.; Veedu, R. N.; Wengel, J. Photoligation of Self-Assembled DNA Constructs Containing Anthracene-Functionalized 2′-Amino-LNA Monomers. *Bioorg. Med. Chem.* **2011**, *19* (24), 7407−7415.
(79) Del Amo, D. S.; Wang, W.; Jiang, H.; Besanceney, C.; Yan, A.; Levy, M.; Liu, Y.; Marlow, F. L.; Wu, P. Biocompatible Copper(I) Catalysts for *in Vivo* Imaging of Glycans. *J. Am. Chem. Soc.* **2010**, *132* (47), 16893−16899.