Account

Chemical Modification of Nucleic Acids toward Functional Nucleic Acid Systems

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Nucleic acids are virtually omnipresent; they exist in every living being. These macromolecules constitute the most important genetic storage material: the genes. Genes are conserved throughout the evolution of all living beings; they are transmitted from the parents to their offspring. Many interdisciplinary research groups are interested in modifying nucleic acids for use in a wider variety of applications. These modified oligonucleotides are used in many diverse fields, including diagnostics, detection, and therapeutics. In this account, we summarize our research efforts related to modified nucleic acid systems. First, we discuss our syntheses of modified oligonucleotides containing fluorescent tags for use as molecular probes (molecular beacons) to detect single-nucleotide polymorphisim (SNP) in nucleic acids and to distinguish between the B and Z forms of DNA. We also describe our research efforts into oligonucleotides functionalized with steroid derivatives to enhance their cell permeability, and the synthesis of several calix[4]arene-oligonucleotide conjugates possessing the ability to form defined triplexes. In addition, we have performed systematic studies to have an understanding about the functional groups necessary for a given nucleoside to behave as an organo or hydrogelator. The aggregation properties of a number of nucleoside-based phospholipids have been examined in different solvents; some of these derivatives are potential candidates for use as nucleoside-based liposomes. Finally, we also describe our research efforts toward the preparation of isoxazole- and isoxazoline-containing nucleoside derivatives and the determination of their antiviral activities.

Key Words : Nucleoside, Nucleotide, Oligonucleotide, Nucleic acid

Introduction

The modification of oligonucleotides has become an area of increased research activity. These modified biopolymers have drawn widespread attention from multidisciplinary research teams. Increasingly, modified analogs of the basic

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constituents of nucleic acid biopolymers – namely, nucleosides, nucleotides, and oligonucleotides – are being used in diverse fields, varying from materials science to therapeutics. For instance, many antiviral drugs are, in fact, modified nucleoside derivatives. Similarly, modified oligonucleotides have been used as molecular probes to better understand the interactions that occur between proteins and nucleic acids at the molecular level; these interactions are responsible for all aspects of cellular or viral gene expression. Synthetic oligonucleotides and their modified analogs can be used successfully to modulate the functions of specific genes.[1] Since their discovery, research into the use of "antisense oligonucleotide" therapeutics has intensified. The antisense oligonucleotides are used to block gene expression by binding especially with complementary sequences of target mRNA. Over the past decade, much effort has been exerted to modify native phosphordiester linkages to improve the nuclease resistance and membrane permeation of naturally occurring oligonucleotides for use in therapeutic applications. Therapeutics based on oligonucleotides are expected to become much more widespread in the future.

The chemist's role in oligonucleotide science has become much more demanding. Oligonucleotides displaying a wide variety of modifications at different parts of their sequences have been synthesized by chemists.[2] The advent of phosphoramidite chemistry, which is the preferred solid phase synthetic method for preparing oligonucleotides, has accelerated the development of oligonucleotides exhibiting modified nucleobases, sugars, and backbone phosphate groups. The biological significance of these modified oligonucleotides as antisene and antigene agents or as aptamers are evaluated, in general, by biologists. The siRNAs are a recent addition to the series of oligonucleotide analogs that inhibit gene functions; they function in a process known as RNA interference (RNAi).[3] A number of research groups, including chemists and biologists working side-by-side, are actively involved in expanding the subject. Other reasons for functionalizing native oligonucleotides are a) to improve their cellular uptake, b) to increase their stability in intracellular fluid, c) to increase their specificity toward a target, and d) to increase their binding affinity toward a target. For this purpose, a number of nucleoside or nucleotide phosphoramidites possessing modified bases, sugars, or backbone units have been introduced into DNA and RNA to provide the corresponding modified analogs.[4]

In this account, we provide a summary of the different types of modified nucleosides and oligonucleotides and their applications.[5,6] In addition, we discuss the effects that such modifications have on the structural, thermodynamic, and hybridization properties of these oligonucleotides and their duplexes with natural complementary oligonucleotides.

Modified Oligonucleotides

A number of modified nucleoside- and non-nucleoside-based phosphoramidite building blocks have been incorporated into oligonucleotides in an effort to improve the properties of the oligodeoxynucleotides (ODNs), including their fluorescence, cell permeability, and three-dimensional structure. Several oligonucleotides modified with chromophores have long been tested as probes for the detection and isolation of target nucleic acids. In this section, we describe the synthesis of several such oligonucleotides containing fluorene and pyrene units as fluorescent moieties, and their potential for use as probes for the detection of single-nucleotide polymorphism (SNP) and the B-to-Z DNA transition. The use of lithocholic acid as a suitable building block to enhance cell permeability is also described. In addition, we discuss our research initiatives into the synthesis of pentraerythritol-based phosphoramidites and their subsequent incorporation into oligonucleotides.

Fluorescent oligonucleotides for probing DNA/RNA. Fluorescence-based techniques have long been used as powerful tools to investigate biological materials, both *in vitro* and *in vivo*. Different kinds of fluorophores exhibiting good quantum yields have been attached to oligonucleotides to provide fluorescent oligonucleotides for detection and diagnostics use in molecular biology. Because of their potential for application, there is a strong demand for fluorescent oligonucleotides. This demand has, in turn, triggered an intense amount of research effort into the preparation of a

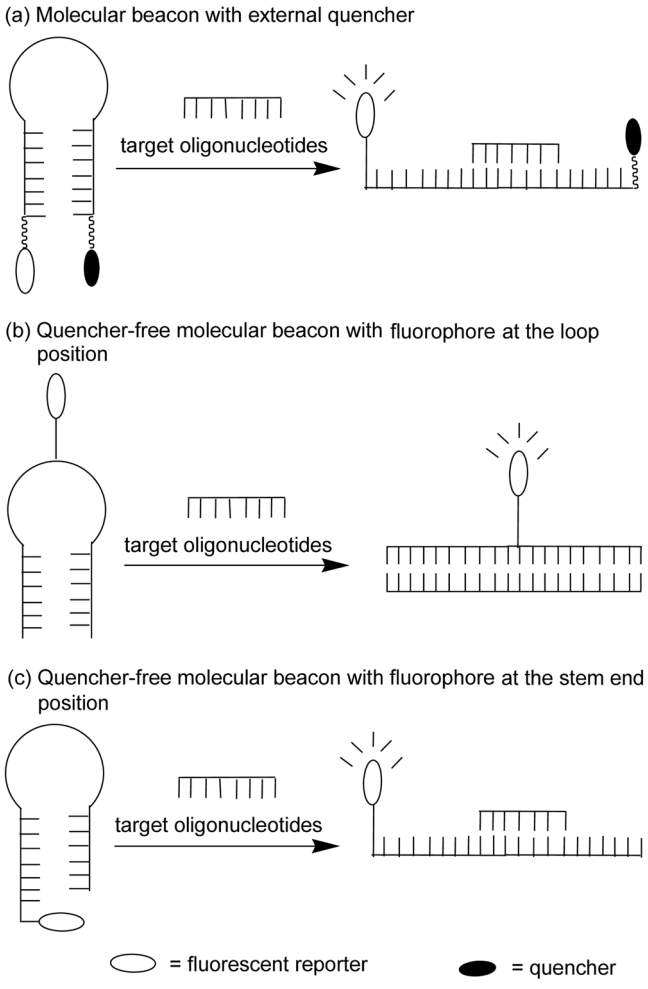
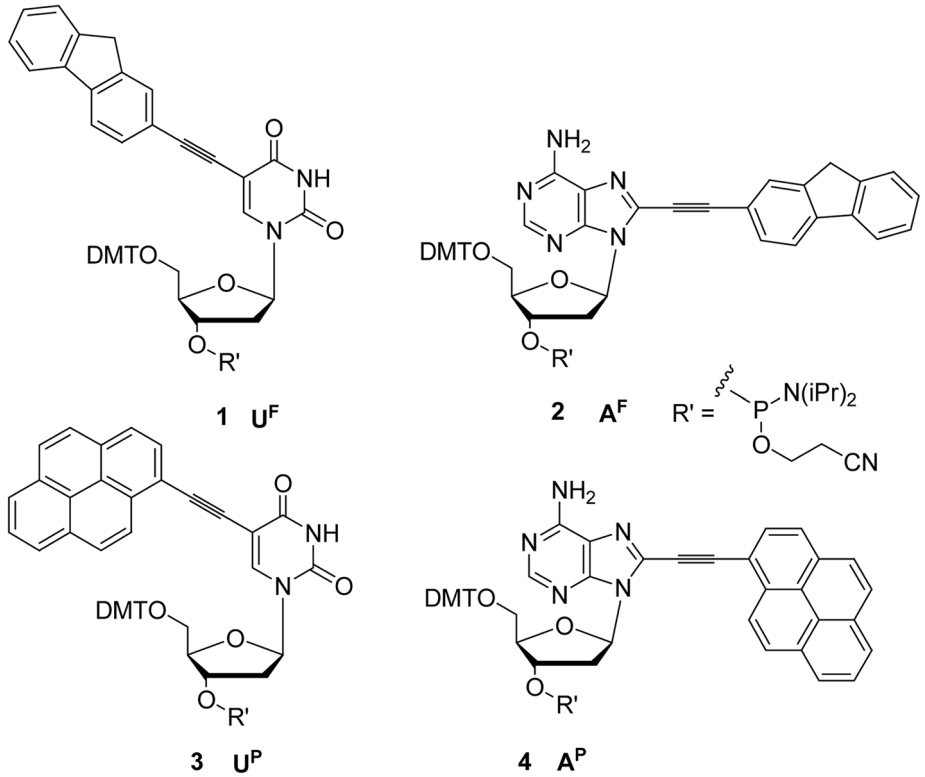


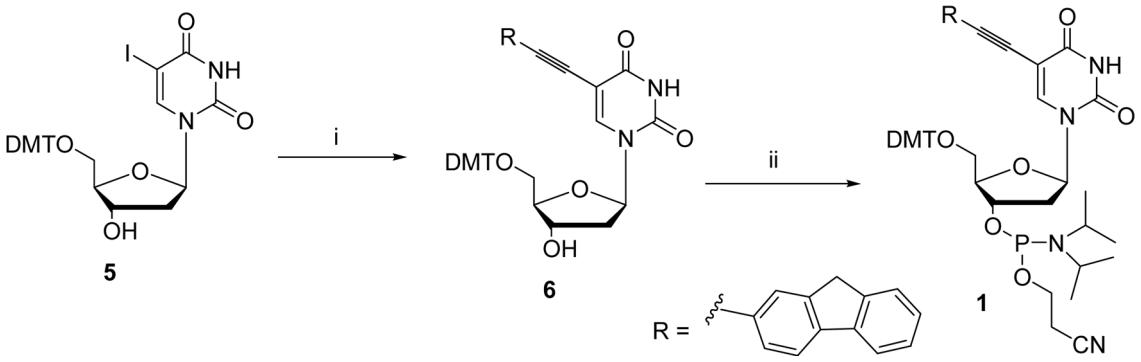
Figure 1. Different types of molecular beacons.



wide range of oligonucleotides containing various fluorescent tags.[7] Although the fluorescent moieties can be appended to different parts of the oligonucleotides, they are most often bonded to the bases. The different types of fluorescent nucleosides and the synthetic methods used to obtain them have recently been reviewed.[8]

The fluorescent oligonucleotides are also used as molecular bacons (MBs) to detect target nucleic acids and to probe SNP in oligonucleotides.[9] Figure 1 provides schematic representations of the structures and functions of the MBs. In a conventional MB, a doubly end-labeled oligonucleotide, which is capable of forming a stable hairpin (stem-loop) structure, is functionalized with a fluorescent reporter dye and a quencher at its two ends (Fig. 1a). In the absence of the target oligonucleotide, fluorescence resonance energy transfer (FRET) quenches the fluorescence of the reporter dye internally. In the presence of the target, the stem opens and the quenching effect is diminished. The target is, thus, recognized by the change in fluorescence intensity.

In our laboratory, we have synthesized oligonucleotide sequences containing the modified nucleoside building blocks **1**-**4**. We have demonstrated that the resulting fluorescent oligonucleotides function as quencher-free MBs of the types in Figures 1b and 1c to recognize base-mismatched oligonucleotides. The phosphoramidites **1**-**4** were designed based on the rationale that substitutions at the C-5 position of uridine and the C-8 position of adenine do not perturb their DNA base pairing ability significantly and, thus, these modifications should have very little influence on the stability of the resulting DNA duplexes.[10] As a representative example, Scheme 1 presents the synthetic method used to attach a fluorene unit covalently to the C-5 position



Scheme 1. Synthesis of a fluorene-labeled uridine derivative; (i) 2-ethynylfluorene, $(PPh_3)_2PdCl_2$, CuI, Et_3N/THF (1 : 3), 45-50 °C, 2 h, 80%; (ii) 2-cyanoethyldiisopropylchlorophosphoramidite, 2-methylmorpholine, CH_2Cl_2, rt, 30 min, 74%.

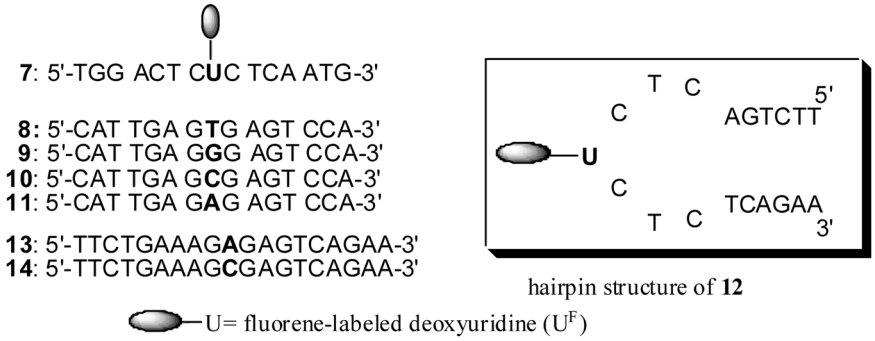


Figure 2. Fluorescent oligonucleotides containing the building block U^F and its hairpin structure.

of deoxyuridine. The fluorene-labeled phosphoramidite **1** was synthesized from the corresponding 5-iodo-5'-dimethoxytrityl-2'-deoxyuridine (**5**). Palladium-catalyzed Sonogashira coupling[11] with 2-ethynylfluorene[12] resulted in the formation of **6**, which was subsequently converted into the corresponding phosphoramidite building block **1**.

Incorporation of this fluorene-labeled deoxyuridine into the central position of the ODN **7** was effected using the standard protocols of automated DNA synthesis.[13] These modified oligonucleotides were characterized by MALDI-TOF mass spectrometry. Initially, we investigated the fluorescence intensity changes of the duplexes containing fluorene units upon the hybridization of fully matched and one-base-mismatched sequences. When ODN **7** binds to a target ODN, its fluorescence intensity is enhanced when perfectly matched base pairing exists, but it is dramatically quenched when it encounters one-base-mismatched pairing. The absorption maxima of the single-base-mismatched duplexes, **7•8**, **7•9**, and **7•10** are slightly red-shifted relative to that of the fully matched duplex **7•11**, *i.e.*, from 334 nm to 339, 339, and 338 nm, respectively. These results suggest that the fluorene units in the single-base-mismatched duplexes presumably interact with one or more of the nucleobases.

On the basis of these results, we designed the oligonucleotide model system **12** as a new type of MB that consists of a six-base-pair stem and a seven-base loop sequence possessing a fluorene-labeled deoxyuridine core (Fig. 2). As target strands, we synthesized the unmodified ODN **13**, which has a fully matched sequence, and **14**, which has a one-base-mismatched sequence.

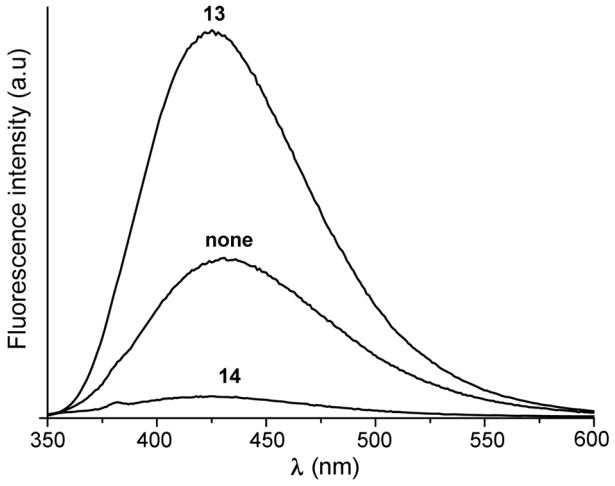


Figure 3. Emission spectra recorded at 20 °C of the duplexes of hairpin **12** (1.5 mM) with either **13** or **14**. Fluorescence spectra were recorded using an excitation wavelength of 340 nm. The spectrum labeled "none" is that of hairpin **12** alone.

The hybridization properties of **12** with **13** and **14** were studied by means of fluorescence measurements (Fig. 3). The hybridization of hairpin **12** with its complementary ODN **13** and single-base-mismatched ODN **14** in solution lead to a 2.2-fold enhancement and a 0.15-fold decrease, respectively, in emission intensity relative to that observed for hairpin **12**. Therefore, the total discrimination factor is 14.7 for the recognition of a single (A/C) base mismatch. The discrimination factor for this MB upon hybridization is high enough for a variety of applications. Thus, hairpin ODN **12** (Fig. 2) functions as new type of MB that requires no fluorescence quencher unit. Our MBs are useful probes that distinguish between their target and one base-mismatched (A/C) DNA sequences.[10]

Similarly, oligonucleotides containing another fluorophore – in this case, pyrene moieties – can also be used to design quencher-free MBs. The pyrene-labeled nucleoside phosphoramidites **3** and **4** were synthesized using a procedure similar to the one described for the fluorene analogs **1** and **2**.[14,15] In these pyrene-labeled deoxyuridine U^P (**3**) and deoxyadenosine A^P (**4**) units, the pyrene is substituted at the C-5 and C-8 positions, respectively, of U and A. The building block **3** was subsequently incorporated into oligonucleotide **15**.

15: 5'-TGG ACA CU^{P}C GGA ATG-3'
16: 5'-CAT TCC GTG TGT CCA-3'
17: 5'-CAT TCC GGG TGT CCA-3'
18: 5'-CAT TCC GCG TGT CCA-3'
19: 5'-CAT TCC GAG TGT CCA-3'

The "smart" single-stranded ODN **15** exhibited a typical pyrene monomer emission (λ_{max} = 408, 434 nm). Very interestingly, the emission intensities of the single-base-mismatched duplexes with ODNs **16**-**18** are reduced considerably relative to that of the single-strand ODN **15**. On the other hand, the emission maximum of the duplex of fully matched sequence **19** shifted from 434 to 444 nm. The

fluorescence efficiency of duplex **15•19** is 5.6 times stronger than that of duplex **15•16**; this fluorescence change can be observed by the naked eye. Thus, similar to the fluorene-labeled ODN **12**, the pyrene-labeled ODN **15** is also a sensitive probe that discriminates between perfect and one-base-mismatched base pairing through changes in fluorescence intensity. These findings suggest a means to design highly sensitive probes for SNP detection.[14]

20: 5'd-CAT TCC G**A^P**G TGT CCA-3'
21: 5'd-CAT TCC G**U^P**G TGT CCA-3'
22: 5'd-TGG ACA C**A^P**C GGA ATG-3'
23: 5'd-CAT TCC C**U^P**C TGT CCA-3'
24: 5'd-CAT TCC A**U^P**A TGT CCA-3'

We undertook further experiments to better understand the dominant noncovalent interactions that are responsible for such fluorescence changes in fluorescent oligonucleotides, especially in pyrene-containing oligonucleotides. In general, if two pyrene-labeled ODNs form a duplex, noncovalent interactions such as hydrogen bonding and interstrand π–π stacking will be responsible for the fluorescence changes, but one of them will be more dominant than the other. To determine which is more dominant, we synthesized the oligonucleotides **20**-**24**, which contain the pyrene-labeled deoxyuridine and deoxyadenosine building blocks **3** and **4**, as models.[15] These oligonucleotides were designed in such a way that opposing nucleobases in the resulting duplexes feature the pyrene units, *i.e.*, they form U^P–A^P, U^P–U^P, or A^P–A^P couples (Fig. 4).

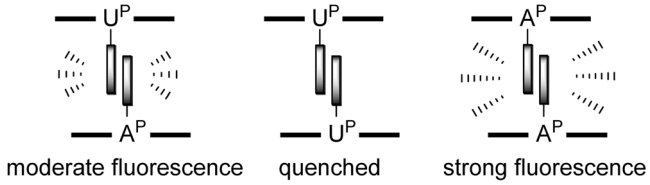


Figure 4. Fluorescent base pairs based on pyrene–pyrene stacking interactions.

Figure 5 displays the principal differences between the fluorescence properties of the oligonucleotides.[15] The hybridization of the matched duplexes **15•20**, **21•22**, and the mismatched duplex **20•22** leads to red-shifted excimer emissions because of π–π interactions occurring between the two pyrene units. A characteristic feature of these excimer bands is that they contain no monomer emission band, presumably because each pyrene unit is linked to its base through an ethynyl unit, which induces rigidity, and therefore the stacking interactions are very strong. Surprisingly, this strong and stable interstrand stacking compensates for the loss of hydrogen bonding in duplexes **15•20** and **21•22**. In addition, Figure 5 indicates that duplexes **21•22**, **15•21**, and **20•22**, which all feature interstrand stacking interactions, exhibit strikingly different fluorescence properties. When the U^P unit bonds with the A^P unit, such as in duplexes **15•20** and **21•22**, a moderately intense fluorescence is emitted. When oligonucleotides containing the nucleobase A^P (A^P-oligo) form duplexes with another A^P-oligo, as in the case of duplex **20•22**, the fluorescence intensity is increased, but in the case of duplexes containing the U^P–U^P couple, the florescence emission is quenched (*e.g.*, in the case of duplex **15•21**). In the case of the mismatched duplexes **22•23**, **22•24**, **15•23**, and **15•24**, we observed two distinct monomer bands. We assume that if no hydrogen bonding interactions exist between the bases neighboring the U^P and A^P units, as is the case of the duplexes **22•23**, **22•24**, **15•23**, and **15•24**, such interstrand stacking interactions are not allowed. To elucidate the detailed three-dimensional structures of these duplexes, we used computer modeling to calculate possible structures for duplexes **15•20**, **15•21**, and **20•22**. The pyrene moiety in duplex **15•21** exists in a highly polar environment, *i.e.*, in the major groove, whereas the pyrene units in duplexes **15•20** and **20•22** are located in the minor groove (*i.e.*, hydrophobic environments).

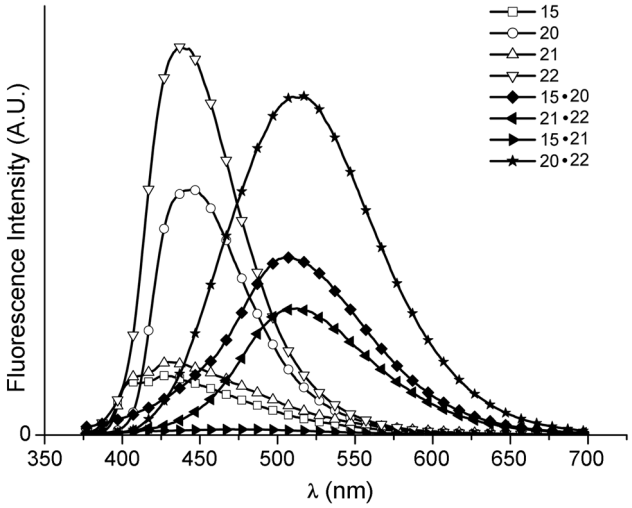


Figure 5. Emission spectra recorded at 20 °C of ODNs and their duplexes (at 1.5 mM concentrations); λ_{ex} = 366 nm. Measured in a buffer of 100 mM NaCl, 20 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.2).

From these experiments, we conclude that the fluorescent nucleobase analogues within the modified ODNs allow strong interstrand stacking interactions between the two pyrene units, which compensate for the loss of hydrogen bonding, and, therefore, they can exhibit different emission intensities (pyrene excimer emissions). These findings may provide new insight into the design of new probes and nucleobase analogues that will have applications in molecular biology.

It is well established that physiochemical interactions between DNA bases and polynucleic aromatic fluorophores strongly quench the fluorescence of the latter. By taking advantage of this phenomenon, we developed a new type of hairpin-based MB, wherein the fluorescent nucleoside building block is substituted at the 5'-terminus of the oligonucleotide. Figure 1c provides a schematic representation of the design and function of this MB. The important

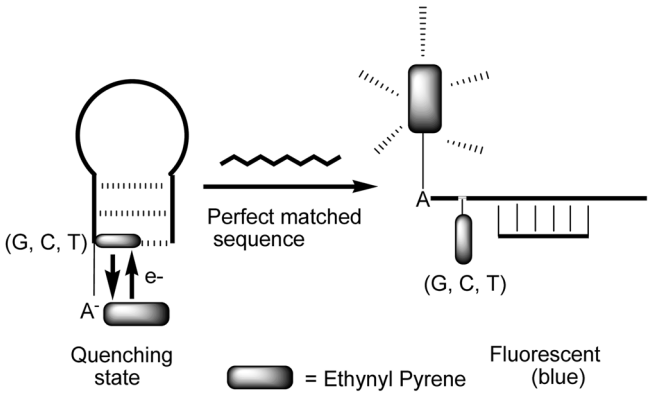


Figure 6. Design and function of quencher-free end-stacking molecular beacons formed using ODNs **25** and **26**.

factors behind the choice of pyrene and fluorene moieties are that a) they possess high quantum yields and efficient planar aromatic structures that enhance π–π stacking and b) procedures for preparing the corresponding fluorescent nucleoside phosphoramidites **1**-**4** are well-established in our laboratory. Because of these salient factors, we synthesized the ODNs **25** and **26**, which contain, respectively, the A^P and U^P nucleoside building blocks at their 5'-termini (Fig. 6).[16]

25: 5'd-**A^{P}G**CGAG AAGTTAG**A**ACCTATG CTCG**C**
26: 5'd-**U^{P}G**CGAG AAGTTAG**A**ACCTATG CTCG**C**
27: 5'd-CATAGGT**T**CTAACTT-3'
28: 5'd-CATAGGT**A**CTAACTT-3'
29: 5'd-CATAGGT**G**CTAACTT-3'
30: 5'd-CATAGGT**C**CTAACTT-3'

The UV–Vis spectra indicate that a strong absorption band difference occurs at *ca.* 420 nm for ODN **25** upon its conformational change, but not for ODN **26**. This absorption band arises from the ground state interactions of the fluorophore unit. We chose to excite the system at 420 nm because of the difference between the absorption intensities of hairpin (closed) and matched duplex (open) states of

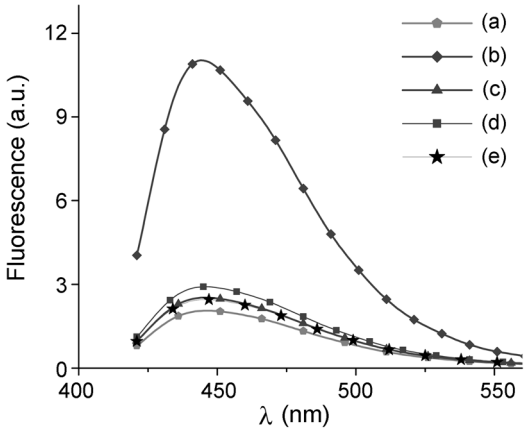


Figure 7. Fluorescence emission spectra of (a) **25**, (b) **25•27**, (c) **25•28**, (d) **25•29**, and (e) **25•30** recorded at 37 °C (100 mM Tris-HCl buffer, 1 mM $MgCl_2$; pH 8); λ_{ex} = 420 nm.

ODN **25**. In the resulting fluorescence spectra of ODN **25** and ODN **26**, we observe a sharp difference between the fluorescence of the hairpin ODN **25** and its "fully matched" duplexes with complementary strands (*e.g.*, the duplex **25•27** in Fig. 7), whereas the fluorescence differences of ODN **26** and its fully matched duplex with its complementary strands (*e.g.*, **26•27**) were much weaker (data not shown).

As indicated in Figure 7, our approach makes it possible to discriminate between matched (ODN **27**) and single-base-mismatched (ODNs **28**-**30**) sequences. The T_m value of the duplex plays a vital role in the discrimination process. The T_m value of the perfectly matched duplex, **25•27** (47 °C) is higher than those of mismatched duplexes **25•28**, **25•29** and **25•30**, which range from 30 to 33 °C. So, at experimental temperature (37 °C), the duplex formed by perfectly matched sequence is stable and the other duplexes are unstable.

The difference in the spectral property must arise from the electronic environment of the fluorophore, *i.e.*, the ethynyl pyrene unit. The A^P moiety is quenched only in the hairpin state. Thus, the stability of the hairpin stem arising from π-stacking and the photoinduced energy transfer (PET) between the pyrene-labeled 2'-deoxynucleotide units (A^P or U^P in **25** or **26**) and their neighboring bases are the two main factors that affect the operation of these novel MBs.[17] The fluorescence can be quenched through PET from the fluorophore to terminal natural C, T, and G bases, but not to an A base. Thus, A^P can distinguish A from C, T, and G at the 5'-end of the hairpin. The ODN **25** allows the ready detection of single nucleotide alterations and does not require the presence of an additional modified fluorescence quencher or linker unit. Thus, it has been demonstrated clearly that pyrene-labeled deoxyuridine and deoxyadenosine units are useful unnatural fluorescent nucleoside analogues.[18] By making use of these peculiar spectral properties, new molecular beacon probes can be designed to recognize SNP in oligonucleotides. This process involves the synthesis of a fluorescent-unit-containing oligonucleotide in its closed (hairpin) state and utilization of its change in λ_{max} upon its transition to an open state. This change in λ_{max} discriminates between the matched and mismatched ODN sequences. This hairpin configuration is attractive because the synthesis of such an MB is relatively simple and inexpensive; *e.g.*, it does not require two distinct processes to prepare the fluorophore and quencher.

Fluorescent G,C-alternating oligonucleotides containing A^P and U^P (*i.e.*, the building blocks **3** and **4**) at dangling positions have been used as novel probes to detect B-to-Z DNA transitions visually, as indicated in Figure 8.[19] This approach is based on the fact that fluorescence quenching occurs through PET in B-DNA, but not in Z-DNA, because of a change in the electronic environment of the nonpolar aromatic fluorophore attached at the terminus of the "dangle" position. That is to say, the dangle end stacking ability of the A^P unit is altered upon the transition from B- to Z-DNA because of a conformational change. The B and Z forms have opposite helicities, and the diameter of Z-DNA

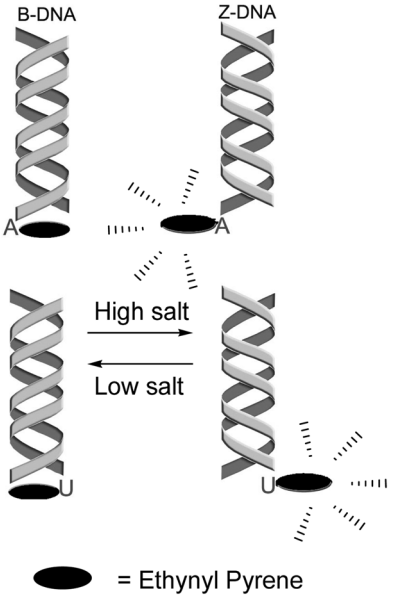


Figure 8. Design of a probe for monitoring the B-to-Z DNA transition.

(18 Å) is much narrower when compared with that of B-DNA (20 Å). To minimize the effect of the stacking interactions in dsDNA, the A^P and U^P moieties were placed at dangling positions at the ends of otherwise base-matched duplexes.

31: 5'd -**A**^{P} GC GCG CGC G
32: 5'd -CG CGC GCG C**U**^{P}
33: 5'- GC GCG CGC G
34: 5'- CG CGC GCG C

We then used circular dichroism (CD) spectroscopy to monitor the B-to-Z-DNA transitions of the ODNs. The CD spectra (Fig. 9) display the dramatic consequence of the conformational change from B- to Z-DNA that occurred upon changing the salt concentration. In 0.1 M NaCl, the **31•34** duplex exists mainly in the B-DNA conformation, but

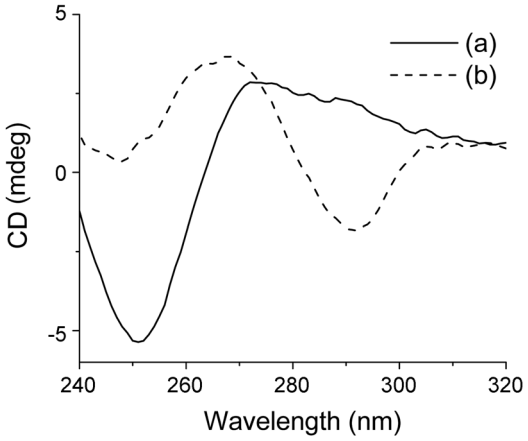


Figure 9. Circular dichroism spectra recorded for the **31•34** duplex in the presence of (a) 0.1 M NaCl and (b) 5 M NaCl. The spectra were recorded at 20 °C in a buffer of 100 mM Tris–HCl (pH 7.2). The duplex concentration was 1.5 μM.

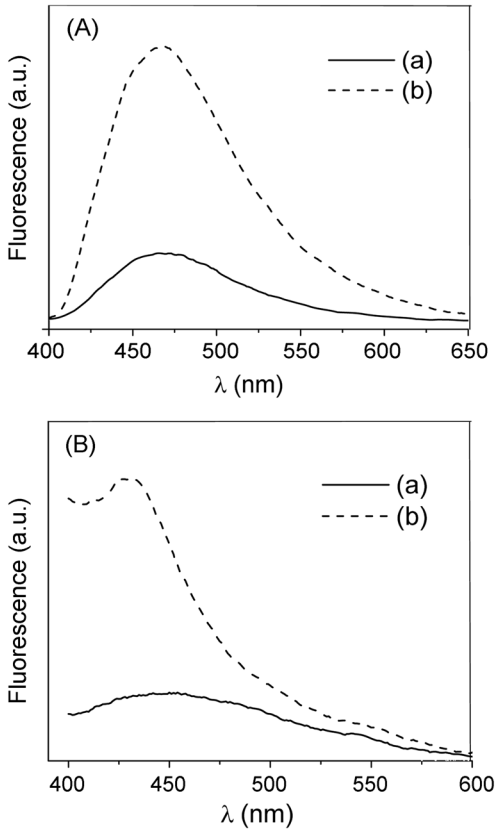
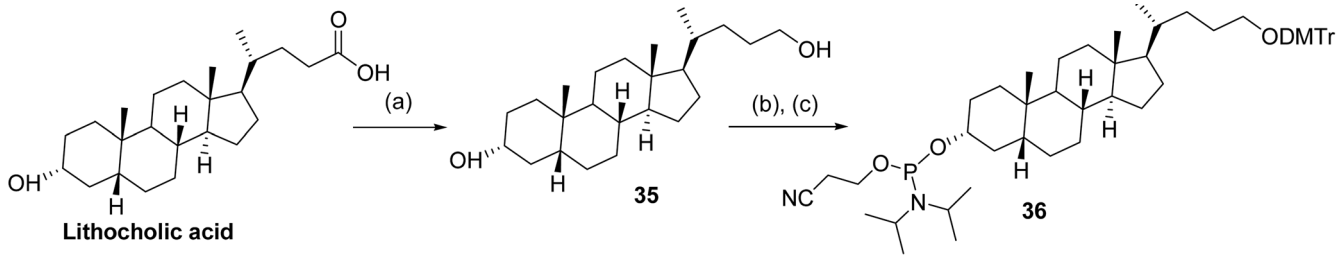


Figure 10. Fluorescence spectra of the duplexes (A) **31•34** and (B) **32•33** [(a) B- and (b) Z-DNA forms]. These spectra were recorded at 20 °C in a buffer of 100 mM Tris–HCl (pH 7.2). The duplex concentration was 1.5 μM and λ_{ex} = 386 nm.

in 5 M NaCl it exists in the Z-DNA form. The duplex **32•33** underwent a similar B-to-Z-DNA transition.

We investigated the fluorescence emission properties of the B- and Z-DNA duplex states of the pyrene-modified ODNs **31** and **32** (Fig. 10). We observed dramatic fluorescence changes for the **31•34** and **32•33** duplexes; in general, their Z-DNA forms exhibited a marked increase in fluorescence relative to those of the B-DNA forms. The changes in fluorescence emission that occur during the B-to-Z-DNA conformational change are believed to arise from changes in the electrostatic interactions (through PET and terminal π-stacking) between the A^P and U^P moieties and their neighboring nucleobases. A strongly donating or accepting terminal π-stacking moiety is usually critical for fluorescence quenching[20] in the B-DNA duplex state; in Z-DNA, end stacking is very difficult to achieve because of the relatively narrow duplex conformations and opposite helicity. Such end stacking of hydrophobic A^P and U^P units enhances the stability of the B-DNA form. We believe that this property allows our system to discriminate between the Z- and B-DNA forms, but the exact mechanism of this phenomenon remains to be established.

Oligonucleotides with non-nucleoside phosphoramidite building blocks. Cholesterol, an important member of the family of cholane derivatives, is known for its ability to interact with, and penetrate through, cell membranes.[21] In



Scheme 2. Synthesis of phosphoramidite derivative **36**: a) $LiAlH_4$ (4.6 equiv), THF, 4 h, 96%; b) DMTrCl (1.9 equiv), DMAP, Et_3N, pyridine, 89%; c) chloro-(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine (1.5 equiv), DIPEA (3 equiv), CH_2Cl_2, 54%.

most cholesterol-modified ODNs, the cholesterol units have been linked to either the 5' or 3' position. Lithocholic acid, another member of the cholane family, is a hydrophobic secondary bile acid that is a substrate of the nuclear Pregnane X receptor (PXR). We chose to use cholane-3,24-diol (3α,5β) units as suitable building blocks for inclusion into ODNs to improve their cellular permeability.

We synthesized the lithocholic acid-based phosphoramidite **36** as a structural scaffold to assemble hairpin-shaped ODNs.[22] Scheme 2 displays the synthesis of **36** in three steps from lithocholic acid. The key intermediate, cholan-3,24-diol (3α,5β) (**35**), was obtained readily from lithocholic acid after reduction with $LiAlH_4$.

Subsequently, building block **36** was used to develop oligonucleotides capable of forming stable double- or triple-stranded nucleic acid systems, depending upon the number of hairpin moieties present in the ODNs.[23] The ODNs containing one hairpin moiety (**37** and **38**) formed duplexes, whereas the ODNs containing two hairpin moieties formed stable triplex structures, as indicated in Figure 11. Thus, the lithocholic acid-based ODNs (L-ODN) **37**-**42** can perform as good mimics of DNA hairpin structures. It should be borne in mind that stable triplexes of this kind can be used as tools to study the biological roles and genetic applications of naturally occurring triplexes. The modified L-ODNs **37**-**42** were synthesized in high coupling efficiencies using an automated DNA synthesizer. Their structures were confirmed through MALDI-TOF mass spectral analysis. All of the ODNs containing one or two hairpin moieties – **37** (Homo-LD), **38** (Homo-ND), **39** (Homo-LT), **40** (Homo-NT), **41**

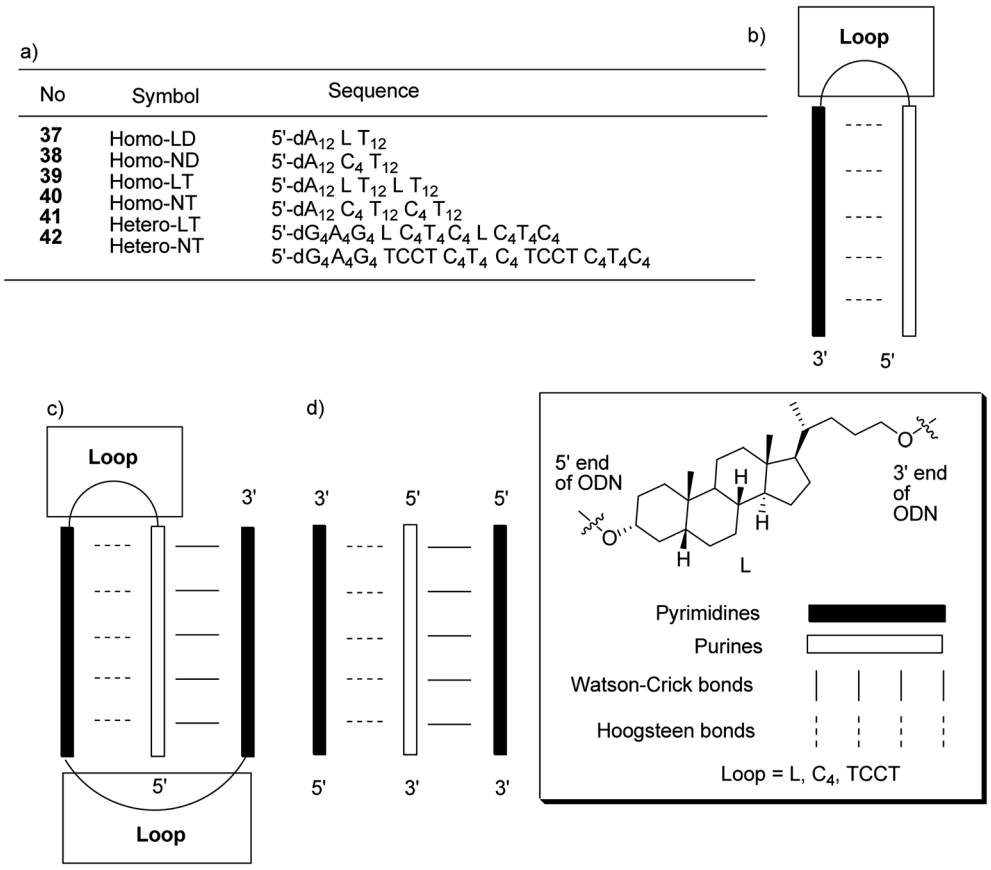
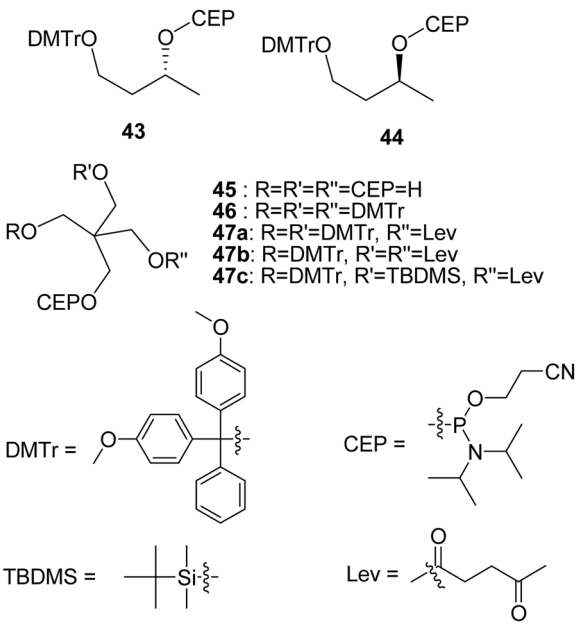


Figure 11. Possible duplex and triplex structures of the lithocholic acid-based ODNs **37**-**42**.

(Hetero-LT), and **42** (Hetero-NT) – exist as palindromers. Thus, these modified ODNs were expected to form duplexes or triplexes that feature intramolecular hydrogen bonding patterns, as indicated in Figures 11b and 11c. Moreover, the triplexes formed by the modified ODNs (Fig. 11c) are more stable relative to the triplexes formed by the unmodified single-stranded ODNs (Fig. 11d). The enhanced stability in the latter is due solely to the presence of the loop.

The secondary structures of L-ODNs **37-42** were confirmed by determining their values of T_m and by performing semi-empirical analyses of CD spectroscopic data.[23] These analyses revealed that the L-ODNs adopt hairpin structures featuring double-helix formation through intramolecular hydrogen bonding between complementary sequences of ODNs; *i.e.*, they mimic natural DNA hairpin structures. These ODNs can adopt triplex structures when they assemble with an added third strand. Also, the triplexes formed by these modified ODNs are highly stable because of intramolecular hydrogen bonding between their complementary sequences. The L moiety provides greater stability to the hairpin structures than that which exists in natural or unmodified DNA hairpin structures.[23]

We also investigated whether the incorporation of cholane-3,24-diol (3α,5β) into L-ODNs enhances their cellular uptake through improved interactions with cell membranes and nuclei.[22] The cellular uptake experiment was performed by comparing the results for 5'd-T_{12}-L-T_{12} with those of the unmodified ODN, 5'd-T_{12}-C_4-T_{12}; the experiment was undertaken in HeLa cells. The natural dye fluorescein (FITC) was attached to both ODNs as a fluorescence label. Using confocal microscopy, we observed that the permeability of the L-ODN was much higher than that of the natural ODN. In addition, we found that 5'd-T_{12}-L-T_{12}-FITC localized mainly at the nucleus, which is consistent with the fact that L is a substrate of nuclear receptor. Thus, the L scaffold serves a double purpose: it mediates the formation of stable hairpin structures and increases cell permeability.[22]



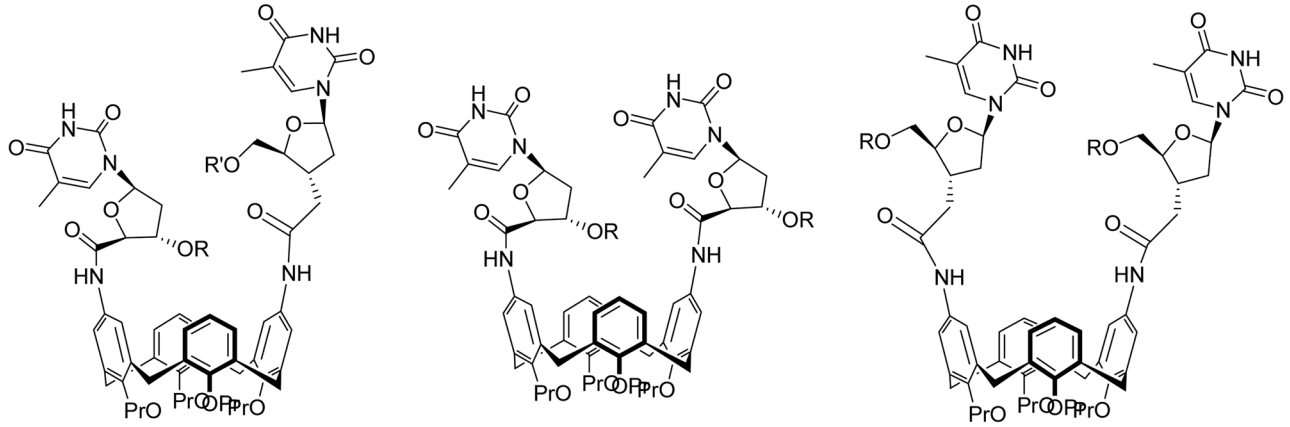
We speculated that a change in chiral center at the 3'-position of single nucleotide unit in ODNs may have profound effect on its binding ability, and duplex stability. As a result, we synthesized ODN-12mers containing an optically pure non-nucleoside phosphoramidite building block, namely, *R* (**43**) or *S* (**44**) epimer of 1,3-butane diol-3'-*O*-phosphoramidite.[24] However, contrary to our expectation, ODNs containing *R* and *S* epimers showed the same T_m values, thereby showing that a change in chirality of only one nucleotide building block do not have any effect on its duplex stability.

Branched ODNs have been used recently in the construction of programmed nanostructures. In particular, hetero-sequences containing branched ODNs are often used as the vertices of such nanostructures, including tetrahedra and cubes. This situation necessitates the availability of suitable building blocks that form both triple- and quadruple-branched ODNs. For this purpose, we synthesized pentaerythriol-based phosphoramidites –both symmetrical (**46**) and unsymmetrical (**47a-47c**)– as non-nucleoside phosphoramidite building blocks.[25] Three different protecting groups, namely, dimethoxytrityl (DMTr), *tert*-butyldimethylsilyl (TBDMS), and levulinyl (Lev), were used to protect the different hydroxyl groups of pentaerythritol (**45**). At present, we are using these compounds to prepare branched ODNs having homo- and hetero-sequences.

Calixarene-oligonucleotide conjugates (calixoligonucleotides). Calixarene is a versatile host molecule because of its directional preorganization toward various guest molecules. Calix[4]arene derivatives have been utilized as building blocks in multifunctional enzyme models.[26] Recently, they have been coupled with various bioactive groups including nucleobases (adenine, thymine, uracil),[27] and guanosine[27c] to develop biologically active synthetic receptors and enzyme mimics. We chose calix[4]arene as a preorganized platform to assemble nucleoside and oligonucleotide analogs to obtain ODNs possessing hairpin structures. Thus, we synthesized the calix[4]arene nucleoside hybrids **48-51** as basic building blocks to obtain calix[4]arene-oligonucleotide conjugates.[28]

These calix[4]arene nucleoside hybrids (also called calixnucleosides) meet the general criteria required for modification of oligonucleotides; thus, they can be used as basic building blocks for the construction of hairpin-type ODNs. It is known that the hairpin ODNs recognize complementary DNA or RNA sequences through triplex formation.[29]

The syntheses of these hetero- (**48**, **49**) and homo- (**50**, **51**) coupled calixnucleosides was achieved through peptide coupling between the amino functional groups of para-1,3-diaminocalix[4]arene and the carboxylic acid groups of thymidine nucleosides. These products were characterized fully by mass spectrometry, ^{1}H and ^{13}C NMR and IR spectroscopy, and elemental analyses. The X-ray crystallographic analysis of the homo-coupled calixnucleoside **51** revealed an interesting hydrogen bonding pattern between the thymine bases and the amide linkages. The solid state



48: R = H, R' = H
49: R = TBDMS, R' = TBDPS

50: R = TBDMS

51: R = TBDPS

structure indicates that the calix[4]arene moiety possesses a pinched-cone conformation because of four independent sets of intermolecular hydrogen bonds between the thymine bases and the amide linkages.[28] We prepared the calix[4]-arene-oligonucleotide hybrids (calixoligonucleotides) **53**-**54** (Table 1) by following the protocols of solid phase oligonucleotide synthesis (using an automated DNA synthesizer).[30] The calixoligonucleotides were separated, purified by RP-HPLC, and characterized using MALDI-TOF mass spectrometry.

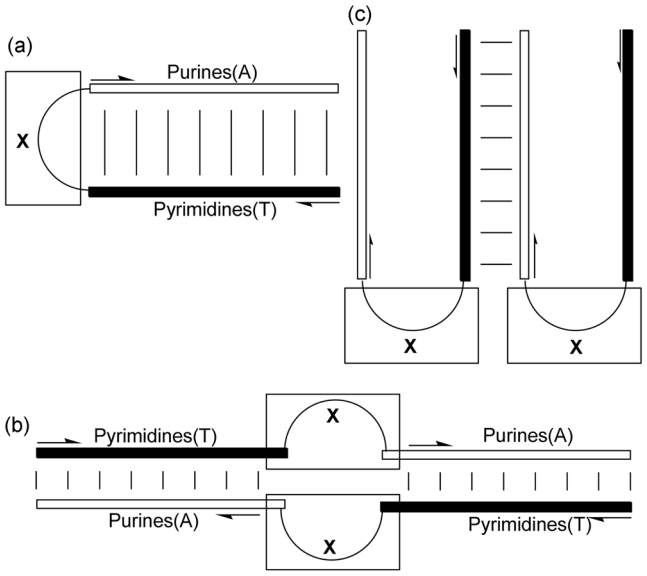
The three possible structures that can be formed by the calixoligonucleotides are shown in Figures 12a-c; structure (a) results from intramolecular hydrogen bonding of the ODNs, while structures (b) and (c) result from intermolecular hydrogen bonding. Based on thermal denaturation experiments, monitored using UV spectroscopy at 260 and 284 nm (Table 1), and circular dichroism spectra of the calix[4]arene-oligonucleotide hybrids, we suggest that the modified oligonucleotides adopt V-shaped conformations as depicted in Figure 12c. This conformation occurs primarily as a result of the presence of the calixnucleoside scaffold (represented by the unit **X** in Table 1), making such units suitable for use as building blocks in the construction of programmed oligonucleotide nanostructures.[30,31] The secondary structures were confirmed by determining T_m values and by comparing analyses made by HPLC, PAGE, and CD spectroscopy.[30]

We used HPLC to confirm that the structures of the calixoligonucleotides were different from those of linear ODNs. In addition, preliminary optical microscopy experiments performed on the calixoligonucleotides suggested that they might be present in double-helix form with intermolecular base pairing, similar to the V structure displayed in Figure 12c, rather than as linear double helices formed through intermolecular base pairing (as in Figure 12b). Through PAGE analysis, we confirmed that the calixoligonucleotides can adopt hairpin structures with double-helix formation through intramolecular hydrogen bonding between complementary sequences of ODNs; *i.e.*, they mimic natural DNA hairpin structures.[30] From the values of T_m of the ODNs, it is

Table 1. Values of T_m of the duplexes formed by ODNs **52**-**55**

Number	Sequence	T_m (260 nm)	T_m (284 nm)[a]
52	5'd-A_4GATATCAA**X**TTGATATCT$_4$	40, 70 °C	40 °C
53	5'd-T_{12}**X**-T_{12}	No T_m	n.d.
54	5'd-A_{12}**X**-T_{12}	39 °C	n.d.
55	5'd-A_{12}**C$_4$**-T_{12}	68 °C	29 °C

apparent that intermolecular base pairings are more favorable than intramolecular ones. The CD spectra exhibit the distinct characteristics of B-form DNA and indicate that the calixoligonucleotides act in a manner similar to that of natural ODNs. The calixoligonucleotides aggregate in solution, with the driving forces of intermolecular base pairing of the ODN units and hydrophobic interactions of



X = Loop of the ODN containing calixnucleoside building block

Figure 12. Possible structures adopted by calixoligonucleotides: (a) hairpin structure, intramolecular base pairing; (b) bulged duplex, intermolecular base pairing; (c) V-shaped aggregates, intermolecular base pairing.

the calix[4]arene residues. Thus, we have demonstrated that calixoligonucleotides possess V-shaped architectures and that calixnucleosides can be used as efficient turning points in long ODN sequences.[30,31]

Modified Nucleosides and Their Applications

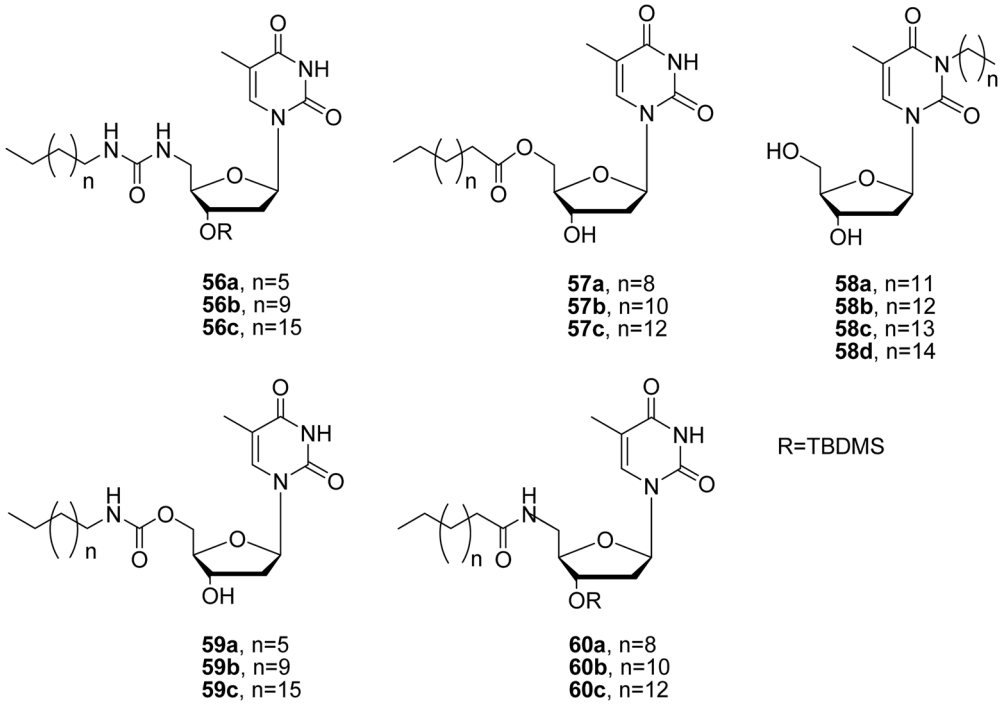
In this section we summarize the outcomes of our research efforts into the preparation of modified nucleosides for use in various applications, such as organo/hydrogelators and the formation of vesicles.

Modified nucleosides for organogelation. The gelation of organic solvents by low-molecular-weight compounds has received an increasing amount of attention in recent years.[32] There are several examples of organogel formation by prebiotic molecules, such as amino acid derivatives,[33] dipeptides,[34] and carbohydrates.[35] We envisioned that nucleosides would be promising materials for preparing new organogelators; thus, we synthesized the thymidine-based organogelators **56-60**.[36,37] We studied these derivatives in detail in an effort to establish a relationship between their structure and gelation ability in different solvents. We determined the microstructures and micro-networks of the gels by recording their SEM images, FT-IR spectra, and DSC traces. The salient features of our design are a) that the basic nucleoside structure, consisting of a hydrophilic sugar moiety and a thymine nucleobase, remains intact and so that its hydrogen bonding ability remains unaffected and b) that the hydrophobic alkyl groups are attached to the nucleoside (5'-position) through four different linking units – namely, urea, amide, carbamate, and ester bonds – and through 3'-*N*-alkylation. In some cases, a bulky silicon unit at the 3'-position was introduced for improved solubility.

The thymidine-based organogelators **56-60** were synthesized through a short series of simple reactions[36] and the products were obtained in moderate-to-quantitative yields. Gelation tests were conducted using a "stable-to-inversion-of-the-container" method with *ca*. 20 organic solvents of varying polarity. Among these solvents, cyclohexane, *n*-pentane, *n*-hexane, and *n*-heptane were organic liquids that formed robust and translucent gels with the urea- and amide-linked gelators, but the carbamate- and ester-linked gelators formed opaque gels with toluene, 1,2,3,4-tetrahydronaphthalene, propylene carbonate, and 3-methoxypropionitrile. Similarly, the 3'-*N*-alkylated gelators formed opaque gels in benzene, CCl_4, toluene, methylene chloride, 1,2,3,4-tetrahydronaphthalene, cyclohexane, and *n*-hexane. Most of our products had MGC values of *ca*. 2.0 wt% in their respective solvents.[36,37] The different gelling abilities are attributed to differences in the types of hydrogen bonding interactions. In this study, we demonstrated for the first time that it is possible to synthesize novel thymidine-based organogelators through simple modification of the sugar and base moieties. In addition, we concluded that thymidine is a promising molecule with which to obtain new gelators having different gelation abilities. For example, the SEM images in Figure 13 display the gels of compounds **58** and **60**, which possess different 3-D network structures.

In collaboration with Shinkai's research group, we undertook studies into the mode of aggregation and the gelation properties of organogelator **57c** upon addition of its complementary and non-complementary polynucleotide sequences **61** and **62**, respectively, in different organic media.[38]

At first, we added the complementary polymer, poly(A), into the organogel of **57c**, but it was virtually insoluble in nonpolar solvents. Hence, to dissolve poly(A) into organic



56a, n=5
56b, n=9
56c, n=15

57a, n=8
57b, n=10
57c, n=12

58a, n=11
58b, n=12
58c, n=13
58d, n=14

59a, n=5
59b, n=9
59c, n=15

60a, n=8
60b, n=10
60c, n=12

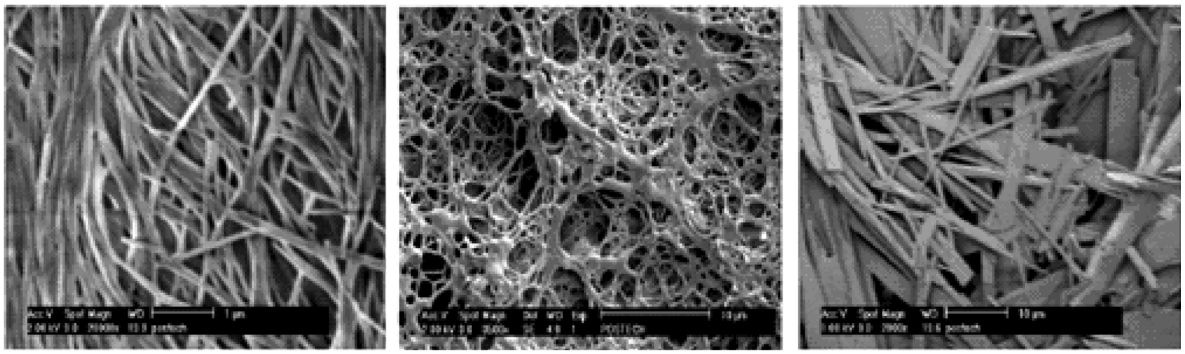
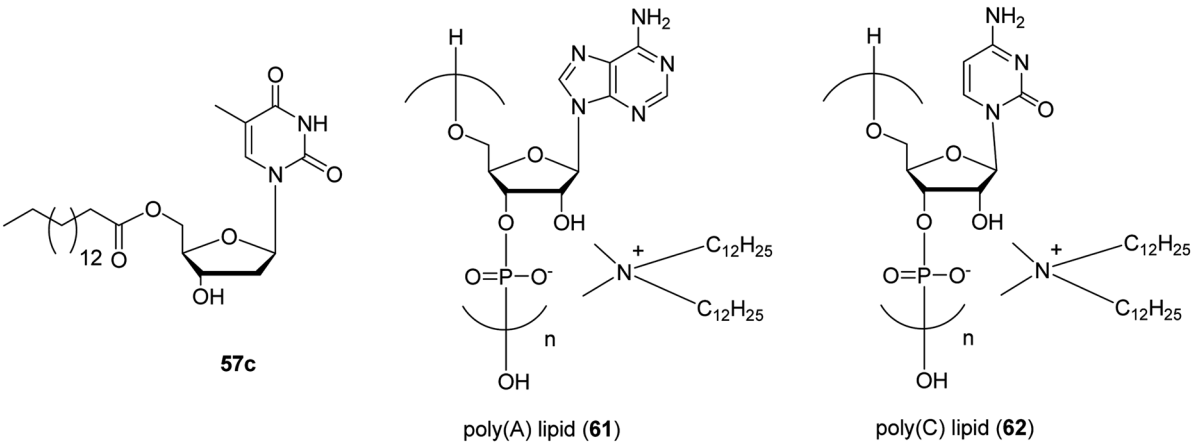


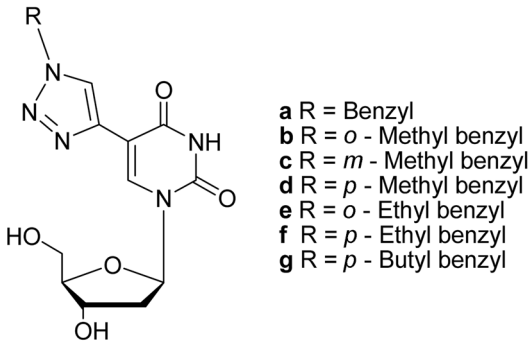
Figure 13. SEM images of (a) **58a**/CCl_4, (b) **60c**/octane, and (c) **58c**/toluene systems.

media, we prepared a suspension of poly(A)/lipid through ion-pair exchange using a cationic surfactant, didodecyldimethylammonium bromide (DDAB).[39] Similarly, we prepared a poly(C)/lipid suspension as a non-complementary polynucleotide. Interestingly, when an equal amount of poly(A)/lipid was dispersed within the **57c**/benzene gel and the mixture heated to obtain a sol phase, the opaque gel of **57c** transformed upon cooling into a transparent gel. In contrast, the original opaque gel was obtained from the mixture of **57c** and poly(C)/lipid. This peculiar observation is explained as arising from a change in the macroscopic aggregation structure upon the addition of poly(A)/lipid and forming complementary hydrogen bonds. Thus, the thymidine-based organogelator **57c** appears to interact with its complementary poly(A)/lipid in the gel phase, thereby inducing a drastic change in its aggregation morphology and, hence, its gelation properties. There are only a few reports describing similar two-component gel systems containing nucleobase-appended gelators and their complementary DNA or RNA strands,[40] but in all of these reports the gelation was performed in aqueous media because of the higher solubility of DNA and RNA. In contrast, we found that poly(A)/lipid acts as a modifier of the gelation properties of **57c** in organic media, wherein hydrogen bonding interactions can exert a very strong attractive force. We believe that the use of such organogel systems will become a more general approach to the modification of the gelation mode of low-molecular-weight gelators, *i.e.*, through the addition of suitable polymeric compounds that can interact with the gel through the concept of molecular recognition.

Modified nucleosides for hydrogelation. Research efforts into hydrogelators are also being undertaken intensively. In fact, owing to the significance of hydrogelators in many pharmaceutical, clinical, agricultural, cosmetics, and food products,[41] they are generally studied to a greater extent than are organogelators. We have also investigated the design of hydrogelators based on biologically important molecules, such as biotin,[42] for their potential use in sustained drug release.

Similarly, we sought to extend our earlier experience with nucleoside-based organogelators (Section 3.1) into suitably derivatized nucleoside derivatives that would form hydrogels.[43] Indeed, we synthesized low-molecular-weight, triazole ring-appended 2'-deoxyuridine hydrogelators **63** that gelate pure water at concentrations as low as 0.2 wt% (MGC values); these compounds display different modes of aggregation, as evidenced by the resulting network structures that we monitored through the recording of SEM images and FT-IR spectra.[43]



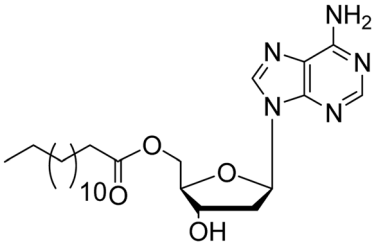


63

In the case of our organogels, we introduced long hydrocarbon chains and bulky protecting groups into thymidine derivatives. For the hydrogelators, in contrast, we controlled the balance between hydrophilicity and hydrophobicity by introducing substituted heterocyclic rings. 2'-Deoxyuridine itself is very hydrophilic because of its many polar functional groups that are capable of forming hydrogen bonds. Hence, control over the hydrophilic/hydrophobic balance requires the introduction of hydrophobic moieties, but in such a way that they also increase the ability of the molecule to aggregate. We attempted to modify the structure of 2'-deoxyuridine by introducing benzyltriazole ring derivatives at its C-5 position.[43] Heterocyclic moieties, especially those containing nitrogen atoms, are often introduced in the synthesis of polymeric and monomeric hydrogelators.[44] The triazole units increase the hydrophilicity of the compounds; the benzyl units increase both the hydrophobicity and degree of aggregation of the molecules through π–π stacking interactions between their aromatic rings.[45] We found that compound **63f** was the best among the set **63a**-**g** at forming hydrogels in pure water; its MGC was 0.2 wt%. Compounds **63a**, **63d**, and **63e** also formed stable hydrogels. Thus, it is possible to successfully prepare low-molecular weight hydrogelators based on 2'-deoxyuridine. These hydrogelators self-assemble through hydrogen bonding interactions and display aggregation properties that depend on the nature of these hydrogen bonds.[43]

The organogels are used as novel media to produce various silica structures, such as linear,[46] lamellar,[47a] and helical[47b] fibrous structures, through sol-gel polymerization. To date, transcription into silica *via* the polymerization of tetraethoxysilane (TEOS) has been limited to superstructures of molecules that possess cationic charge, in the form of metal cations,[46,47] or hydrogen bonding sites in the form of primary amines or combinations of primary and secondary amines.[48] In contrast, the compound **63a** does not possess either direct positive charge or an amino groups, which are capable of forming efficient hydrogen bonds. Moreover, the amide-type NH group of **63a** has difficulty in acting as a direct driving force for sol-gel transcription[49] because the amide-type NH groups form intermolecular hydrogen bonds between gelator units and because their protonation is more difficult than that of amines. Irrespective of these features, the hydrogel formed by the 2'-deoxyuridine derivative **63a** could be transcribed successfully into the silica structure through sol-gel polymerization of TEOS under acidic conditions.[50] We have found that an acidic proton can protonate the molecular aggregates (gel fibers) of **63a** in its gel phase, thus providing the driving force necessary for the transcription process.

It is known that the values of pK_b of triazole derivatives are relatively high,[51] such that the nitrogen atoms of the triazole group of gelator **63a** should be partially protonated under acidic conditions. In contrast, when we added a small amount of benzyl amine as base to the hydrogel of **63a**, the gel structure disappeared immediately. These results indicate that the gelation ability of **63a** is influenced by the pH of the medium. Preliminary results that we obtained using other gelators that do not contain positive charges or amino groups indicate that this phenomenon is quite general.



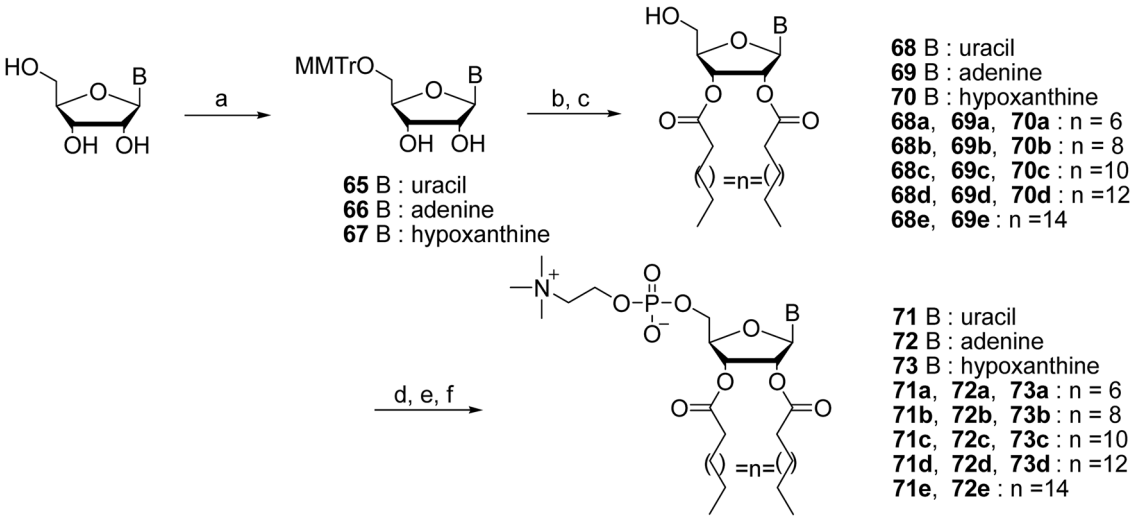
64

We have also synthesized a two-component gel system based on the concept of Watson-Crick hydrogen bonding of complementary base pairs. The nucleoside derivatives **57b** and **64** form two-component organogels in various organic solvents with values of MGC of 1-2 wt%.

Modified nucleosides as liposomes. The preparation of self-assembled supramolecular architectures that mimic the desired structures and functions of natural biological entities is of great interest. Phosphocholine lipid bilayers comprise one such class of supramolecular assembly that has attracted a great deal of research interest among chemical biologists. Phosphocholine bilayers are important constituents of cell membranes and, hence, a thorough understanding of these molecular assemblies is paramount when attempting to control cellular functions.

A number of phospholipids containing hydrophobic tail moieties and hydrophilic (glycerol) head groups have been studied extensively.[52] Recently, however, the conventional glycerol backbones have been replaced with various other units in the anticipation that such modified phospholipids would provide new opportunities for 1) forming new supramolecular structures and 2) attaching macromolecules or ligands for biological targeting. For example, several uridine-based phosphocholine amphiphiles have been synthesized and their supramolecular characteristic investigated.[53] Some of these derivatives formed molecular aggregates such as vesicles, fibers, hydrogels, and organogels in different media.

In our laboratory, we also became interested at the same time in the preparation of nucleoside-based phosphocholines



Scheme 3. (a) MMTr-Cl, pyridine, r.t., 9 h (uridine), 20 h (adenosine), 18 h (inosine); (b) EDC, DMAP, decanoic/lauric/myristic/palmitic/stearic acid, CH_2Cl_2, r.t., 4 h; (c) 80% acetic acid, 60 °C, 3 h; (d) DIPEA, 2-chloro-1,3,2-dioxaphospholane, THF, r.t., 15 min; (e) Br_2, 0 °C, 1 h; (f) 40% Me_3N, $CHCl_3$/*i*PrOH/CH_3CN (3 : 5 : 5), r.t., 3 days

that mimic cell membranes.[54] As a result, we synthesized a new class of amphiphiles based on nucleosides of the type **71**-**73**. These amphiphiles possess both molecular recognition units (towards complementary nucleic acids) and long, functionalized, fatty acid side chains as lipid moieties. These novel phospholipids containing uridine, adenosine, and inosine nucleobases were obtained through an efficient six-step reaction sequence starting from their corresponding nucleosides (Scheme 3). The 5'-*O*-protected nucleosides were treated with the fatty acids in the presence of EDC and DMAP to yield the corresponding 2',3'-diesters **68**-**70**. Phosphocholine groups were introduced into the 5'-positions of the diesters in three steps to obtain the corresponding phospholipids **71**-**73** in quantitative yields. The self-assembly of these derivatives in aqueous solution and the hydrodynamic radii of their aggregates were studied using various spectroscopic and microscopic methods.

The liposomes of compounds **71**-**73** were prepared under conditions that favor their self-assembly. They formed vesicles in a buffer solution comprising 30 mM Tris/HCl, 20 mM KCl, and 0.1 mM EDTA, which was adjusted to pH 8 at room temperature. The morphologies of the vesicles were observed using transmission electron microscopy (TEM); Figure 14 displays the resulting micrographs. From these images, it is clear that the liposomes aggregated into spherical closed liposomes. Each of the liposomes formed from the nucleoside-based phospholipids **71**-**73** had a spherical morphology, even though they all featured different nucleobases. From these results, we conclude that the morphologies of the nucleoside-based phospholipids are independent of the nucleobase. The most significant effect on the liposomes' structures arose from changes in the nature of the nonpolar alkyl chains. The vesicles formed from compounds containing longer alkyl chains were larger that those formed from the corresponding derivatives possessing smaller alkyl chains. This finding highlights the fact that the molecular structure of the lipid has the most important effect on the morphology and physical properties of the aggregates.

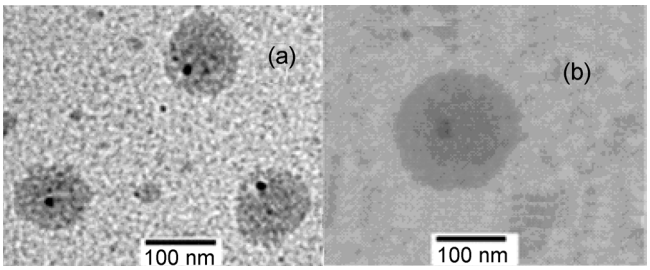


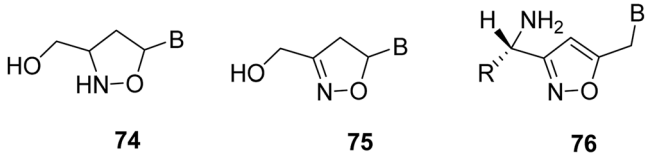
Figure 14. TEM images of negatively stained liposomes formed from dispersions of nucleoside-based phospholipids (0.3 mM) in Tris–HCl (30 mM)/KCl (20 mM)/EDTA (0.1 mM) at pH 8. Micrographs of (a) adenosine-based liposome **72b** and (b) uridine-based liposome **71b**.

To confirm the location of the nucleobases within the liposomes, and to study their molecular recognition properties, we treated dispersions of the liposomes with functionalized dyes that are specific toward certain base sequences. We recorded the confocal microscopy images of the liposomes formed from **71c** using a fluorescein isothiocyanate-2'-deoxynucleoside trimer (*e.g.*, FITC-5'd-AAA) and a hexachlorofluorescein-2'-deoxynucleoside trimer (Hex-AAA). Analysis of the images indicated that hydrogen bonding interactions existed between the uridine and complementary nucleotide trimer in a selective manner. That is, the liposome formed from **71c** binds both to the FITC- and Hex-linked adenosine trimers (*i.e.*, both of these dyes can be accommodated by the liposome). To rule out the possibility that this binding might be indiscriminate (*e.g.*, that it is the dye that binds and not the adenosine trimer unit), we irradiated a solution containing the liposome of **71c** and a mixture of the dyed oligonucleotide trimers FTIC-AAA, Hex-CCC, and Hex-GGG. The liposome selectively recognized the AAA unit over the CCC and GGG units (*i.e.*, selective hydrogen bonding existed between the uracil and

adenine bases). From these findings, we believe that liposomes of uridine-based phospholipids may recognize the poly(A) tail of mRNA. These nucleoside-based phospholipids possess large backbones that increase the spacing between the head and tail units, and they have increased hydrodynamic radii relative to other phospholipids prepared with different backbones.

Nucleosides and Oligonucleotides Containing Heterocycles

The introduction of different types of modified nucleosides into naturally occurring nucleotides and oligonucleotides is one of the most well studied areas in the field of nucleic acid chemistry. The principle aim of such modifications is to augment the natural properties of the nucleic acid. Recently, we reviewed the different types of modifications that have been undertaken to date to the base, sugar, and backbone units of nucleic acids.[4] In this section, we describe the successful introduction of nucleoside building blocks containing isoxazole and isoxazoline ring systems.



74 **75** **76**

Nucleosides containing heterocycles in place of the sugar. Potential antiviral drug candidates. Several nucleoside analogs containing heterocycles have been used as antiviral agents, especially, anti-HIV drugs. For instance, replacement of the nucleoside ribose moiety with a heterocyclic system has resulted in the preparation of an excellent anti-HIV drug, 3TC. Such modifications remain a promising strategy toward antiviral lead compounds.[55] Adams and coworkers prepared heterocyclic nucleoside analogues through cycloaddition reactions between 1-vinylthymine and 1,3-dipoles.[56] Isoxazolidinyl nucleosides **74** and dihydroisoxazole nucleosides **75** have been synthesized and their anti-HIV-1 activities investigated.[57] Based on these reports and our own earlier experience with 1,3-dipolar cycloadditions,[58] we synthesized novel isoxazole nucleosides of the type **76**. The principle step of this efficient synthetic method involves nitrile oxide cycloaddition with a propargylic dipolarophile; Scheme 4 displays a representative reaction.[59,28b] The products were obtained in moderate-to-quantitative yields. Using this synthetic protocol, we successfully prepared seventeen different nucleoside derivatives containing isoxazole rings in place of the sugar (ribose) moiety, different nucleobases (thymine, uracil, and 5-fluorouracil), and methylene linkers between them.

We investigated the antiviral activities of these heterocyclic nucleoside analogues using different bioassays. The bioassay results revealed that among the seventeen isoxazole nucleoside analogs that we screened, compounds **79a** and **79b** exhibited potent anti-polio activities (Table 2). In particular, compound **79a** displayed antiviral activity superior to that even of the reference drug, ribavirin, in terms of its EC_{50} value. Unfortunately, because these compounds displayed poor selectivity indexes (SI), they could not be recommended for further clinical trials.[28b]

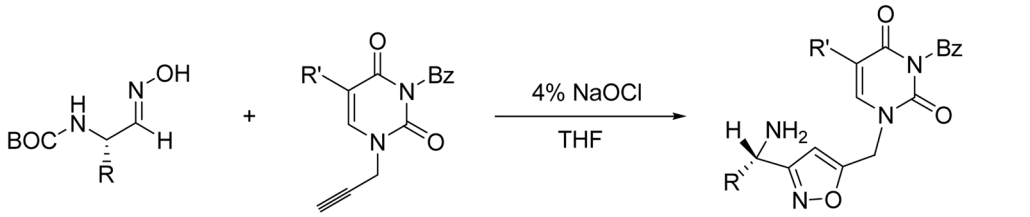
Preparation of oligonucleotides containing di- and trithymidines. Antisense oligonucleotides are used for the treatment of diseases at the level of gene expression. A number of new antisense oligonucleotides possessing different types of modifications in their backbone or phosphodiester linkages have been synthesized.[60] In addition, replacement of the phosphodiester linkage of the natural oligonucleotide with a neutral heterocyclic linkage renders them more stable against enzymes and more rigid. In our laboratory, we prepared thymidine derivatives, containing carboxylic acid (**80**) and amide groups (**81**) at the 3'-position, as basic building blocks for backbone-modified oligonucleotides. We used X-ray crystallographic data to elucidate the hydrogen bonding patterns of compounds **80** and **81**.[61]

Similarly, we prepared the thymidine derivative **82** in six steps from thymidine in an overall yield of 40.5%.[62] Recrystallization of the final product from dichloromethane/diethyl ether/*n*-hexane (1 : 1 : 1) gave enatiomerically pure

Table 2. Antiviral activities in terms of EC_{50} values

	Anti-polio activity (EC_{50} μg/mL)		Selectivity index (SI) (CC_{50}/EC_{50})	
Compound	COX.B3[a]	VSV[b]	COX.B3[a]	VSV[b]
79a	10.61	7.94	2.46	3.29
79b	98.07	80.02	1.02	1.25
Ribavirin[c]	92.45	15.62	5.58	27.26

[a]Coxsakie B virus type 3. [b]Vesicula Stomatitis virus. [c]Average values.

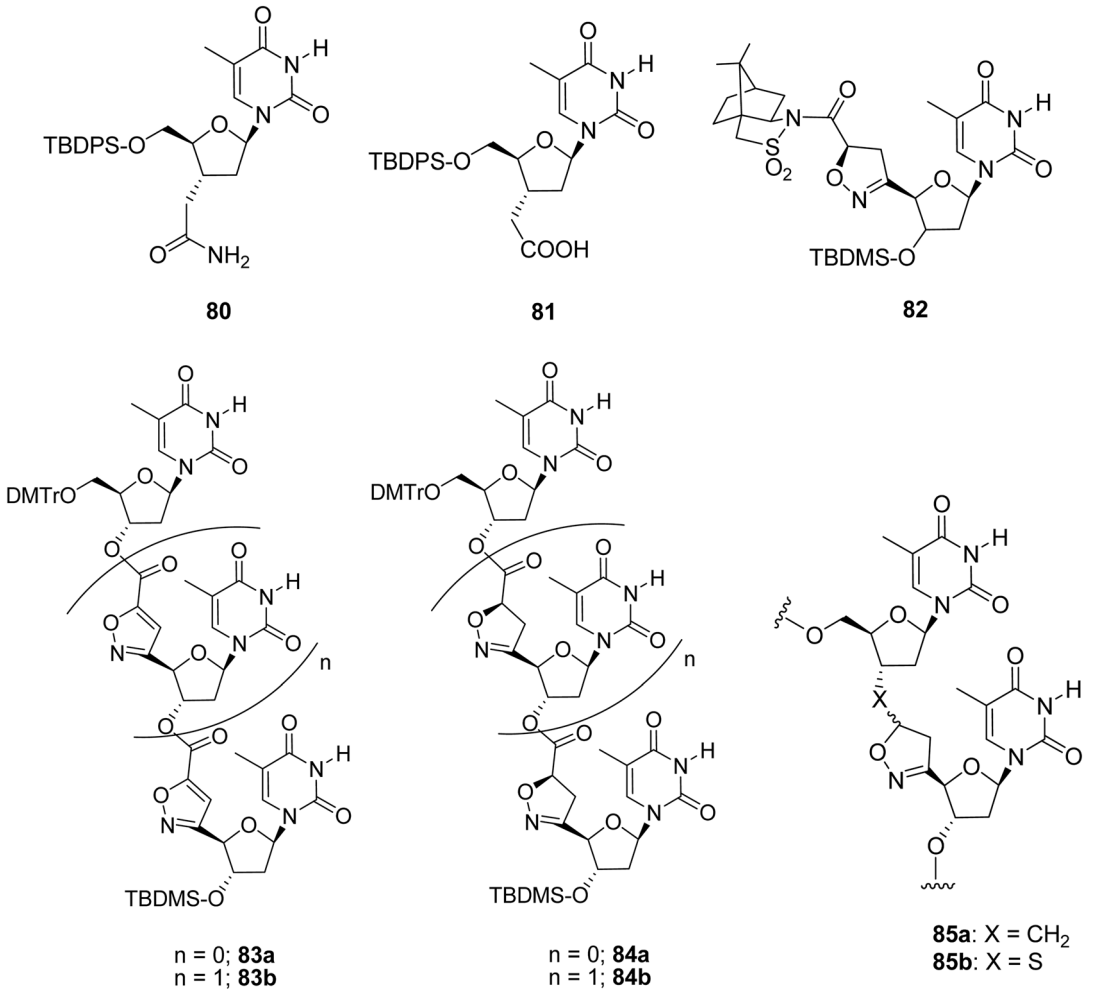


77a; R = $CH_2(C_6H_5)$
77b; R = $CH(CH_3)_2$

78a; R = CH_3
78b; R = H

79a; R = $CH_2C_6H_5$, R'=CH_3 (71%)
79b; R = $CH(CH_3)_3$, R'= H (74%)

Scheme 4. Synthesis of heterocyclic nucleosides.



82. An X-ray diffraction-grade single crystal of **82** was grown upon slow evaporation of its EtOH/EtOAc solution. X-Ray crystallographic analysis of **82** reveals that it self-assembled to form a cyclic dimer through hydrogen bonding between pairs of thymine bases. We designed an efficient synthetic approach toward the monomeric units, namely, di- or tri-nucleotides containing isoxazoline or isoxazole heterocycles in place of their phosphodiester linkages.[63] This method involves a dipolar cycloaddition reaction (similar to that presented in Scheme 4) followed by facile chain-extension steps, whereby the thymidine derivative **82** is subsequently converted into the di- and tri-nucleotides **83** and **84** in moderate yields.

In **83a** and **83b**, the phosphodiester backbones are replaced by an isoxazole ring system; in **84a** and **84b**, they are replaced by isoxazoline rings. Using a similar synthetic protocol, we prepared two different diastereoisomers of TT dinucleotides (**85a**; X = CH_2; **85b**; X = S) containing the isoxazoline moiety. These building blocks were subsequently incorporated into oligonucleotides by using the usual phosphoramidite method; we then studied their biological activities.[64] We successfully prepared four dodecanucleotides incorporating these TT dinucleotides in the middle of their DNA sequences; we studied their melting temperatures (T_m values) by measuring the change in their UV absorbance.

Summary

In this account, we describe our research efforts related to modified nucleic acid systems. Initially, we explored the applications of these modified nucleosides in the field of materials science; for example, a systematic study into the gelation properties of nucleosides provided us with some understanding into the functional groups necessary for a given nucleoside to behave as an organo or hydrogelator. We have also demonstrated that isoxazole- and isoxazoline-modified nucleoside analogs can possess potential antiviral activity; unfortunately, our failure to improve the selectivity index prevented us from submitting these materials to further clinical trials. We also studied the solution properties of several nucleoside-based phospholipid derivatives, which led us to conclude that suitably derivatized nucleoside phosphocholines, such as **71** to **73**, can indeed self-assemble into the form of spherical vesicles; the morphologies of these aggregates differ substantially. In addition, these vesicles can form complexes with complementary nucleotide trimers through hydrogen bonding; this feature makes them attractive alternatives to glycerol- and carbohydrate-based phospholipids. These results may provide new insight into the tailoring of vesicle properties for specific pharmaceutical and industrial applications.

The calixnucleoside derivatives reported herein are good building blocks for the synthesis of calix[4]arene/oligonucleotide conjugates (calixoligonucleotides) with defined structures (hairpin) and the ability to form triplexes. A series of steroid (cholane)-containing oligonucleotides had better cell permeability than did their naked oligonucleotide analogs. The highlight – and, perhaps, the most important aspect – of our research into modified oligonucleotides containing fluorescent tags was our use of them as molecular probes for SNP typing. Some of these fluorescent oligonucleotides, also called "molecular beacons," could discriminate between perfectly matched and single base mismatched sequences. In fact, some of these fluorescent oligonucleotides can also recognize conformational changes in DNA strands, *e.g.*, B to Z transitions. In addition, we have synthesized many siRNAs and are presently studying their cell permeability and gene silencing activities. We are excited to continue discovering the future applications of nucleosides, nucleotides, and oligonucleotide derivatives in a diverse range of fields.

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