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Templated synthesis of nylon nucleic acids and characterization by nuclease digestion

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Abstract

Nylon nucleic acids containing oligouridine nucleotides with pendent polyamide linkers and flanked by unmodified heteronucleotide sequences were prepared by DNA templated synthesis. Templatation was more efficient than the single-stranded synthesis: Coupling step yields were as high as 99.2%, with up to 7 amide linkages formed in the synthesis of a molecule containing 8 modified nucleotides. Controlled digestion by calf spleen phosphodiesterase enabled the mapping of modified nucleotides in the sequences. A combination of complete degradation of nylon nucleic acids by snake venom phosphodiesterase and dephosphorylation of the resulting nucleotide fragments by bacterial alkaline phosphatase, followed by LCMS analysis, clarified the linear structure of the oligo-amide linkages. The templated synthesis strategy afforded nylon nucleic acids in the target structure and was compatible with the presence heteronucleotides. The complete digestion procedure produced a new species of DNA analogues, nylon ribonucleosides, which display nucleosides attached via a 2'-alkylthio linkage to each diamine and dicarboxylate repeat unit of the original nylon nucleic acids. The binding affinity of a nylon ribonucleoside octamer to the complementary DNA was evaluated by thermal denaturing experiments. The octamer was found to form stable duplexes with an inverse dependence on salt concentration, in contrast to the salt-dependent DNA control.

Introduction

Recently, the significance of DNA has expanded beyond its essential role in life as the repository of genetic information. Owing to outstanding molecular recognition and self-assembly properties, and relatively simple and stable structure, DNA molecules have been utilized as linkers or templates to assemble many DNA nanostructures^{1,2} as well as self-assembled composites³ with fullerenes,^{4,5} nanoparticles^{6–9} or carbon nanotubes.^{10–13} DNA has also been used to construct hybrid nano-objects or nanodevices such as nanowires,^{14–17} field-effect transistors,¹⁸ optical or electrochemical sensors^{19–22} and nanomechanical devices.^{23–27} It has been used to template a variety of organic reactions.^{28–31} However, far fewer monodisperse and sequence-specific DNA-mediated polymer syntheses are known.^{32–34}

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[†]Electronic Supplementary Information (ESI) available: Full experimental details including: Templated syntheses of nylon nucleic acids, MALDI-TOF MS of nylon nucleic acids, controlled exonuclease digestion of nylon nucleic acids, exhaustive enzymatic digestion of nylon nucleic acids, LCMS analysis of nylon ribonucleoside oligomers, HPLC purification and MALDI-TOF MS analysis of nylon ribonucleoside oligomers, thermal denaturing studies of nylon ribonucleoside oligomer. Supplementary figures S1–S4, S6 and S7, supplementary table S5. See DOI: 10.1039/b000000x/

In previous work, we have reported inter- and intra-molecular^{35,36} amide linkages in DNA molecules, and the use of DNA as a scaffold for the construction of nylon-like polymers.^{37,38} Here, we describe the synthesis and detailed characterization of strands **3–9** (Table 1), some of which were used recently to examine the thermodynamic properties and structures of nylon nucleic acid (NNA) hybrids with complementary DNA and RNA.³⁹ In addition, we describe the isolation and characterization of an interesting new nylon-like oligomer that displays pendent nucleosides and forms stable duplexes with a complementary DNA sequence.

In our previous synthetic approach^{37,38} the 2'-position of an RNA analog nucleoside was derivatized with thioalkyl-diamine or dicarboxylate groups, and the pendent groups were condensed to form a short nylon-like chain crosslinked at every nucleoside to an RNA backbone (Fig. 1). In the earlier design, a 16-mer with four nylon nucleic acid residues flanked by polythymidine, 5'-(dT)₆UcUnnUccUn(dT)₆, was synthesized *via* single-stranded amide ligation from an uncoupled precursor hexadecanucleotide strand. Our notation indicates uridine precursor (i.e., uncoupled) nucleotides containing single amino or carboxyl modifications as **Un** or **Uc**, respectively, and diamine or dicarboxylate modifications are labeled as **Unn** or **Ucc**, respectively (Fig. 2). The coupled products (i.e., nylon nucleic acids) are indicated by an underline at the coupled nucleotides (e.g., UnUc). In this study, the presence of heterobases creates the potential for unwanted coupling reactions between pendent carboxylic acids and the amino groups of adenine, cytosine, and guanine. Another concern (e.g., strands **6–9**) is the potential cross-coupling between distant pendent groups; that is, instead of coupling between adjacent groups to form a linear polyamide linkage, the amino groups of **Unn** or **Un** residues could couple with the carboxyl groups of remote **Ucc** or **Uc** residues to form undesired (i.e., “short-circuit”) amide crosslinks. Given that single-stranded DNA (ssDNA) is very flexible and capable of forming loop structures or even turns of 180°, the chance of such side reactions is a potential issue and increases with additional modified residues incorporated into the strand. Therefore, we decided to prepare nylon nucleic acids **3–9** by employing a complementary DNA template with a hairpin tail (Fig. 2).³⁹ A nucleic acid double helix is much less flexible than single-stranded DNA,⁴⁰ the ~50 nm persistence length of double helical DNA under standard conditions corresponds to about 150 nucleotide pairs. At lengths well below this size, double-stranded DNA (dsDNA) behaves as a fairly rigid polymer, and, therefore, the relatively strict distance between nucleotides is expected to prevent the occurrence of undesired cross coupling. Furthermore, the nucleobase pairs reduce the exposure of reactive positions, diminishing the possibility of cross coupling reactions between pendent carboxyl groups and nucleobases. A 46-mer complementary hairpin DNA molecule was designed as a template because prior experience showed that it was more stable than just a simple complement;⁴¹ the extra nucleotides also facilitate its separation in subsequent gel purification.

Nuclease digestions of the synthesized strands would provide useful characterization of coupled NNA products. Controlled exonuclease digestion followed by MALDI-TOF MS analysis of the mass ladders has been used to determine DNA sequence,^{42,43} locate DNA lesions,⁴⁴ identify DNA secondary structure,⁴⁵ and study the binding of small molecules to DNA.⁴⁶ Another efficient tool for DNA analysis, complete exonuclease digestion combined with LCMS analysis^{47,48} has been applied to the detection of DNA adducts,^{49,50} DNA nucleobase lesions,^{51,52} post-transcriptional modification of RNAs,^{53,54} and DNA-metal complex interactions.⁵⁵ In this study, both exonuclease digestion methods were employed to allow not only the location and identification of the modified regions of nylon nucleic acids (Fig. 1), but also to provide quantification of the base identification and composition. Such experiments can clarify the regiochemistry of the amide ligations in nylon nucleic acids afforded by templated synthesis.

Results and discussion

DNA templated synthesis of nylon nucleic acids

The syntheses of nylon nucleic acid phosphoramidite monomers and precursor strands containing pendent monoamines, diamines, monocarboxylates and dicarboxylates were performed as described earlier,³⁷ except that the strands were cleaved from the solid support and deprotected at 50°C, rather than at room temperature. To test the chemical compatibility of templated nylon nucleic acid synthesis, all four deoxynucleotides (dT, dA, dC and dG) were included. The 18-mer precursor strands were designed to contain a central sequence of thymidines that could be substituted systematically with contiguous pendent uridines (Table 1). This central sequence was flanked on both sides by heteronucleotide sequences. After routine purification by HPLC or gel electrophoresis, both the precursor strands and the complementary hairpin were desalted by reverse-phase HPLC. This procedure is crucial to eliminate side reactions in the ensuing coupling step.

The templated synthesis was initiated by annealing the precursor strands with the complementary DNA hairpin to form a duplex. In the presence of the coupling reagent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), the pendent 2'-mercaptoalkyl amine and carboxylate groups were ligated to form a polyamide linkage. After annealing and coupling, an aliquot of solution was removed and subjected to non-denaturing gel electrophoresis, which confirmed that the oligonucleotides were still in the duplex form. In Fig. 3, uncoupled precursor strand **5a** and the hairpin are shown in lanes 2 and 5 of the electropherogram. A mixture containing a slight excess of hairpin and **5a** resulted in a new band indicating duplex formation shown in lane 3; similar observations were made for hairpin and nylon nucleic acid **5b** in lane 4. The excess of hairpin ensures that amide formation occurs in the context of duplex DNA. Minimal differences in gel mobility were observed for hairpin:**5a** and hairpin:**5b** as expected, since there is no change in net charge. The DMT-MM coupling reagent identified by a screen of available agents as described in the earlier study³⁷ also proved efficacious for coupling reactions here that occur in the presence of aminonucleotides. The coupled nylon nucleic acid molecule was separated from the template hairpin by denaturing gel electrophoresis. A series of nylon nucleic acid sequences containing 1 to 7 amide bonds aligned by all four natural heterobase nucleotides (**3b-9b**) was synthesized by this method.

MALDI-TOF MS analysis of nylon nucleic acids

Synthetic precursor strands and nylon nucleic acids were characterized by MALDI-TOF mass spectrometry. As shown in Table 1, observed masses agreed with theory. The coupling yields were estimated conservatively by integrating all observed peaks and comparing with the product molecular ion (M+1). Coupling reactions using only unpaired single strands of **6a**, **7a** and **8a** were performed to compare with reactions templated by the hairpin. The single-stranded synthesis of **6b** gave the anticipated product in 91 % estimated yield (Supporting information, S1). However, for nylon nucleic acid **7b**, though the desired product peak was still observed in the MALDI-TOF mass spectrum, side peaks appeared in high intensity, which led to only 28 % yield (73 % per amide) for single stranded synthesis (Supporting information, S2). The mass pattern became even broader for nylon nucleic acid **8b**, where the multiple side peaks form a hump with nearly obscured product mass peak (Fig. 4) and the yield dropped further to 21 % for single-stranded coupling (73 % per amide). The poor coupling efficiency of single-stranded synthesis of higher oligomers may originate from the unwanted cross couplings mentioned above, which can form amide bonds either between distant pendent groups or between nucleobases and pendent carboxyl functions. Such reactions may leave adjacent pendent groups intact.

In contrast to single-stranded synthesis, duplex DNA-templated syntheses of nylon nucleic acids **3b** – **7b** afforded about 96 % yield, which corresponds to 96 ~ 99 % yields for each coupling step. However, step yields were slightly lower for oligomers **8**–**9**, so the protocol was modified to include a second addition of DMTMM after 24 h. This method produced **8b** and **9b** in 92 and 94% yield, which corresponds to > 99% step yields for these reactions (e.g., Fig. 5). The MALDI-TOF MS results demonstrate that templated nylon nucleic acid synthesis can afford high product yields even with the formation of multiple amide bonds, consistent with the expectation that DNA templation positions the pendent groups so that adjacent groups react efficiently.

Controlled exonuclease digestion of nylon nucleic acids

The ODN sequence can be determined by mass spectral analysis of the ODN ladders produced by sequential removal of nucleotides with exonuclease. In this method, individual mononucleotides are identified from the mass differences between adjacent peaks corresponding to product ions from enzymatic cleavage.⁵⁶

To locate the modified regions of nylon nucleic acids, controlled exonuclease digestions were performed on the template-synthesized **3b** and **8b** and control strand **1**. The digestion reactions were monitored as a function of time. Phosphodiesterase II (calf spleen phosphodiesterase, CSP), which cleaves from the 5'-terminus, served as the exonuclease. The MALDI-TOF data revealed mass ladders for oligonucleotide sequencing from the 5'-end (Fig. 6). After 2 min of reaction time, the digestion of **3b** reached modified nucleotides **UnUc**. It took 4 min for the digestion of **8b** to move to the modified region. Control DNA **1** showed clear 9–17-mer ladder patterns (the lower mass range was obscured by doubly charged ions) in 0.5 min. With extension of digestion time, 4–9-mer ladders of DNA **1** were observed (Supporting Information S3), and after 1 h no DNA fragments longer than trimer were detected (the masses of 1–3-mer fall in the mass range dominated by matrix ions, Fig. 7A). By contrast, digestions of **3b** and **8b** stopped at modified nucleotides and, even after 4 h, no degradation was detected for either the 5'-**UnUc**TTGTCTAC fragment from **3b** or the 5'-**UcUnnUccUnnUccUn**GTCTAC fragment from **8b** (Fig. 7B, 7C). It has been reported that CSP is inhibited by sugar-modified nucleotides⁵⁶ and so these CSP digestion results are consistent with the 2'-ribose modification present in nylon nucleic acids. Furthermore, these data verify the unmodified DNA flanking sequence on the 5'-terminus.

Complete nuclease digestion of nylon nucleic acids

HPLC or LCMS analysis of nucleoside fragments generated from complete nuclease digestion of nucleic acids can provide precise information about base identity and composition. Thus, if a nylon nucleic acid molecule could be fully degraded by nuclease, a linear fragment consisting of uridine nucleosides linked only at the 2'-position by a polyamide would result. Without phosphodiester linkages, these uridine oligomers protrude from the polyamide backbone via the 2'-sulfide tether. Here, **U2**, **U3a**, **U3b**, **U4**, **U5**, **U6** and **U8** are used to designate the uridine oligomers resulting from the digestion of nylon nucleic acid molecules **3b**, **4b**, **5b**, **6b**, **7b**, **8b** and **9b**, respectively (Fig. 8). By contrast, any incompletely or incorrectly coupled isomers or other byproducts from nylon nucleic acid synthesis would not afford these unique oligomers but would produce other, shorter fragments. The ratio of product nucleosides, both unmodified nucleosides and amide-linked oligouridine, should agree with the base composition of individual strands, based on analysis of the UV chromatogram.

Phosphodiesterase I (snake venom phosphodiesterase, SVP) and bacterial alkaline phosphatase (BAP) served to catalyze the complete digestion of nylon nucleic acids. Reaction products were analyzed by LCMS. Initially, a Zorbax C18 analytical column

(Agilent Technologies) was used to separate the digestion products. For **3b**, **4b**, **5b**, **6b** and **7b**, UV chromatograms showed five peaks (Fig. 9). In order of elution, these peaks were identified as dC, dA, dG, dT⁵⁷ and a strongly retained fragment whose mass values and isotopic patterns were consistent with expectations for **U2**, **U3a**, **U3b**, **U4** and **U5**, respectively. Correlating with the number of uridine residues, the retention times of these oligomers increased in the following order: **U2**<**U3a**≈**U3b**<**U4**<**U5**. Though in different sequences, **U3a** and **U3b** both have three modified residues and therefore displayed very similar retention times. A C8 column was required for the analysis of the more hydrophobic **U6** and **U8** (Fig. 10). Under the same elution conditions, retention times were shorter and the elution order changed to dC, dG, dA, dT⁵⁸ and the uridine oligomers. The ESI-MS of **U6** and **U8** afforded peaks corresponding to both singly- and doubly-charged molecules by adjusting instrument parameters (Fig. 10). The UV chromatogram also showed that **U8** had a slightly longer retention time than **U6**. Integration ratios from all UV chromatograms were measured, and the relative ratios of the five components (dC, dA, dG, dT and oligouridine) were consistent with the base composition of the individual strands (Supporting Information S5). For **6b-9b**, only the expected oligomers from linear linkages were observed, and shorter uridine oligomers were not detected. Modified uridines linked with other nucleosides were not observed for any of the compounds studied. These results demonstrate that templated synthesis produces nylon nucleic acids in high yield with coupling only between pendent groups attached to adjacent nucleotides. This key observation relating to the fidelity of the templated coupling reaction indicates that this approach is a robust route to the target, nylon nucleic acids.

As a control, the precursor strand **6a** was also subjected to complete nuclease digestion and LCMS analysis, affording all eight different nucleosides (dC, dA, dG, dT, Un, Unn, Uc and Ucc, supporting information S6). Thus, complete nuclease digestion, coupled with LCMS analysis, provided both identification and quantification of nucleosides.

Hybridization studies of **U8** to native DNA

After removal of the phosphate groups, the remaining oligoamides display nucleosides (uridine in this case) protruding from the polymer backbone. These digestion products are reminiscent of the peptide ribose nucleic acid molecules (PRNA) reported by Wada,^{59,60} and are more broadly related to peptide nucleic acids (PNA) in general.^{61–63} Given the relationship of our digestion products to PRNA and PNA, the binding affinity of these neutral oligomers to native DNA was examined.

The nylon ribonucleosides **U4**, **U5**, **U6** and **U8** were isolated by preparative reverse-phase chromatography using a Zorbax C8 column and characterized by MALDI-TOF MS. The mass signals were in accord with LCMS measurements (Fig. 9H, Fig. 10F and Fig. 10I, Supporting information S4E). Therefore, these data have provided additional supports for the assigned structures of nylon nucleic acids afforded by templated synthesis.

Because the study of the stable duplex with oligodeoxyadenosine (dA) requires at least a heptamer,^{64,65} the synthesis and purification of **U8** was scaled up. The hybridization behavior of the polyamide-linked **U8** with a phosphate-linked deoxyadenosine octamer (dA₈) was assessed by thermal denaturing experiments. In light of reports^{66,67} that oligonucleotides containing dT formed more stable duplexes than analogous strands containing dU, the phosphate-linked deoxyuridine octamer (dU₈) was synthesized and served as a control for **U8**.

The stability of the **U8**:dA₈ duplex showed a marked inverse dependence on salt concentration (Fig. 11A). In the absence of salt, a cooperative melting curve was observed with a melting temperature (T_m) at about 10 °C. The addition of salt to the sample

destabilized the duplex; T_m decreased to 8.7 °C in 10 mM sodium phosphate buffer and dropped further to 6.5 °C in 100 mM NaCl. No melting transition was detected above 300 mM NaCl. In contrast, the binding affinity of dU₈:dA₈ exhibited the usual nucleic acid trend, showing a direct dependence on salt concentration (Fig. 11B). Under salt-free and low salt (10 mM phosphate) conditions, the UV transition normally associated with association/dissociation of duplex was not observed. With the increase of salt concentration from 100 mM NaCl to 1 M NaCl, a clear enhancement of the UV transition was shown. For unmodified DNA duplexes, it is known that high sodium cation concentration can counteract the negatively charged phosphate backbone and increase helix stability.⁶⁸ For neutral DNA analogs, such as PNA⁶⁹ and amide-linked polythymidine,⁷⁰ it has been reported that increasing the salt concentration impairs binding to DNA; this finding was rationalized as differential stabilization of the phosphate-containing single strand. Nevertheless, the present nylon oligoribonucleoside hybridized with its DNA complement and formed a stable duplex in water and low salt buffer. Furthermore, the nylon oligouridine we obtained exhibited good water solubility, in contrast to the common behavior of PNA. The compound **U8** was found to be soluble at a concentration of at least 3 mM in aqueous solution at ambient temperature; higher concentrations were not tested. This significant solubility, about a factor of 10 greater than expected for analogous naked PNA,^{71,72} can be attributed to the presence of the hydrophilic ribose moieties. Regarding potential stability *in vivo*, the molecule was generated by exhaustive enzymatic digestion of nylon nucleic acids, so the nuclease resistance of this DNA analogue is certain. To our knowledge, this nylon-linked oligonucleoside is the first DNA analogue produced from complete nuclease digestion. It may have applications in special environments unsuitable for unmodified DNA or other DNA analogs, such as binding DNA in low dielectric media.

Conclusions

A series of hybrid nylon nucleic acid strands containing heterobase nucleotides was synthesized using DNA templation. To evaluate the efficiency of synthesis and to clarify the chemical structure of nylon nucleic acid, a variety of analytical techniques were employed. First, MALDI-TOF MS analysis demonstrated that nylon nucleic acid molecules with up to 7 amide linkages were synthesized in very high yield; the efficiency of templated coupling reactions was markedly greater than in single stranded coupling. Second, modified nucleotide regions were identified by controlled exonuclease digestion, coupled with MALDI-TOF analysis. Finally, a combination of complete nuclease digestion with LCMS analysis or HPLC – MALDI-TOF analysis established that the nylon nucleic acids were coupled only to amines/carboxylates from neighboring nucleotides and further verified that the 2'-amide linker formation was in a linear arrangement that was compatible with the presence of hetero-base nucleotides. The success of DNA templated synthesis of nylon nucleic acid provides a practical strategy to assemble organic polymers under the direction of DNA; this is another step toward our long-term goal of using the control afforded by nucleic acids^{37,73–76} to direct the topology of polymers with industrial importance. Furthermore, the complete nuclease digestion of nylon nucleic acids produced a new species of DNA analogue, a nylon-like polyamide with pendant nucleosides attached via the 2' position of ribose. Thermal denaturing studies revealed that this nylon ribonucleoside formed stable double helices with complementary DNA molecules under conditions that ranged from salt-free to medium-salt solutions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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57. The LCMS eluent contains 0.1% formic acid (C18 column) to facilitate the coupled ESI-MS analysis, so the elution order of nucleosides is different from that reported for neutral RP-HPLC analysis on a C18 column (dC, dG, dT, dA). (See: Eadie JS, McBride LJ, Efcavitch JW, Hoff LB, Cathcart R. *Anal Biochem.* 1987; 165:442. [PubMed: 2827538] Andrus A, Kuimelis RG. *Current Protocols in Nucleic Acid Chemistry.* 2000:10.6.1–10.6.6. The nucleoside elution order under the slightly acidic conditions is dC, dA, dG and dT on a C18 column, which was confirmed by mass data and also by control experiments with commercial nucleosides and digests of unmodified DNA.
58. On a C8 column the nucleosides elution order under slightly acidic LCMS conditions is dC, dG, dA and dT, which is different from C18 column separation in the same LCMS conditions (dC, dA, dG and dT) and also the neutral HPLC C18 column separation (dC, dG, dT and dA) (For example, compare Fig. 9A with Fig. 10A, where the same digestion products from DNA 1 were separated

on C18 and C8 columns). Commercial nucleosides and digests of normal DNA were used to verify the observation.

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Fig. 1. Schematic illustration of nylon nucleic acid synthesis and complete nuclease digestion to produce nylon ribonucleotides.

Fig. 2.

Templated synthesis of nylon nucleic acid **9b**. A DNA hairpin template strand with a complementary sequence to the strand **9a** forms a stable duplex. Proximal amines and carboxylates are then connected using chemical ligation to form a nylon backbone (red strip). The nylon nucleic acid **9b** is separated from the hairpin template by denaturing gel electrophoresis.

Fig. 3. Non-denaturing gel electrophoresis of nylon nucleic acid to confirm annealing with the complementary hairpin DNA. Lane **1**: 10 Nucleotide pair marker ladder; Lane **2**: Uncoupled precursor strand **5a**; Lane **3**: Hairpin:**5a** (before adding coupling agent, 1.1 eq added hairpin DNA); Lane **4**: Hairpin:**5b** after coupling (note minimal effects on mobility, because the net charge is not altered); and Lane **5**: Hairpin.

Fig. 4. Comparison of MALDI-TOF mass spectra of (A) the uncoupled precursor strand **8a**; (B) DNA templated coupling product nylon nucleic acid **8b** (coupling reagent, DMTMM, was added only once); (C) DNA templated synthesis of nylon nucleic acid **8b** (coupling reagent, DMTMM, was added twice at an interval of 24 h); and (D) single-stranded synthesis of nylon nucleic acid **8b** (without template).

Fig. 5. MALDI-TOF mass spectra of (A) the precursor strand **9a** and (B) DNA templated synthesis of nylon nucleic acid **9b**.

Fig. 6. MALDI-TOF mass spectra of phosphodiesterase II (CSP) digests of DNA **1** at 0.5 min (A), nylon nucleic acid **3b** at 2 min (B) and **8b** at 4 min (C). The signals corresponding to doubly charged ions are marked with an asterisk (*).

Fig. 7. MALDI-TOF mass spectra of phosphodiesterase II (CSP) digests of DNA **1** at 1 h (A), nylon nucleic acid **3b** at 4 h (B) and **8b** at 4 h (C).

Fig. 8.

Structures of uridine oligoribonucleosides resulting from the complete nuclease digestion of nylon nucleic acids.

Fig. 9. LCMS analysis of DNA **1** and nylon nucleic acids **3b** – **7b** complete nuclease digestion products and MALDI-TOF mass spectrum of **U5**. (A) UV chromatogram of DNA **1** digest; (B) UV chromatogram of **3b** digest; (C) UV chromatogram of **4b** digest; (D) UV chromatogram of **5b** digest; (E) UV chromatogram of **6b** digest; and (F) UV chromatogram of **7b** digest. Items (G) ESI mass spectrum and (H) MALDI-TOF mass spectrum of **U5** are shown as examples. For mass spectra of **U2**, **U3a**, **U3b** and **U4** see supporting information.

Fig. 10.

LCMS analysis of DNA **1**, **2** and nylon nucleic acids **8b**, **9b** complete nuclease digestion products and MALDI-TOF mass spectra of **U6** and **U8**. (A) UV chromatogram of DNA **1** digest; (B) UV chromatogram of **8b** digest; (C) UV chromatogram of DNA **2** digest; (D) UV chromatogram of **9b** digest; (E) ESI mass spectrum of **U6** eluate from the LC separation with target mass of quadrupole ion trap control set to $[M]$, both mono-ionic peaks ($[M+1]^+$, $[M+Na]^+$) and bi-ionic peaks ($[M+2H]^{2+}$, $[M+2Na]^{2+}$) are shown; (F) MALDI-TOF mass spectrum of **U6**; (G) ESI mass spectrum of **U8** with target mass of quadrupole ion trap control set to $[M]$, only mono-ionic peak ($[M+Na]^+$) is shown; (H) ESI mass spectrum of **U8** with target mass of quadrupole ion trap control set to $[M/2]$, both dicationic peaks ($[M+2H]^{2+}$, $[M+2Na]^{2+}$) and tricationic peak ($[M+3H]^{3+}$) are shown; (I) MALDI-TOF mass spectrum of **U8**, peak corresponding to a fragment (molecular ion minus one uracil base) is marked with an asterisk (*).

Fig. 11.

Melting curves under the indicated salt conditions for (A) U8: dA_8 , and (B) dU8: dA_8 . $[A] = A_T/A_{\text{max}}$. (A_{max} are the absorbance at 25 $^{\circ}\text{C}$ for (A) and the absorbance at 40 $^{\circ}\text{C}$ for (B)). Duplex concentration: 37 μM .

Table 1

Oligonucleotides used in this study and MALDI-TOF MS analysis.

ODNs	Sequence ^a
1	5'-GCATAGTTTTTTGTCTAC
2	5'-GCATATTTTTTTTCTAC
3a	5'-GCATAGTTUnUcTTGTCTAC ^b
3b	5'-GCATAGTTUnUcTTGTCTAC ^b
4a	5'-GCATAGTTUcUnnUcTGTCTAC ^c
4b	5'-GCATAGTTUcUnnUcTGTCTAC ^c
5a	5'-GCATAGTTUnUccUnTGTCTAC ^b
5b	5'-GCATAGTTUnUccUnTGTCTAC ^b
6a	5'-GCATAGTUcUnnUccUnTGTCTAC ^b
6b	5'-GCATAGTUcUnnUccUnTGTCTAC ^b
7a	5'-GCATAGTUcUnnUccUnnUcGTCTAC ^b
7b	5'-GCATAGTUcUnnUccUnnUcGTCTAC ^b
8a	5'-GCATAGUcUnnUccUnnUccUnGTCTAC ^b
8b	5'-GCATAGUcUnnUccUnnUccUnGTCTAC ^b
9a	5'-GCATAUcUnnUccUnnUccUnnUccUnTCTAC ^c
9b	5'-GCATAUcUnnUccUnnUccUnnUccUnTCTAC ^c

^aODN **1** is an unmodified DNA control for **3a/3b-8a/8b**, **2** is an unmodified DNA control for **9a** and **9b**. ODNs **3a-9a** are uncoupled precursor strands with 2, 3, 3, 4, 5, 6 or 8 modified nucleotides incorporated, and **3b-9b** contain corresponding nylon nucleic acid sequences.

^bRef 39.

^cMALDI-TOF MS (calculated, found): **4a** 5840.8, 5838.9; **4b** 5804.8, 5803.9; **9a** 6594.6, 6594.6; **9b** 6468.6, 6468.6.