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# Solid-phase Synthesis of Positively Charged Deoxynucleic Guanidine (DNG) Modified Oligonucleotides Containing Neutral Urea Linkages: Effect of Charge Deletions on Binding and Fidelity

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**Abstract**—A solid-phase synthesis for a DNA analogue with a mixed guanidinium and urea backbone is reported. This material is nearly identical in structure to deoxynucleic guanidine (DNG) but the neutral urea internucleoside linkages can be used to attenuate the overall positive charge on the oligomer. The opposite charge attraction between urea containing DNG oligomers (DNGUs) and complimentary DNA can be controlled so that the affinity of DNG for DNA does not overwhelm the base-pairing discrimination necessary for specific binding. Octameric DNGU containing between 1 and 3 urea substitutions covered the range between very tight and very weak bonding. Each deletion of a positive charge reduced the thermal denaturation temperature ($T_m$) by approximately 5 °C. Mismatches in the DNA oligomers reduced the $T_m$ values by 3 to 5 °C for each of the DNGU oligomers. DNGUs were found to bind in a 2:1 fashion to complimentary DNA in the same manner as DNG. © 2000 Published by Elsevier Science Ltd.

## Introduction

Oligonucleoside analogues capable of arresting cellular processes at the translational and transcriptional levels via recognition and binding to complementary RNA or DNA are known, respectively, as antisense and antigene agents.[1–3] Important goals in designing antisense compounds include increasing the binding affinity to the base sequence while maintaining fidelity of recognition, resistance to degradation by nucleases, and effective membrane permeability. A recurring theme in many of these antisense compounds is the incorporation of neutral internucleoside linkages that eliminate the mutual repulsion between the negatively charged phosphate diester backbones in duplex DNA. These unnatural linkages are likely to be resistant to nucleases and may also be membrane permeable. Oligonucleosides linked by amides,[4] phosphonates,[5] carbamates,[6] methylenemethylimino (MMI),[7] heterocycles,[8] and acetals[9] are representative of this approach. Another approach is to replace the phosphoribose backbone entirely, such as in the cases of PNA,[10,11] PHONA,[12] or PNAA.[13]

In recent years, approaches involving the incorporation of positive charges in antisense oligomers have been developed. Positive charges can be added to the bases[14] or the sugar rings to give zwitterionic DNA. The phosphate backbone can be alkylated with alkylamines to produce positively charged phosphate triester linkages between the nucleosides.[15] Another promising approach is to replace the internucleoside phosphate linkage with a positively charged, achiral guanidinium group.[16] The guanidinium linkage is resistant to nucleases[17] and the positive charges of the backbone may give rise to cell membrane permeability through electrostatic attraction of the oligonucleoside to the negatively charged phosphate groups of the cell surface. Micelles featuring positively charged surfaces have been reported to carry impermeable compounds such as DNA oligomers through the lipid bilayer of cells.[18]

In recent years, a number of publications have reported the synthesis and properties of DNG thymidyl pentamers (DNG-5 in Chart 1) and have shown than these pentamers bind very strongly in a 2:1 fashion to complimentary homopolymeric adenyl DNA (poly(dA)) but do not bind to homopolymers of the other DNA bases.[19,20] Dimeric DNG compounds have been developed that allow the inclusion of DNG linkages into DNA oligomers via

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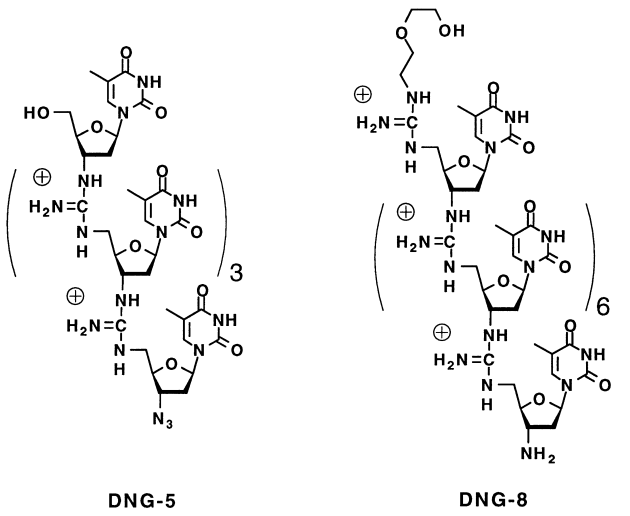
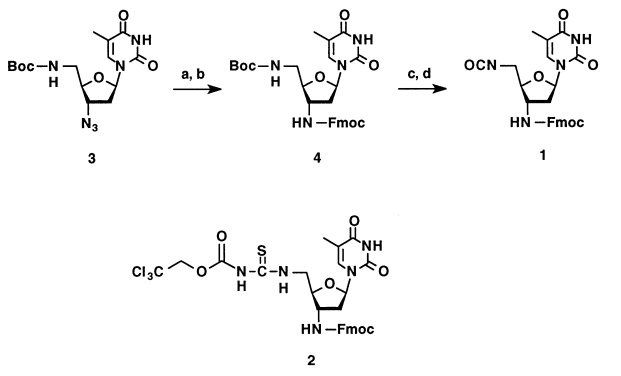



**Chart 1.** DNG oligomers.



**Scheme 1.** Synthesis of monomers for solid-phase synthesis of DNGUrea oligomers: (a) $H_2$, 10% Pd/C, EtOH; (b) 9-fluorenylmethyl-chloroformate, dioxane, 10% sodium carbonate; (c) 20% TFA/DCM; (d) carbonyl diimidazole, TEA, DCM.

standard DNA solid-phase synthesis and the properties of these oligomers are being investigated.[17,21] Recently, a facile solid-phase synthesis of DNG oligomers has been developed that enables the rapid synthesis of DNG oligomers longer than the previous limit of five units. Using this method, a DNG thymidyl octamer (DNG-8 in Chart 1) was synthesized and demonstrated to bind strongly to DNA adenyl octamers in a 2:1 fashion and yet cytidyl mismatches in the DNA weakened the association, demonstrating that sequence specificity was maintained.[22]

Because of the very strong binding affinity of DNG oligomers for DNA, the possibility exists that sequence specific base pairing may be lost to the overpowering electrostatic attraction between the positively charged backbone of the DNG and the negatively charged DNA as longer oligomers are employed. A solution to this eventual possibility is to reduce the overall charge on the oligomer by including neutral internucleoside linkages among the positively charged guanidinium linkages. There are many candidates for such a linkage as one could utilize any of the neutral internucleoside linkages previously reported. To study the effect of charge deletion of the binding affinity of DNG with DNA, the urea internucleoside linkage was chosen because it is isosteric with DNG. The only variable in the oligomers will be the difference in the overall positive charge. This paper reports the solid-phase synthesis of charge deleted urea substituted thymidyl DNG octamers, referred to as DNGU, and the effect of these reduced charges on the binding affinity and fidelity with complementary and mismatched short DNA oligomers.

## Results and Discussion

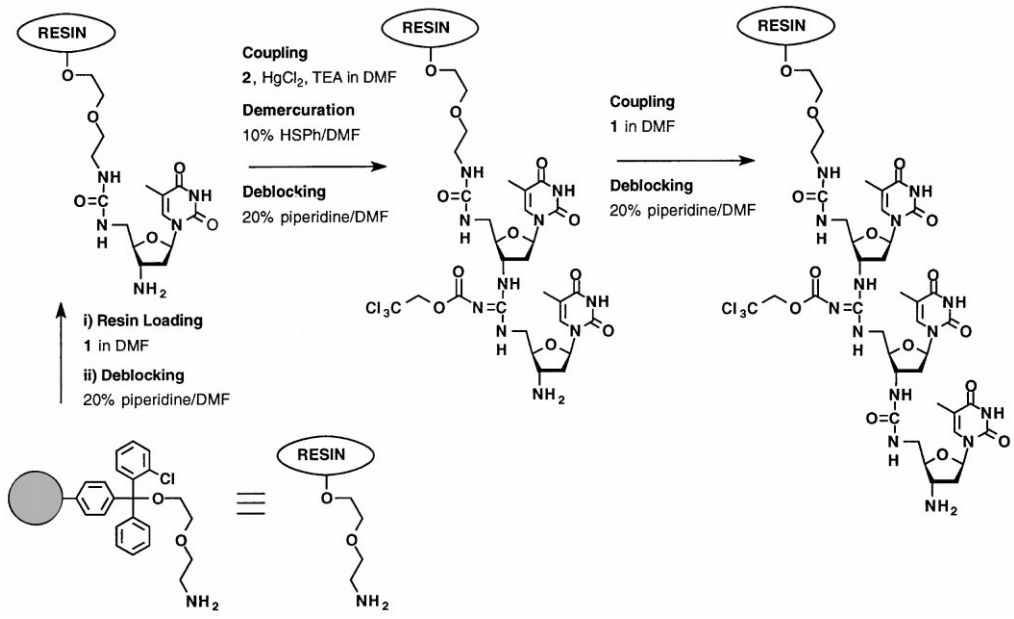
### Solid-phase synthesis of DNG/urea oligomers

Monomers for the various solid-phase synthesis approaches were synthesized as outlined in Scheme 1. The syntheses of **2** and **3** have been described previously[16,22] and the details for the synthesis of **4** and **1** are described in Experimental.

A commercially available 2% cross-linked polystyrene 2-(2-aminoethoxy)ethanol 2-chlorotrityl resin was used as the solid support. The amino functionality allows the loading step to use the same chemistry as the subsequent coupling steps and the chlorotrityl linker may be cleaved under mild acidic conditions. The synthesis cycle proceeds in the 5′–3′ direction as outlined in Scheme 2. The solid-phase synthesis of the guanidinium linkages in the oligonucleotides is based on the previously reported solid-phase synthesis of DNG oligomers[22] where an activated carbodiimide is created in situ by the addition of mercuric chloride and triethyl amine (TEA) to the protected thiourea monomer **2**. This mixture is poured over the resin beads where the 3′-amine on the terminal end of the growing oligomer attacks the carbodiimide to form a trichloroethoxy (troc) protected guanidinium. After the coupling step, mercury precipitate entrained in the resin is removed by a brief wash with 10% thiophenol in DMF. Using 6 equiv of monomer **2** and agitating for 2 h, coupling yields of 98% are observed.[22] The troc group is stable to the coupling conditions and is easily removed by cadmium or zinc powder and acetic acid.[23]

The urea linkages require no protection and are incorporated using the well known isocyanate route,[24,25] as outlined in Scheme 2. The monomer **1** is dissolved in DMF and this solution is poured directly over resin beads where the 3′-amine on the terminal end of the growing oligomer attacks the isocyanate and directly forms the urea linkage. Using 6 equiv of monomer **1** and agitating for 6 h, coupling yields of 92% are observed. After each coupling step the Fmoc protected 3′-amino group on the terminus of the oligomer is removed with 20% piperidine in DMF to give the free 3-amino group which is available to continue the extension of the oligomer.

Five examples of urea substituted DNG oligomers were synthesized on the resin and are detailed in Table 1. After the solid-phase synthesis was complete the oligomers were cleaved from the resin with 3% trifluorotacetic acid (TFA) in dichloromethane (DCM) and the filtrate was precipitated with cold ether. This gave the crude troc protected oligomers that were purified by reverse-phase HPLC chromatography. The products were characterized by electrospray mass spectrometry while in the protected




**Scheme 2.** Examples of the coupling steps in the synthesis cycle that give rise to a protected guanidinium linkage and a urea linkage in the oligonucleotide. By combining each of these two cycles in a given order, the desired oligonucleotide can be synthesized.

form. This allowed unambiguous assignment of the composition of urea and troc-protected guanidinium linkages in each oligomer. After deprotection, the small mass difference between urea and guanidinium groups would have made such an assignment more difficult.

The purified protected oligomers were then individually dissolved in acetic acid and a small amount of powdered cadmium was added. The heterogeneous mixture was agitated vigorously for 30 min and the solid was removed by centrifugation and decanting the acetic acid supernatant. The supernatant was evaporated in vacuo and the residue was desalted by using a cation exchange column with volatile ammonium acetate as the elutant. The fractions containing the deprotected oligomer were combined and evaporated. The residue was dissolved in water and evaporated two more times to remove all the ammonium acetate. The yield of desired oligomer compared to the theoretical maximum based on resin loading was between 25 and 40% for the five oligomers.

**Table 1.** DNGUrea and DNA oligomers

| Oligo | Sequence |
|---|---|
| DNGU-1 | $HO(CH_2)_2O(CH_2)_2$-**u**TgTgTgTgTgTgTgT-$NH_2$ |
| DNGU-2 | $HO(CH_2)_2O(CH_2)_2$-**u**Tg**u**Tg**u**Tg**u**TgT-$NH_2$ |
| DNGU-3 | $HO(CH_2)_2O(CH_2)_2$-**u**Tg**u**Tg**u**Tg**u**TgTgT-$NH_2$ |
| DNGU-4 | $HO(CH_2)_2O(CH_2)_2$-**u**TgTgTg**u**TgTgTgT-$NH_2$ |
| DNGU-5 | $HO(CH_2)_2O(CH_2)_2$-**u**TgTg**u**TgTg**u**TgTgT-$NH_2$ |
| DNG-8 | $HO(CH_2)_2O(CH_2)_2$-gTgTgTgTgTgTgTgT-$NH_2$ |
| DNA I | HO-ApApApApApApApA-OH |
| DNA II | HO-**C**pApApApApApApA-OH |
| DNA III | HO-**C**pApApApApApApA**C**-OH |
| DNA IV | HO-**C**p**C**pApApApApA**C**p**C**-OH |
| DNA V | HO-ApApAp**C**p**C**pApApA-OH |
| DNA VI | HO-ApApAp**C**pApApApA-OH |

The cation exchange HPLC chromatograms of the purified DNGU oligomers are presented in Figure 1. The retention times for each oligomer are dependent on the number of positive charges present on the oligomers. The oligomers with lower overall charge such as DNGU-2 and-3 elute earliest and retention times increase with the increasing content of guanidinium linkages as demonstrated with DNGU-5 and-4. The longest retention time among the DNGUrea oligomers is for DNGU-1 that has seven guanidinium groups.

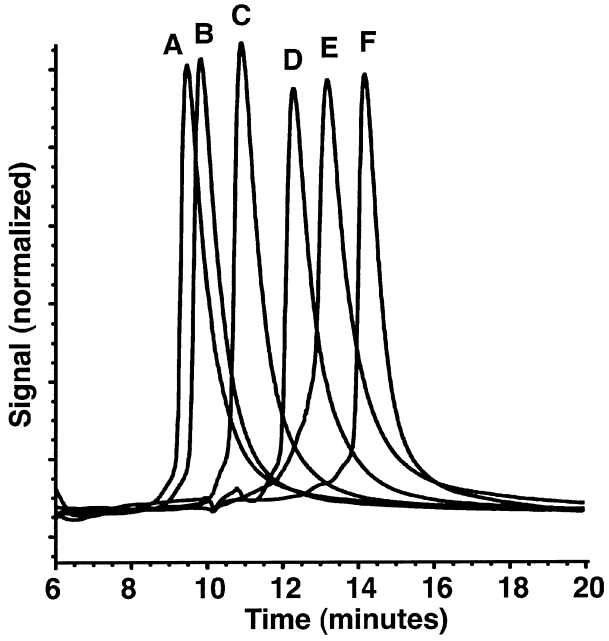



**Figure 1.** Analytical cation exchange HPLC of DNGUrea oligomers. Scale is normalized. A: DNGU; B: DNGU-2; C: DNGU-5; D: DNGU-4; E: DNGU-1; F: DNG-8.

### Binding of DNGUrea oligomers to DNA

Due to the planar nature of guanidinium groups, five of the atoms between each pair of linked nucleotides will be co-planar. The result is that the guanidinium backbone of DNG oligomers is more rigid than the phosphate diester backbone of DNA. It has been reported that including urea linked dinucleotides in otherwise DNA oligomers reduces the binding affinity of these modifies oligonucleosides for complimentary DNA due to the unfavorable steric contribution of the rigid urea linkages.[26] Since urea groups are isosteric with guanidinium groups, the same steric penalty can be expected for guanidinium linked oligonucleotides. When DNG dinucleotides are included in otherwise DNA oligomers, the binding affinity for DNA changes very little due to the opposite charge attraction of the guanidinium groups for the phosphate backbone of the complimentary DNA compensating for the steric penalty.[21]

In the case of homo-oligomers of DNG, the positive charge attraction for the backbone of target DNA more than compensates for the steric cost of the DNG backbone and very high binding affinities are observed. Octameric thymidyl DNG-8 binds to octameric adenyl DNA (DNA-1) in a 2:1 fashion with a thermal denaturation temperature ($T_m$) of 63 °C.[22] Theoretical dynamics computation of DNG oligomers base-pairing to a complimentary DNA oligomer indicate that the guanidinium internucleoside linkages are very similar in structure to calculated urea structures.[27,28]

To investigate the contribution of the positive charge to the association of DNG oligomers with DNA, the reduced charge oligomers DNGU-1 to 5 were synthesized as described above. These oligomers are very similar to the homoguanidinium oligomer DNG-8 due to the similar structure of the urea substitutions to the structure of the guanidinium groups that they replace. Figure 2 shows the CD spectrum for the DNGU oligomers compared with that for the original DNG-8. It can be seen that these spectra are very similar indicating no major changes in backbone structure or base orientation in the oligomers.

Job or continuous variation plots were performed to measure the stoichiometry of association (Fig. 4). The absorbance at 260 nM was measured for samples containing a constant total concentration of 2 μM oligonucleoside varying [DNGU] between 0 and 100% with a given DNA oligomer making up the remainder. The samples were mixed and allowed to equilibrate for 12 h at room temperature. The inflection point in the plot indicates the stoicheometry of the DNGU:DNA complexes. Job plots of oligomers DNGU-1,-2,-3,-4, and-5 complexed with DNA-**I** in 10 mM phosphate buffer and 0.1 M KCl salt for ionic strength are shown in Figure 3. The Job plot for DNGU-1 with DNA-**I** shows a clear inflection at 66% DNGU-1 indicating a 2:1 association of the DNGU oligomer with the complimentary DNA target.

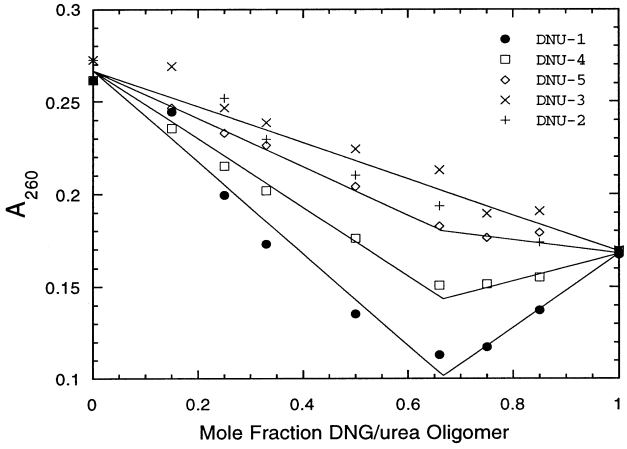



**Figure. 3.** Job plot of DNGU oligomers with DNA oligomer **I** plotted by %. [Total oligomer] = 2.0 uM, [$KHPO_4$] = 10 mM, [KCl] = 0.1 M, pH 7.4, observed at 260 nM.

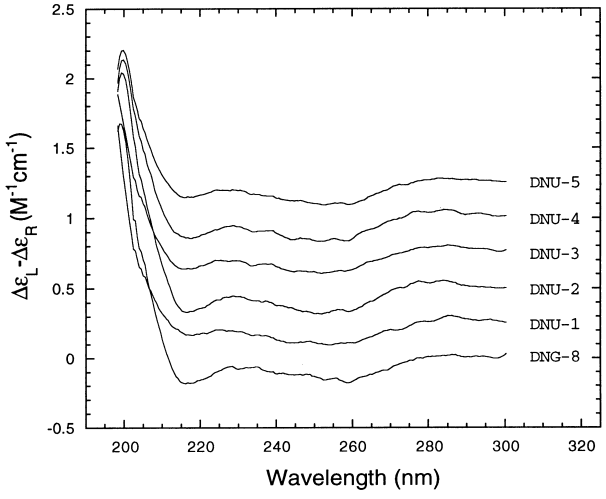



**Figure 2.** CD spectra of DNG-8 compared to DNGU-1 through DNGU-5. Solution conditions: $2.0\times10^{-6}$ M in oligomer, 0.1 M KCL, 10 mM phosphate buffer, pH 7.0 at 24 °C. The spectra are separated by the value of 0.25 for clarity.

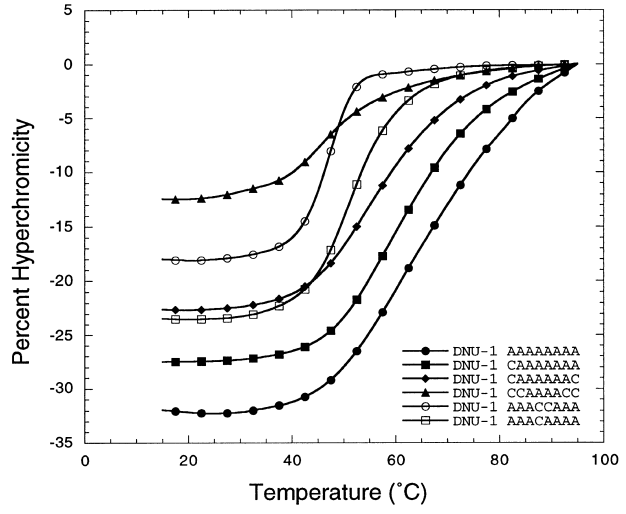



**Figure 4.** Thermal denaturation plots for DNA association with DNG: 1.25 μM DNA oligomer, 2.5 μM DNGU-1, 10 mM $KHPO_4$ buffer, pH 7.0, 0.1 M KCl. Rate of heating was 0.2 °C/min. DNA oligomer **I**, —●—; **II** —■—; **III** —◆—; **IV**, —▲—; **V** —○—, **VI** —□—.

DNGU-4 and DNGU-5 also demonstrate 2:1 binding with weaker deflections indicating that the base pairing is not as strong or organized in the case of DNGU-1. The lower positive charge of these two oligomers results in the binding affinity being lower. In the case of the least positively charged oligomers DNGU-2 and DNGU-3, the inflection is too small to be detected indicating very weak binding.

Melting experiments were carried out to determine the strength of association of the various DNGU oligomers for complimentary and mismatched DNA oligomers. The thermal denaturation temperatures ($T_m$) were determined by hyperchromicity changes as annealed samples of 2:1 DNGU:DNG oligomers were heated from 15 to 95 °C at a rate of 0.2 °C/min. The absorbance of the sample was measured at 260 nM and the hyperchromicity calculated from the maximum absorbance at 95 °C. The Tm values were determined from the first derivative of the thermal denaturation curves and are presented in Table 2.

In the case of the most positively charged DNGU-1 oligomer, which contains seven positively charged guanidinium groups, the affinity for complimentary DNA-**I** is very strong with a $T_m$ value of 61.5 °C. Figure 4 shows the melting curves for the 2:1 combination of DNGU-1 and DNA oligomers. Each cytidyl mismatch in the octameric DNA oligomers reduces the Tm value by approximately 4 °C. The hyperchromicity due to organized base pairing also is reduced with increasing mismatch content in the DNA oligomers. For the most part the melting curves are broad indicating that many intermediate species are produced as the denaturation proceeds.

In the case of DNGU-4, which possesses a urea substitution in the center of the oligomer, the $T_m$ values are reduced by 4 °C for each of the complimentary and mismatch DNA oligomers which contain the cytidyl mismatches at their termini. Figure 5 shows the melting curves for the 2:1 combination of DNGU-4 and DNA oligomers. The curves rise more sharply than those for the DNGU-1 case indicating that less intermediate species are formed during the melting process. The reduction in $T_m$ for DNA oligomers containing cytidyl mismatches in the center is not as great as for the terminal mismatched DNA oligos. The site of deletion of the positive charge is opposite the mismatches and does not add to the weakening of the association as that site is not base paired in the first place. The hyperchromicity for the DNGU-4:DNA triplexes is comparable to that for DNGU-1 as one charge deletion does not seem to impair organized base pairing.

**Table 2.** Tm values for DNGU/DNA association

| | DNGU-1 | DNGU-2 | DNGU-3 | DNGU-4 | DNGU-5 |
|---|---|---|---|---|---|
| DNA-I | 61.5 | 42 | 42 | 57.5 | 52 |
| DNA-II | 58 | 44 | 37.5 | 53.5 | 49.5 |
| DNA-III | 54 | — | 32 | 50 | 45 |
| DNA-IV | 45.5 | — | 30 | 44 | 39.5 |
| DNA-V | 47 | — | 26 | 46 | 38 |
| DNA-VI | 53 | — | 30 | 52 | 47 |

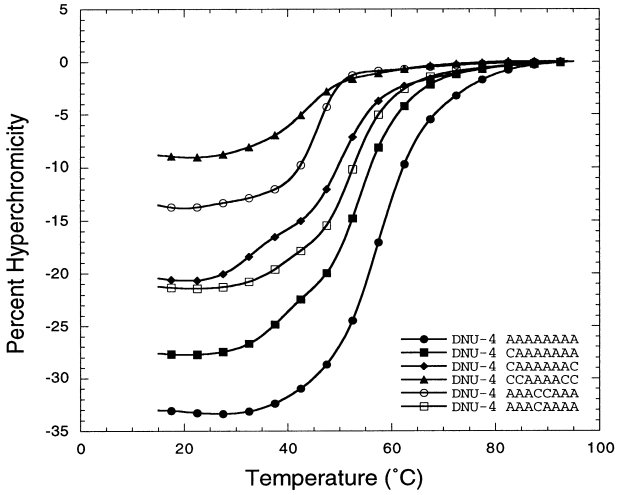



**Figure 5.** Thermal denaturation plots for DNA association with DNG. 1.25 μM DNA oligomer, 2.5 μM DNGU-4, 10 mM $KHPO_4$ buffer, pH 7.0, 0.1 M KCl. Rate of heating was 0.2 °C/min. DNA oligomer **I**, —●—; **II** —■—; **III** —◆—; **IV** —▲—; **V** —○—, **VI** —□—.

Figure 6 shows the melting curves for DNGU-5. The melting curves are still sharper though the Tm values and hyperchromicities have dropped significantly. The reduction in $T_m$ with each terminal cytidyl mismatch remains in the range of 3–4 °C. In the case of DNA-IV the melting curve is too shallow to determine a $T_m$ indicating very weak binding. In the case of DNGU-3 (Fig. 7) the melting curves are shallow and the $T_m$s could not be determined for DNAs-IV and V. The only sharp and clear melting curve was that for DNGU-3 with DNA-I, the complementary DNA oligo. In the case of DNGU-2 (Fig. 8) all the melting curves were shallow and broad. Only the complementary DNA-I and single mismatched DNA-II showed significant hyperchromicity.

As summarized in Figure 9, as urea substitutions are added and the overall charge on the DNGU oligomer is

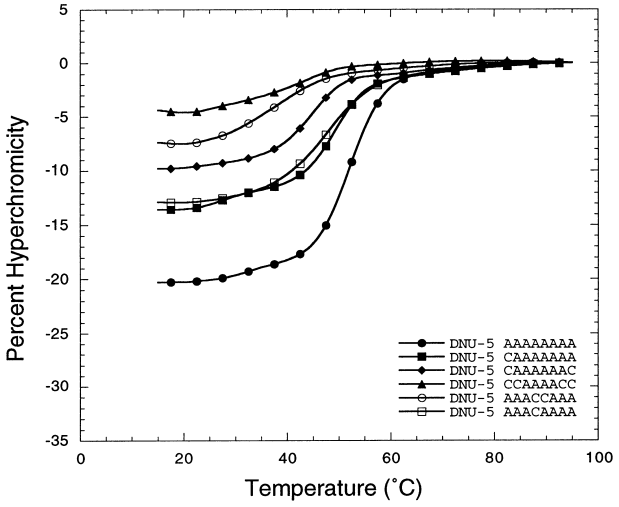



**Figure 6.** Thermal denaturation plots for DNA association with DNG: 1.25 μM DNA oligomer, 2.5 μM DNGU-5, 10 mM $KHPO_4$ buffer, pH 7.0, 0.1 M KCl. Rate of heating was 0.2 °C/min. DNA oligomer **I** —●—; **II** —■—; **III** —◆—; **IV** —▲—; **V** —○—, **VI** —□—.

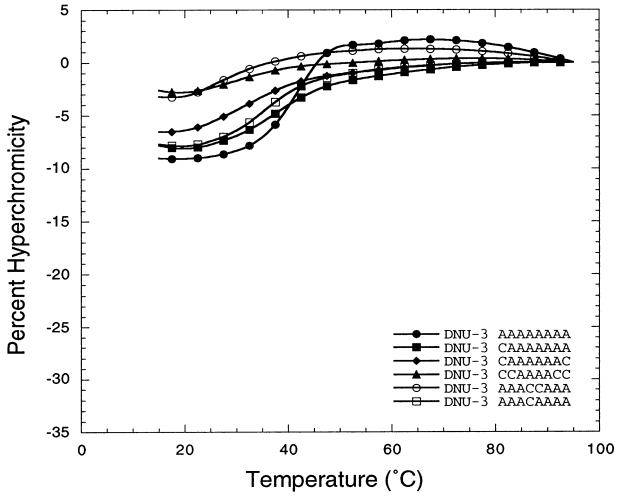



**Figure 7.** Thermal denaturation plots for DNA association with DNG: 1.25 μM DNA oligomer, 2.5 μM DNGU-3, 10 mM $KHPO_4$ buffer, pH 7.0, 0.1 M KCl. Rate of heating was 0.2 °C/min. DNA oligomer **I** —●—; **II** —■—; **III** —◆—; **IV** —▲—; **V** —○—, **VI** —□—.

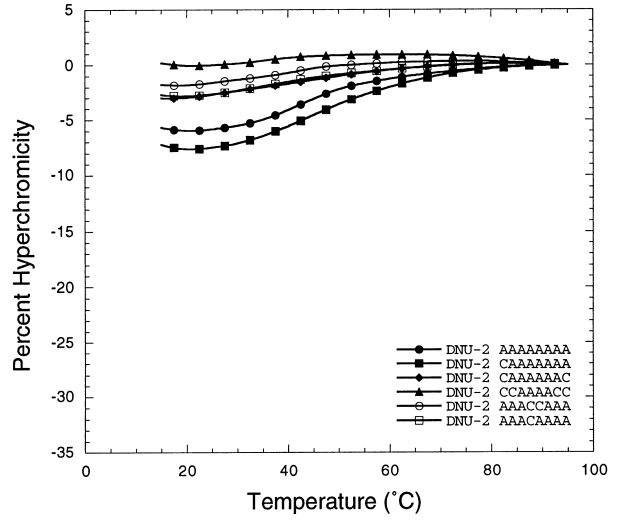



**Figure 8.** Thermal denaturation plots for DNA association with DNG: 1.25 μM DNA oligomer, 2.5 μM DNGU-2, 10 mM $KHPO_4$ buffer, pH 7.0, 0.1 M KCl. Rate of heating was 0.2 °C/min. DNA oligomer **I** —●—; **II** —■—; **III** —◆—; **IV** —▲—; **V** —○—, **VI** —□—.

reduced, the $T_m$ value for the DNGU:DNA 2:1 association is reduced. The reduction in $T_m$ is roughly equivalent across the range of DNA mismatched oligomers as shown by the trends in $T_m$ for internal and external mismatches being fairly parallel for each DNGU oligomer. Each cytidyl mismatch reduced the $T_m$ value by about 4 °C for external mismatches and 6 °C for internal mismatches.

## Conclusion

DNG oligomers with reduced overall charge but nearly identical structure can be synthesized efficiently by solid-phase methods. Replacement of internucleoside guanidinium linkages by urea linkages results in the elimination of a positively charged group but no significant change in structure of the oligomer. These new

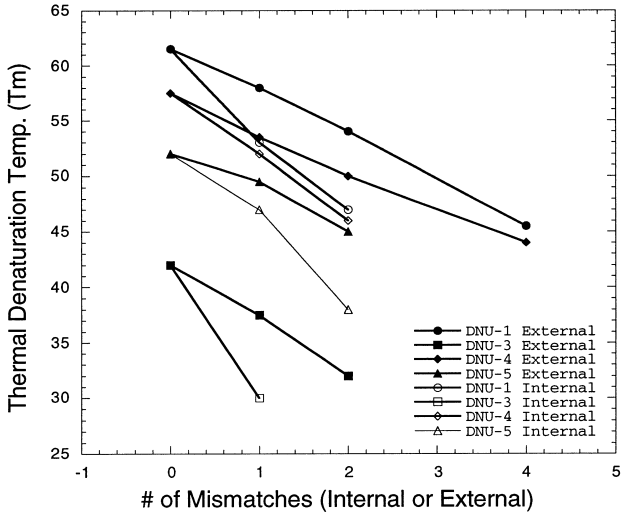



**Figure 9.** Plot of $T_m$ values against number of internal and external mismatches in the target DNA oligomers for each of DNGU-1, -3, -4, and -5.

DNGU oligomers possessed a range of affinity for complementary DNA target oligomers depending on the overall positive charge and the placement of the charges. Affinity of DNG for DNA can be fine-tuned by insertion of urea linkages without altering any other properties. Sensitivity to base mismatches is fairly constant among the DNGU oligomers. With proper arrangement of neutral urea linkages, longer DNG oligomers can be synthesized that will not overwhelm base-pairing by their strong opposite charge attraction.

## Experimental

### Materials

HPLC grade solvents and sodium carbonate were obtained from Fisher Scientific. Anhydrous solvents, triethyl amine, 9-fluorenylmethylchloroformate, mercury(II) chloride and carbonyl diimidazole were purchased from Aldrich and used without further purification. The 2% cross-linked polystyrene 2-(2-aminoethoxy)ethanol 2-chlorotrityl resin (0.31 meq/g substitution) was purchased from Novabiochem. All DNA oligomers were purchased prepurified from the Biological Resource Center at UCSF. Compound **2** was synthesized as described previously.[22]

### General

Hydrogenations were carried out with a Parr hydrogenator equipped with a 500 mL hydrogenation vessel. Analytical reverse-phase HPLCs were performed on a Hewlett Packard 1050 system equipped with a quaternary solvent delivery system and UV detector set at 260 nM and a 2.1×250 mm C18 ODS-hypersil reverse phase column purchased from Altech. A gradient from 100% eluent A (100 mM triethylammonium acetate, pH 7.0) to 50% eluent A, 50% eluent B (acetonitrile) over 15 min with a flow rate of 1.3 mL/min was used. Analytical cation exchange HPLCs were performed with the same

system with a 2.1×250 mm SCX ion exchange column purchased from Altech. A gradient from 100% eluent A (100 mM TRIS buffer, pH 7.0) to 100% eluent B (100 mM TRIS, 1.0 M guanidinium hydrochloride, pH 7.0) over 20 min with a flow rate of 1.3 mL/min was used. Preparative reverse-phase HPLC were performed on the same system with a 10×250 mm C8 ODS-hypersil reverse-phase column purchased from Altech. A gradient from 66% eluent A (100 mM triethylammonium acetate, pH 7.0), 33% eluent B (acetonitrile) to 20% eluent A, 50% eluent B over 15 min with a flow rate of 3.0 mL/min was used. Preparative cation exchange HPLCs were performed with the same system with a 10×250 mm SCX ion exchange column purchased from Altech. A gradient from 66% eluent A (water), 34% eluent B (3 M ammonium acetate) to 100% eluent B over 15 min with a flow rate of 3.0 mL/min was used. $^{1}H$ and $^{13}C$ NMR spectra were obtained on a Varian Unity 400 spectrometer at 400 and 100 MHz, respectively. UV spectra were obtained on a Cary 100 Bio UV–vis spectrophotometer equipped with a temperature programmable cell block. IR spectra were in KBr pellets with a Perkin–Elmer 1300 spectrophotometer. TLC was carried out on silica gel (Kieselger 60 $F_{254}$) glass backed commercial plates and visualized by UV light.

### Melting studies

Thermal denaturation ($T_m$) plots were obtained by observing the absorbance at 260 nM of a solution of the oligomers in 1 cm path-length quartz cuvettes as the temperature was raised 0.2 °C/min from 15 °C to 95 °C. All samples had been previously annealed by cooling from 90 to 15 °C at 0.2 °C/min and stored at 20 °C for 2 days. Samples consisted of 2.5 μM DNGU and 1.25 μM DNA oligomer with 10 mM potassium phosphate buffer at pH 7.0 and either 0.1 M KCl for ionic strength control. For $T_m$ determinations hyperchromicity was used. Data were recorded every 0.5 °C. Samples were covered with mineral oil to prevent evaporation.

### Job plots

Job[29] or continuous variation plots were obtained by mixing samples at various ratios of DNG oligomer to DNA oligomer while maintaining the total concentration of nucleoside base at 2 μM. All solutions contained 10 mM potassium phosphate buffer at pH 7.0 and 0.1 M KCl. Solutions were mixed and left to equilibrate at room temperature for 24 h. DNA oligomer concentrations were determined spectrophotometrically using extinction coefficients provided by the manufacturer. The value of $e_{268} = 8700\ M^{-1}\ cm^{-1}$ was used for the extinction coefficient (per nucleoside base) of DNGU.[30]

### Circular dichroism spectra

CD spectra were obtained on an OLIS RSM circular dichroism spectrophotometer. Scans were run from 350 to 210 nM taking a measurement every 1 nM. The integration time for each data point was 1.4 s. Ten scans were made of each sample and then averaged and smoothed using a 15-point exponential fitting algorithm. Samples were held in a 1 cm path length cuvette and the temperature was maintained at 20 °C.

## Methods

**5′-*t*-Butoxycarbamoyl-3′-(9-fluorenylmethoxycarbamoyl)-3′,5′-deoxythymidine (4).** To a solution of **3** (4.49 g, 12.3 mmol) in 100 mL of 95% ethanol was added 50 mg of 10% Pd/C and the mixture was hydrogenated at 50 PSI for 2 h then filtered through Celite. The filtrate was evaporated to dryness using a rotary evaporator and kept under high vacuum overnight. The solid residue was dissolved in a mixture of 100 mL dioxane and 50 mL of 10% sodium carbonate in water. This mixture was cooled in an ice bath and 9-fluorenylmethylchloroformate (5.16 g, 20.0 mmol) was added slowly. The mixture was left to stir in the ice bath for one hour until TLC analysis indicated that the reaction was complete. A crystalline precipitate had appeared during the reaction and 50 mL water were added until it complete dissolved. The mixture was extracted with ethyl acetate (3×100 mL) and the organic layer was dried with sodium sulfate and evaporated to a white foam which was purified by silica gel flash column chromatography (5×20 cm column with 0 to 5% gradient of methanol in ethyl acetate). Fractions containing the product, **4**, were collected and evaporated to give 4.25 g white foam product (61% yield). TLC (3:1, EtOAc: hexane)) $R_f$ 0.35; $^{1}H$ NMR (400 MHz DMSO-$d_6$) δ 1.76 (3H, s, thymine-$CH_3$), 2.13 (m, 1H; 2′-H), 2.22 (m, 1H; 2′-H), 3.22 (m, 1H; 5′-H), 3.35 (m, 1H; 5′-H), 3.80 (m, 1H; 4′-H), 4.10 (m, 1H; 3′-H), 4.20 (t, $J=7$ Hz, 1H; Fmoc-CH), 4.26 (m, 2H; Fmoc-$CH_2$), 6.14 (t, $J=6.8$ Hz, 1H; 1′-H), 7.30 (t, $J=7$ Hz, 2H; Fmoc-2″-H), 7.38 (t, $J=7$ Hz, 2H; Fmoc-3″-H), 7.55 (s, 1H; 6-H), 7.67 (m, 2H; Fmoc-1″-H), 7.79 (d, $J=8$ Hz, 1H; Fmoc-NH), 7.87 (d, $J=7$ Hz, 2H; Fmoc-4″-H), 9.83 (t, $J=5$ Hz, 1H; 5′-NH), 11.30 (s, 1H; thymine-NH); $^{13}C$ NMR (100 MHz, DMSO-$d_6$) δ 12.1, 36.0, 46.7, 51.7, 65.5, 73.8, 80.7, 83.3, 94.9, 109.9, 120.1, 125.1, 127.1, 127.6, 136.2, 140.8, 143.8, 150.4, 152.0, 155.8, 163.7, 179.6; IR (KBr pellet) 3315, 3067, 1688, 1533, 1449, 1260, 1151, 1084, 1034, 758, 739; MS (FAB) $m/z$ 563 $(M+H)^+$); HRMS $m/z$: 563.24752 $(M+H)^+$, calcd for $C_{30}H_{34}N_4O_7$ 563.25057.

**5′-Isocyano-3′-(9-fluorenylmethoxycarbamoyl)-3′,5′-deoxythymidine (1).** To a mixture of **4** (1.26 g, 2.24 mmol) in 20 mL of dichloromethane (DCM) was added 10 mL of trifluoroacetic acid. The solution was stirred for 20 min at room temperature and then evaporated to dryness using a rotary evaporator and kept under high vacuum overnight. The residue was a pale-yellow foam. This was slurried in 20 mL DCM, cooled in an ice bath and 0.31 mL of triethylamine (TEA) were added. Upon addition of the TEA the mixture became a clear solution. To this mixture was added carbonyl diimidazole (0.36 g, 2.24 mmol) and the mixture was stirred at room temperature for 60 min. The DCM solution was washed with 100 mL of water and dried with sodium sulfate. The solvent was evaporated and the residue was purified by silica gel flash column chromatography (5×20 cm column

with 0 to 5% gradient of methanol in ethyl acetate). Fractions containing the product, **1**, were collected and evaporated to give 1.06 g white foam product (95% yield). $^1$H NMR (400 MHz DMF-$d_7$) δ 1.85 (3H, s, thymine-$CH_3$), 2.41 (m, 1H; 2′-H), 2.55 (m, 1H; 2′ -H), 3.74 (m, 1H; 5′-H), 3.79 (m, 1H; 5′-H), 4.12 (dd, 1H, 4′-H, $J=6$ Hz, $J=10$ Hz,), 4.32 (m, 1H, 3′-H), 4.20 (m, 3H; Fmoc-CH, Fmoc-$CH_2$), 6.31 (t, $J=7$ Hz, 1H; 1′-H), 7.35 (t, $J=7$ Hz, 2H; Fmoc-2″-H), 7.43 (t, $J=7$ Hz, 2H; Fmoc-3″-H), 7.72 (m, 3H; 6-H, Fmoc-1″-H), 7.88 (d, $J=7$ Hz, 2H; Fmoc-4″-H), 7.90 (d, $J=8$ Hz, 1H; Fmoc-NH), 11.35 (s, 1H; thymine-NH); IR (KBr pellet) 1692, 1542, 1477, 1449, 1371, 1319, 1287, 1264, 1094, 759, 739; MS (FAB) $m/z$ 489 $(M+H)^+$; HRMS (FAB) $m/z$ 489.17092 $(M+H)^+$, calcd for $C_{26}H_{24}N_4O_6$: 489.17741.

### Solid-phase syntheses

**Resin loading.** In a typical synthesis, 15 mg of the commercially available 2% cross-linked polystyrene 2-(2-aminoethoxy)ethanol 2-chlorotrityl resin were placed in a screw capped reaction tube fitted with a coarse glass frit filter and a stopcock and swelled in dry DMF for 2 h. Then the DMF was removed by filteration and enough DMF was added to just barely cover the beads. 22 mg of **1** were placed in a small vial and dissolved in 1 mL of dry DMF. This was transferred via syringe to the reaction tube. The tube was agitated for 6 h and then the resin was filtered and washed with DMF (3×3 mL).

**Guanidinium coupling.** To insert a troc-protected guanidinium linkage into a growing oligomer the previously reported procedure was used[22] and is outlined briefly here. 22 mg of **2** were placed in a small vial and dissolved in 1 mL of dry DMF. This was transferred via syringe to the reaction tube. Then 0.5 mL of a 50 mM $HgCl_2$ solution in DMF and 0.5 mL of a 100 mM TEA solution in DMF were added as described above. After two hours the resin was filtered and washed briefly treated with a 20% thiophenol solution in DMF (2 mL for 10 s) and the beads instantly returned to their original yellow color as the black precipitate was removed. The supernatant was removed by filtration the beads and the resin was washed with DMF repeatedly (5×3 mL).

**Urea coupling.** To insert a urea linkage into a growing oligomer the same method for resin coupling was used as with the resin loading step above with the exception that each coupling was carried out over 2 instead of 6 h.

**Deblocking.** To unmask the terminal 3′-amino group after the resin loading and each coupling step, a solution of 10% piperidine in DMF was poured over the beads and the reaction vial was agitated for 20 min. The supernatant was removed by filtration and the beads were washed with DMF (3×2 mL).

**Characterization of DNGU oligomers.** The synthetic oligomers were characterized by HPLC and mass spectrometry. After cleavage from the resin each oligomer was purified by reverse-phase HPLC and the purified product was analysed electrospray MS. Since the troc protecting groups are present, the composition of the oligomer can be unabiguously determined. The oligomers were then deprotected by cadmium/acetic acid and the product was purified by cation exchange HPLC. The resulting oligomers were then confirmed by electrospray MS.

**DNGU-1.** For the troc-protected oligomer, reverse-phase analytical HPLC gives a retention time of 13.5 min, electrospray MS shows the peaks from the multiply charged product ($m/z$ 865, 1152, 1728; calcd for $C_{113}H_{137}Cl_{21}N_{40}O_{41}$ (M + 4H): 865, (M + 3H): 1153, (M + 2H): 1729); for the deprotected oligomer, cation exchange analytical HPLC gives a retention time of 13.2 minutes, electrospray MS shows the product ($m/z$ 744; calcd for $C_{92}H_{128}N_{42}O_{27}$ (M + 3H): 744).

**DNGU-2.** For the troc-protected oligomer, reverse-phase analytical HPLC gives a retention time of 4.9 min, electrospray MS shows the peaks from the multiply charged product ($m/z$ 977, 1466; calcd for $C_{104}H_{131}Cl_{12}N_{37}O_{38}$, (M + 3H): 978, (M + 2H): 1467); for the deprotected oligomer, cation exchange analytical HPLC gives a retention time of 9.5 min, electrospray MS shows the product ($m/z$ 744; calcd for $C_{92}H_{127}N_{37}O_{30}$ (M + 3H): 745). 744; calcd for $C_{92}H_{128}N_{42}O_{27}$ (M + 3H): 744).

**DNGU-3.** For the troc-protected oligomer, reverse-phase analytical HPLC gives a retention time of 6.3 min, electrospray MS shows the peaks from the multiply charged product ($m/z$ 977, 1465; calcd for $C_{104}H_{131}Cl_{12}N_{37}O_{38}$, (M + 3H): 978, (M + 2H): 1467); for the deprotected oligomer, cation exchange analytical HPLC gives a retention time of 9.8 min, electrospray MS shows the product ($m/z$ 1115; calcd for $C_{92}H_{127}N_{37}O_{30}$ (M + 2H): 1116).

**DNGU-4.** For the troc-protected oligomer, reverse-phase analytical HPLC gives a retention time of 10.9 min, electrospray MS shows the peaks from the multiply charged product ($m/z$ 1094, 1640; calcd for $C_{110}H_{135}Cl_{18}N_{39}O_{40}$, (M + 3H): 1095, (M + 2H): 1642); for the deprotected oligomer, cation exchange analytical HPLC gives a retention time of 12.3 min, electrospray MS shows the product ($m/z$ 744; calcd for $C_{92}H_{129}N_{39}O_{28}$ (M + 3H): 744).

**DNGU-5.** For the troc-protected oligomer, reverse-phase analytical HPLC gives a retention time of 8.09 min, electrospray MS shows the peaks from the multiply charged product [$m/z$ 1037, 1553; calcd for $C_{107}H_{133}Cl_{15}N_{38}O_{39}$, (M + 3H): 1037, (M + 2H): 1555]; for the deprotected oligomer, cation exchange analytical HPLC gives a retention time of 10.9 min, electrospray MS shows the product [$m/z$ 744; calcd for $C_{92}H_{128}N_{38}O_{29}$ (M + 3H): 744].

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