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Template-Directed Oligonucleotide Strand Ligation, Covalent Intramolecular DNA Circularization and Catenation Using Click Chemistry

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Abstract: The copper-catalyzed azide−alkyne cycloaddition reaction has been used for the template-mediated chemical ligation of two oligonucleotide strands, one with a 5′-alkyne and the other with a 3′-azide, to produce a DNA strand with an unnatural backbone at the ligation point. A template-free click-ligation reaction has been used for the intramolecular circularization of a single stranded oligonucleotide which was used as a template for the synthesis of a covalently closed DNA catenane.

Introduction

There is currently considerable interest in the properties and applications of oligonucleotide (ODN) constructs with unusual and unique topologies.[1−4] DNA nanostructures which are held together only by Watson−Crick base pairing suffer from the disadvantage that they can be disassembled by heat or various denaturing agents. Therefore they have to be analyzed under nondenaturing conditions, and this complicates their characterization and purification.[5] Moreover, it limits their value as building blocks that can be stored for subsequent use in the assembly of larger more complex nanostructures. Chemical methods for the synthesis of DNA constructs in which the strands are covalently interlocked offer a potential solution to this problem. Ideally such chemistry should be clean, fast, easily switched on and off, functional under template-mediated or template-free conditions, and amenable to large scale synthesis. Photoinduced reversible cycloaddition reactions have been explored in this context,[6−8] and there is considerable scope for developing alternative chemistries to stabilize DNA nanostructures.

The copper-catalyzed azide−alkyne cycloaddition (CuAAC) reaction, the best example of click chemistry,[9] appears to fulfill all the necessary criteria. It functions efficiently in aqueous media and has previously been used to immobilize DNA on electrode surfaces and chips[10,11] and to cyclize peptides on resins.[12] It has also been used to link oligonucleotide strands on surfaces.[13] It is catalyzed by Cu[I] to produce a 1,2,3-triazole linkage between the two reactants.

We now report an efficient method of oligonucleotide ligation (click ligation) that produces an unnatural extended DNA backbone linkage (Figure 1). For this purpose one of the reacting ODNs contains a 3′-azide and the other a 5′-alkyne. The ligated oligonucleotide product is obtained within 2 h at room temperature using equimolar ratios of the two participating ODNs. The ligation can be forced to proceed with or without template simply by varying the concentrations of the reactants. If the alkyne and azide are located in the same oligonucleotide, circularization occurs even in the absence of a template ODN. The circularized ODN can then be used in the formation of a double strand DNA catenane.

Results and Discussion

Azide-ODN-2 was prepared (Scheme 1) by labeling 3′-amino-modified ODN-1 with 4-azidobutyric acid NHS ester **2** in bicarbonate buffer at pH 8.75. The resultant 3′-azide-labeled oligonucleotide was then purified by reversed-phase HPLC and characterized by MALDI-TOF MS [$M + H^+$ calcd, 5387; found, 5387]. All ODN sequences are given in Table 1.

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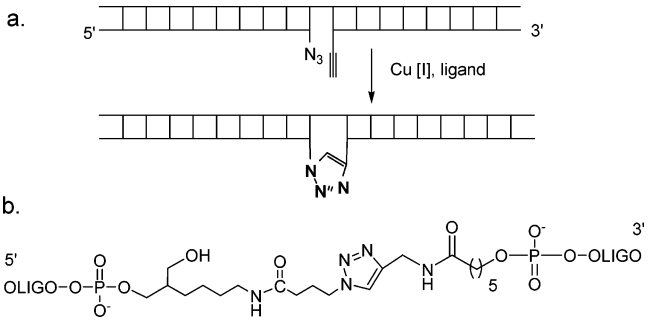
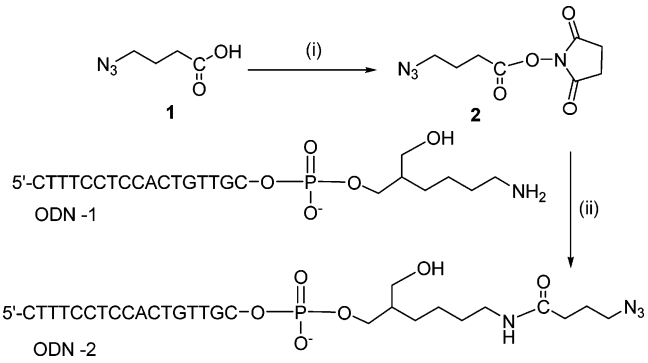


Figure 1. Template-mediated click-ligation of two ODNs: (a) schematic and (b) chemical structure at ligation point.

Scheme 1 [a]



[a] Conditions: (i) NHS, DCC, DCM, 63%; (ii) 0.5 M Na_2CO_3/$NaHCO_3$ buffer (aq, pH 8.75), DMSO.

An orthogonal approach was developed for 5′-alkyne-labeling of ODNs to ensure that it is possible to label a single oligonucleotide with both alkyne and azide. The alkyne-bearing phosphoramidite monomer **6** was synthesized according to Scheme 2. This monomer was incorporated into ODN-3 at the 5′-terminus by standard automated oligodeoxynucleotide synthesis methods to give ODN-4, which was purified by reversed-phase HPLC and characterized by MALDI-TOF MS [M + H^+ calcd, 4817; found, 4817].

For the oligonucleotide ligation reactions, the Cu[I] click catalyst was prepared in situ from Cu[II] sulfate and sodium ascorbate. All ligation reactions were carried out in 0.2 M aqueous NaCl to ensure complete formation of duplex with the template ODN-5. Unfortunately under these conditions extensive degradation of all ODNs occurred, even with degassed buffers (Figure 2a). This problem has been encountered previously.[13] However, the water-soluble tris-triazolylamine Cu[I]-binding ligand[14] greatly reduced degradation and when greater than 5-fold excess of ligand relative to Cu[I] was employed very little decomposition was observed. Click ligation reactions of azide ODN-2 and alkyne ODN-4 in the presence of template ODN-5 were carried out at 10.0, 2.0, and 0.4 μM concentrations of each oligonucleotide, with and without template, and with and without Cu[I]-catalysis/ligand. In all cases no reaction was observed without the catalyst/ligand (Figure 2b). At 10.0, 2.0, and 0.4 μM in the presence of catalyst/ligand and template ODN-5 a near quantitative conversion to the click ligated ODN-6 was observed by denaturing anion-exchange HPLC (Figure 2c). At high ODN concentrations (>10 μM) formation of ODN-6 was observed without template (up to 50% conversion) but no significant ligation was observed at 2.0 μM and 0.4 μM ODN concentration in 3 h at room temperature in the absence of template ODN-5 (Figure 2d). In summary, in the presence of catalyst/ligand the intermolecular reaction is easily controlled by varying oligonucleotide concentration. It can be conducted in a template-mediated manner over a wide concentration range, and below 2.0 μM the reaction does not proceed in the absence of a template ODN. In addition, the reaction can be initiated at any time simply by the addition of ligand-bound catalyst to the participating ODNs or quenched by gel-filtration.

Click-ligated ODN-6 was purified by anion-exchange HPLC, desalted, and characterized by MALDI TOF MS [M + H^+ calcd, 10204; found, 10205]. To investigate the properties of the click ligation junction of ODN-6, two 18-mers ODN-7 and ODN-8 were prepared and melting experiments were carried out using a Roche LightCycler and SYBR Green I DNA fluorescent binding dye. ODN-7 is complementary to the central region of ODN-6 and ODN-8 is the exact complement of ODN-7. Duplex melting temperature of the 18-mers ODN-7/ODN-8 (normal duplex) and ODN-7/ODN-6 (click ligated duplex) were 69 and 58 °C, respectively. The lower melting temperature of the duplex containing the click ligated ODN is almost certainly an effect of the extended 22-atom unnatural backbone at the site of ligation. Clearly there is great scope for varying the nature and length of this simply by changing the alkyne and azide employed to label the ODNs.

Having established the optimum conditions for oligonucleotide click-ligation chemistry, the approach was applied to the assembly of nanoscale ssDNA closed circular constructs and dsDNA pseudohexagons with each side consisting of a single turn of a B-DNA helix. In our case click ligation was employed to "permanently" link the two DNA strands by covalently joining the 5′- and 3′-ends of each ODN in the duplex. For this purpose a 70-mer (ODN-9) and a 72-mer (ODN-11) were prepared using standard automated DNA synthesis and both were labeled with 3′-azide and 5′-alkyne as described above. It is noteworthy that 5′-alkynyl ODNs containing 3′-amino functions can be labeled with azide NHS ester **2** without a major side reaction occurring between the 5′-alkyne and the active ester **2**. This is because triazole formation is very slow in the absence of Cu[I]. Several criteria were used in the design of the sequences for each of the six 10-mer sides of this pseudohexagon; each side is orthogonal to any part of the other five sides (≤4 consecutive matching bases), each has a similar GC-content and the sequences were screened to avoid the possibility for hairpin formation. Furthermore, to minimize the possibility of fraying ends, each side has at least one terminal GC base pair. The ssDNA closed circle (ODN-10) was prepared (Figure 3) by the intramolecular click self-ligation of ODN-9 using tris-triazolylamine Cu[I]-catalysis.

Having previously established that the intermolecular click-ligation reaction does not occur in the absence of the template ODN up to at least 2.0 μM, reaction conditions were chosen so that only cyclic product and no linear dimeric product would be produced.

Accordingly, ODN self-circularization reactions were carried out at 0.4 μM and monitored by denaturing polyacrylamide gel-electerophoresis (Figure 4 lane a). This clearly showed the formation of a cyclic product (ODN-10) with lower gel-mobility than the linear starting ODN-9 as previously reported for

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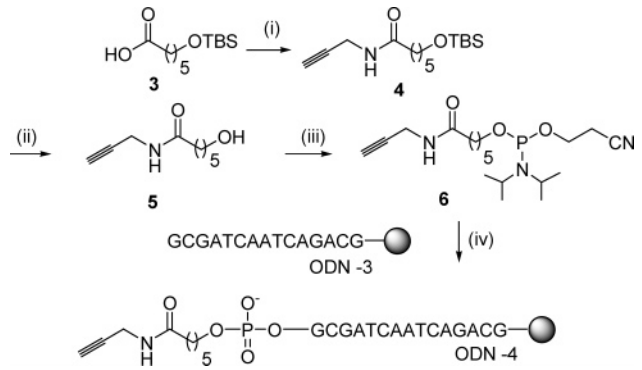
Table 1. Oligonucleotides Used in This Study

entry	sequences (5′-3′) (***K*** = alkyne, ***Z*** = azide, ***NH$_2$*** = amino C7, ***X*** = 1,2,3-triazole linker)
ODN-1	CTTTCCTCCACTGTTGC***NH$_2$***
ODN-2	CTTTCCTCCACTGTTGC***Z***
ODN-3	GCGATCAATCAGACG
ODN-4	***K***GCGATCAATCAGACG
ODN-5	TTTTTCGTCTGATTGATCGCGCAACAGTGGAGGAAAGTTTTT
ODN-6	CTTTCCTCCACTGTTGC***X***GCGATCAATCAGACG
ODN-7	ATTGATCGCGCAACAGTG
ODN-8	CACTGTTGCGCGATCAAT
ODN-9	***K***CCATACATACTTCCACAGCATCTTGATTAGCGTCTTCGATGGTATCTTGGCTCTACAGTTGAGGAGGATG***Z***
ODN-10	Circular ssDNA of oligonucleotide ODN-9
ODN-11	***K***TGACGCTAATCTTGATGCTGTGGTTGTATGTATGGTTCATCCTCCTCTTCTGTAGAGCCTTGATACCATCGT***Z***
ODN-12	dsDNA pseudohexagonal catenane
ODN-13	***K***TGAGGAGGATGT***Z***
ODN-14	Circular ssDNA form oligonucleotide ODN-11
ODN-15	Circular ssDNA form oligonucleotide ODN-13
ODN-16	AGGCTCTACAGAAGAGGAGGATGA

Scheme 2 [a]



[a] Conditions: (i) propargylamine, EDC, HOBt, DIPEA, DCM, 75%; (ii) TBAF/THF, 63%; (iii) 2-*O*-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite, DIPEA, DCM, 55%; (iv) oligonucleotide synthesis.

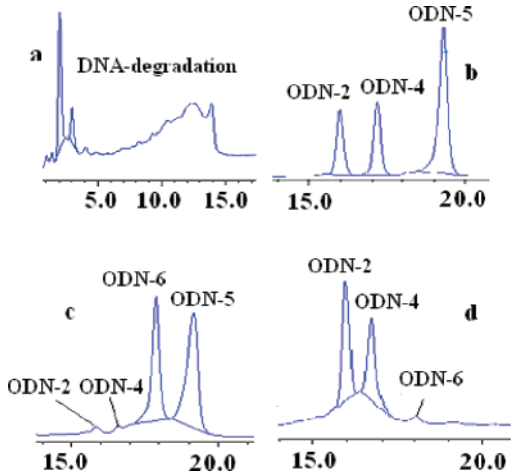


Figure 2. Denaturing anion-exchange HPLC chromatograms (UV absorbance vs time) of the reaction between ODN-2 and ODN-4 under different conditions: (a) oligonucleotide degradation caused by uncomplexed Cu[I] if no ligand is used; (b) no reaction in the absence of Cu[I] catalysis; (c) ODN-2 and ODN-4 at 0.4 μM and ODN-5 at 0.44 μM + 0.28 mM ligand + Cu[I] (0.4 mM sodium ascorbate, 40 μM Cu[II]); the reaction is almost complete within 2 h; (d) ODN-2 and ODN-4 at 0.4 μM + 0.28 mM ligand + Cu[I] (0.4 mM sodium ascorbate, 40 μM Cu[II]) in the absence of template ODN-5; no significant reaction was observed.

noncovalently closed pseudohexagons by nondenaturing gel-electrophoresis.[5] Only one major product was formed and no further reaction was observed even after extended times. The 70-mer circular ssDNA ODN-10 was purified by extraction from the gel into sterile water. Template-mediated oligonucleotide

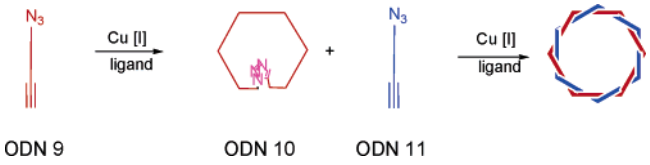


Figure 3. Formation of double-stranded pseudohexagon from single-stranded cyclic template ODN-10 and linear ODN-11. In this representation of the catenane the two strands are entwined six times.

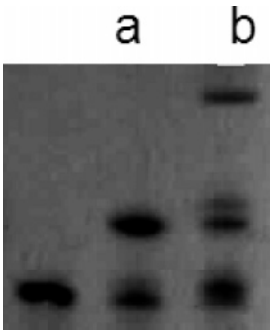


Figure 4. Denaturing 8% PAGE gel: (lane a) Formation of single-stranded closed circular ODN-10 (upper spot) from ODN-9 (lower spot) at 0.4 μmol ODN concentration. (lane b) Formation of covalently closed pseudohexagonal duplex (upper spot) from ODN-10 and ODN-11. Linear ODNs and single-stranded closed circles are also present. Linear ODN-9 (no Cu[I] catalysis) is shown on the left for reference.

circularization has been carried out previously by enzymatic and chemical methods, but non-template-mediated circularization of long oligonucleotides has proved difficult.[15,16]

To further demonstrate that the circular products and not linear or cyclic dimers are formed under these conditions,[12] oligonucleotide ODN-13 (12-mer) was circularized with high efficiency, as shown by HPLC (Figure 5), and the resulted pure circular ODN-15 was characterized by MALDI TOF MS [M + H^+ calcd, 4316; found, 4317].

Conclusive proof of circularization was obtained by annealing the circular ODN-14 (72-mer) to a complementary ODN-16 (24-mer) to create a cleavage site for the restriction enzyme MboII. A partial enzyme digestion produced a single product migrating at the same position on gel as the linear ODN-11 (72-mer) (Figure 6). In contrast when the linear ODN-11 (72-mer) was annealed to the same ODN-16 (24-mer) and cut with MboII,

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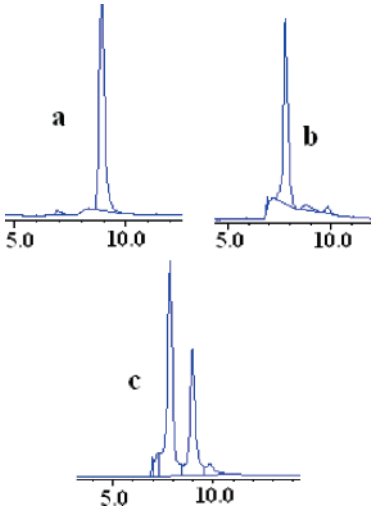

Figure 5. Reversed-phase HPLC analysis of click ligation reaction (UV absorbance at 275 nm vs time): (a) linear oligonucleotide (ODN-13), (b) reaction mixture to produce circular oligonucleotide (ODN-15); (c) mixture of linear oligonucleotide (ODN-13) and reaction mixture (ODN-15).

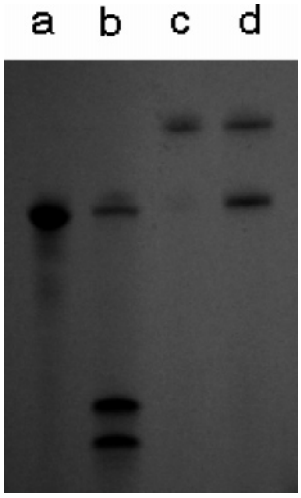

Figure 6. Denaturing 8% polyacrylamide gel: (lane a) ODN-11 (control sample prepared exactly as the sample in lane b but without the addition of the restriction enzyme). (lane b) Digestion of linear oligonucleotide ODN-11 with MboII. (lane c) Circular oligonucleotide ODN-14 (control sample prepared exactly as the sample in lane d but without the addition of the restriction enzyme). (lane d) Digestion of circular oligonucleotide ODN-14 with MboII.

two short fragments were obtained. These were consistent with the expected 34-mer and 38-mer.

Template-mediated formation of a dsDNA pseudohexagon was carried out by mixing the purified circularized ODN-10 with its linear complement ODN-11 (Figure 3). The click-ligation reaction was carried out and a new retarded band appeared on the denaturing polyacrylamide gel (Figure 4 lane b), which is attributed to the formation of the covalently closed linked double stranded catenane constructed from the two click-ligated oligonucleotides. However, it is not possible to deduce if one, a few, or even all six of the double-stranded regions of this pseudohexagon are actually formed. Modeling, energy minimization, and molecular dynamics simulations using the Amber force field in HyperChem suggest that a strained pseudohexagon is possible in which the single-strands are entwined three times. A structure in which all six edges are simultaneously double stranded and the strands are entwined six times can also be constructed but this may be more strained (Figure 7).

Interestingly when linear ODN-9 and ODN-11 were mixed in aqueous buffer and the tris-triazolylamine Cu[I]-catalysis was added, significantly less of this catenated double-stranded species was formed. This is expected since the linear duplex structure will increase rigidity and reduce intramolecular interactions at the termini of the construct. Clearly the gel-purification of ODN-10 is also important, as it serves to remove any impure ODN-9 that might contain nonfunctional termini resulting from incomplete alkyne/azide labeling. This would also limit the yield of the pseudohexagonal duplex. Lane b of Figure 4 shows the presence of a weak band just above ODN-10 that is probably due to circular single-stranded ODN-11.

The linear single-stranded ODN-11 can probably form a duplex with circular ODN-10 without the necessity to initially thread through the center of the ODN-10 circle. After formation of a short double-stranded region, the backbone of ODN-10 could rotate and "wind-in" ODN-11 until the double-stranded pseudohexagon has formed. The covalently closed duplex is described as pseudohexagonal because it contains non-Watson−Crick TT base pairs at the vertices, that is, the two complementary strands ODN-10 and ODN-11 have six fully complementary 10-mer duplexes punctuated by TT hinges. Without these mismatched regions, it would not be possible for this short double-stranded circular DNA structure to adopt such a small radius of curvature. We have no proof that all the Watson−Crick base pairs in the pseudohexagon can form simultaneously, even with TT mismatches, and future strategies will focus on the use of flexible hinges such as hexaethylene glycol in place of thymidines.

Conclusions

A click chemistry approach has been developed and applied to the template-mediated ligation of two oligonucleotide strands against a complementary template. The methodology has also been used for the synthesis of a covalently closed ssDNA circle and a dsDNA pseudohexagon with sides of ca. 4 nm in length. This method is likely to be of value in nanotechnology applications involving DNA scaffolds. In addition, the use of single-stranded covalently closed DNA circles in biological applications warrants further investigation. Such constructs are likely to have increased in vivo stability as they will be resistant to exonuclease degradation.

Experimental Section

All reagents were purchased from Aldrich, Avocado, Fluka, or Link Technologies and used without purification with the exception of the following solvents, which were purified by distillation: THF (over sodium wire and benzophenone), DCM, DIPEA, and pyridine (over calcium hydride). All reactions were carried out under an argon atmosphere using oven-dried glassware with purified and distilled solvents. NAP columns were purchased from GE Healthcare. Water soluble polyhydroxypropyltriazole amine Cu[I] binding ligand was synthesized by the method reported in the literature.[14] Column chromatography was carried out under pressure using Fisher Scientific DAVISIL 60A (35−70 micron) silica. Thin layer chromatography was performed using Merck Kieselgel 60 F24 (0.22 mm thickness, aluminum backed). Compounds were visualized by staining with potassium permanganate solution.

^{1}H NMR spectra were measured at 300 MHz on a Bruker AC300 spectrometer and ^{13}C NMR spectra were measured at 75 MHz.

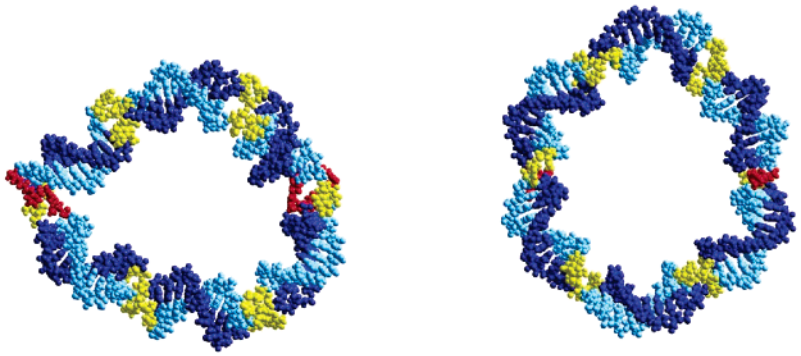

Figure 7. Formation of double-stranded pseudohexagon from single stranded cyclic template ODN-10 and linear ODN-11. HyperChem representations in which the single-strands are entwined three times (left) and six times (right, shown schematically in Figure 3). Unpaired TpT/TpT hinge segments are shown in yellow, click-ligated regions in red, and double-stranded regions in light and dark blue.

Chemical shifts are given in ppm relative to tetramethylsilane, and *J* values are given in Hz and are correct to within 0.5 Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. Multiplicities of ^{13}C signals were determined using DEPT spectral editing technique. ^{31}P NMR spectra were recorded on a Bruker AV300 spectrometer at 121 MHz and were externally referenced to 85% phosphoric acid in deuterated water. Low-resolution mass spectra were recorded using electrospray technique on a Fisons VG platform instrument or a Waters ZMD quadrupole mass spectrometer in acetonitrile (HPLC grade). High-resolution mass spectra were recorded in acetonitrile, methanol, or water (HPLC grade) using electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. MALDI-TOF MS were recorded using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode using oligonucleotide dT standards.[17]

Succinimidyl-4-azidobutyrate, 2. Dicyclohexylcarbodiimide (DCC) (0.89 g, 4.3 mmol) was added to a suspension of 4-azidobutyric acid **1** (0.46 g, 3.6 mmol)[18] and *N*-hydroxysuccinimide (0.49 g, 4.3 mmol) in DCM (20 mL) at room temperature, and the reaction was left to stir. After 4 h saturated aqueous KCl (20 mL) was added, and the organic layer was separated, washed with water, dried over sodium sulfate and filtered. The solvent was removed in vacuo. Upon purification by column chromatography (99:1, DCM/methanol) the title compound was isolated as a colorless oil (0.51 g, 63%). IR (thin film) ν: 2091, 1780, 1731 cm^{-1}. δ_H (300 MHz, $CDCl_3$) 3.37 (2H, t, $J = 6.6$ Hz, N_3CH_2), 2.77 (4H, m, $COCH_2CH_2CO$), 2.66 (2H, t, $J = 7.0$ Hz, $COCH_2$-), 1.94 (2H, m, CH_2); δ_C (75 MHz, $CDCl_3$) 168.9 (CO), 167.9 (CO), 50.0 (N_3CH_2), 28.1 ($COCH_2$-), 25.6 ($COCH_2CH_2CO$), 24.1 (CH_2). *m*/*z* LRMS [ES^+, MeCN] 249 (M + Na^+, 100%). HRMS (M + Na^+) ($C_8H_{10}N_4NaO_4$): calcd, 249.0594; found, 249.0590.

Synthesis of Alkyne Phosphoramidite Monomer. 6-*O*-TBS-Hexan-1-propargylamide, 4. EDC (2.45 g, 12.8 mmol) was added to a suspension of 6-*O*-TBS-1-hexanoic acid **3** (2.1 g, 8.5 mmol),[19] propargylamine (0.52 g, 9.4 mmol), and *N*-hydroxybenzotriazole hydrate (HOBt) (1.44 g, 9.4 mmol) in DCM (20 mL) followed by DIPEA (5.50 g, 42.7 mmol). The reaction mixture was left to stir for 4 h at room temperature then partitioned between DCM and saturated aqueous $NaHCO_3$. The organic layer was separated, washed with water, dried over sodium sulfate and filtered, and the solvent was removed in vacuo. Upon purification by column chromatography (60:40, ethyl acetate/hexane) the title compound was isolated as a colorless oil (1.80 g, 75%). δ_H (300 MHz, $CDCl_3$) 5.73 (1H, bs, NH), 4.0, 3.56 (4H, m, NCH_2, OCH_2), 2.18 (3H, m, $COCH_2$, alkyne-H), 1.65−1.32 (6H, m, 3xCH_2-), 0.84 (9H, s, TBS), 0.0 (6H, s, TBS); δ_C (75 MHz, $CDCl_3$) 172.6 (CO), 79.7, 71.5 (alkyne-C), 64.0, 63.0 (OCH_2, CH_2N), 36.4, 32.5, 29.1 (CH_2), 25.5 (3xCH_3, TBS), 25.4 (CH_2), 18.3 (C−TBS), 0.5 (2xCH_3,-TBS). *m*/*z* LRMS [ES^+, MeCN] 284 (M + H^+, 20%), 306 (M + Na^+, 30%). HRMS (M + Na^+) ($C_{15}H_{29}NNaO_2Si$): calcd, 306.1860; found, 306.1858.

6-Propargylamido-1-hexanol, 5. Compound **4** (0.80 g, 2.8 mmol) was dissolved in TBAF/THF (6 mL, 1:1) at room temperature, left to stir for 1 h then concentrated in vacuo and partitioned between DCM and saturated aqueous $NaHCO_3$. The organic layer was separated, washed with water, dried over sodium sulfate and filtered, and the solvent was removed in vacuo. Upon purification by column chromatography (80:20, ethyl acetate:hexane) the title compound was isolated as a colorless oil (0.30 g, 63%). δ_H (300 MHz, DMSO-d_6) 8.20 (1H, bs, NH), 4.28 (1H, bs, OH), 3.84, 3.38 (4H, m, NCH_2, OCH_2), 3.03 (1H, s, alkyne-H), 2.26 (2H, t, $J = 7.3$ Hz, $COCH_2$), 1.62−1.19 (6H, m, 3xCH_2-); δ_C (75 MHz, DMSO-d_6) 171.8 (CO), 81.3, 72.7 (alkyne-C), 60.6 (OCH_2), 35.1, 32.2, 27.6, 25.2, 25.0 (CH_2N, CH_2); *m*/*z* LRMS [ES^+, MeCN] 170 (M + H^+, 45%), 192 (M + Na^+, 10%). HRMS (M + Na^+) ($C_9H_{15}NNaO_2$): calcd, 192.0995; found, 192.0999.

6-Propargylamidohexan-1-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)-phosphoramidite, 6. Compound **5** (1.0 g, 5.9 mmol) was dissolved in DCM (10 mL) under an atmosphere of argon. DIPEA (2.05 mL, 11.8 mmol) was then added followed by 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.45 mL, 6.5 mmol) dropwise. The reaction mixture was left to stir at room temperature for 2 h then transferred under argon into a separating funnel containing degassed DCM (20 mL). The mixture was washed with saturated aqueous KCl (20 mL), and the organic layer was separated, dried over sodium sulfate and filtered. The solvent was removed in vacuo. Upon purification by column chromatography under argon pressure (60:40 ethyl acetate/hexane, 0.5% pyridine) the product was isolated as a pale yellow oil (1.2 g, 55%); δ_P (300 MHz, DMSO-d_6) 146.5; *m*/*z* LRMS [ES^+, MeCN] 370 (M + H^+, 40%), 392 (M + Na^+, 35%). HRMS (M + Na^+) ($C_{18}H_{32}N_3NaO_3P$): calcd, 392.2073; found, 392.2079.

Oligonucleotides Synthesis. Standard DNA phosphoramidites, solid supports and additional reagents including the C7-aminoalkyl cpg were purchased from Link Technologies or Applied Biosystems Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 0.2 or 1.0 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal (A,G,C,T) monomers was 25 s and the coupling time for the alkyne phosphoramidite monomer **6** was extended to 360 s. 3′-Aminoalkyl oligonucleotides were synthesized starting from C7-aminolink cpg (Link Technologies Ltd). Cleavage of the oligonucleotides from the solid

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support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C.

Synthesis of Azidobutyramide-Labeled Oligonucleotides. To incorporate the ezido group at the 3′-end of C7-aminoalkyl oligonucleotide, 10−50 nmol of the oligonucleotide in 40 μL of 0.5 M Na_2CO_3/$NaHCO_3$ buffer (pH 8.75) was incubated for 4 h at room temperature with 10 μmol of succinimidyl-4-azidobutyrate **2** in 12 μL of DMSO. The crude oligonucleotide was purified by reversed-phase HPLC and desalted by NAP-10 gel-filtration according to the manufacturer's instructions (GE Healthcare).

Template-Mediated Click Ligation of Oligonucleotides. To 950 μL of 0.2 M aqueous NaCl tris-hydroxypropyl triazolyl ligand[14] (1.38 μmol), sodium ascorbate (2.0 μmol) and $CuSO_4 \cdot 5H_2O$ (0.20 μmol) were added sequentially. To the above solution, azide ODN-2 (2.0 nmol), alkyne ODN-4 (2.0 nmol), and template ODN-5 (2.2 nmol) were added, and the reaction mixture was kept at room temperature for 2 h. After completion of the reaction click-ligated ODN-6 was desalted on a NAP-10 column (GE Healthcare), purified by anion-exchange HPLC, desalted again on a NAP-10 column (GE Healthcare), analyzed by MALDI-TOF mass spectrometry [$(M + H^+)$ calcd, 10204; found, 10205.1] and subjected to melting experiments along with unmodified oligonucleotides (18-mer) ODN-7 and ODN-8.

Click-ligation reactions of ODN-2, ODN-4, and ODN-5 were also carried out at 10.0, 2.0, and 0.4 μmolar concentrations with and without template oligonucleotide and also carried out with and without copper catalyst. Dilution was carried out by increasing the volume of 0.2 M aqueous NaCl.

Preparation of Circular ssDNA (ODN-10). To 2500 μL of 0.2 M aqueous NaCl, triazolyl ligand (1.38 μmol), sodium ascorbate (2.0 μmol), and $CuSO_4 \cdot 5H_2O$ (0.20 μmol) were added sequentially. To the above solution, azide−alkyne labeled ODN-9 (1.0 nmole) was added, and the solution was stored at room temperature for 2 h. The reaction mixture was desalted on a NAP-25 column (GE Healthcare) and then lyophilized. Click-ligated single-strand cyclic ODN-10 was purified by denaturing polyacrylamide gel electrophoresis. The circular ssDNA ODN-10 was also prepared in this manner at 1.0 and 4.0 μmolar concentrations.

Following the same procedure, oligonucleotides ODN-11 and ODN-13 were circularized in 2.0 μmolar concentrations to give ODN-14 and ODN-15, respectively.

Preparation of dsDNA Pseudohexagon (ODN-12). To 400 μL of 0.2 M aqueous NaCl, triazolyl ligand (1.38 μmol), sodium ascorbate (2.0 μmol), and $CuSO_4 \cdot 5H_2O$ (0.20 μmol) were added sequentially. A solution of circular ssDNA and azido−alkyne labeled ODN-11 (1.0 nmol) in 100 μL of 0.2 M aqueous NaCl was heated to 90 °C then cooled slowly to 20 °C. This was repeated three times, and the solution was then added to the above prepared catalyst solution at 10 °C and kept at this temperature for 2 h and then for a further 30 min at room temperature. The mixture was desalted on a NAP-10 column (GE Healthcare) and then lyophilized. The reaction product was analyzed by denaturing polyacrylamide gel electrophoresis.

Analysis and Purification of Oligonucleotides. Denaturing Polyacrylamide Gel Electrophoresis (PAGE) Analysis and Purification. Purification of ODN-10, ODN-14, and ODN-15 was carried out by gel electrophoresis. The crude mixture was purified on 8% polyacrylamide/7 M urea gel at constant power of 20 W for 2 h, using 0.09 M Tris-borate-EDTA buffer (pH 8.0). Following electrophoresis the plates were wrapped with cling-film, placed on a fluorescent TLC plate, and illuminated with a UV lamp (254 nm) to visualize the band. The bands were excised, and the gel pieces were crushed and incubated in 3 mL of sterile water at 37 °C (16 h). The tubes were then vortexed and centrifuged, and the supernatants were lyophilized, dissolved in sterile water (1 mL), and desalted using NAP-10 columns (GE Healthcare).

HPLC Purification. Reversed-Phase HPLC. Purification of oligonucleotides ODN-2, ODN-4, ODN-5, ODN-7, ODN-8, ODN-9, ODN-11, ODN-13, and ODN-16 was carried out by reversed-phase HPLC on a Gilson system using an ABI Aquapore column (C8), 8 mm × 250 mm, pore size 300 Å. The system was controlled by Gilson 7.12 software and the following protocol was used: run time, 30 min; flow rate, 4 mL per min; binary system. Gradient (time in mins (% buffer B)): 0 (0), 3 (0), 5 (20), 21 (100), 25 (100), 27 (0), 30 (0). Elution buffers: (A) 0.1 M ammonium acetate, pH 7.0; (B) 0.1 M ammonium acetate with 35% acetonitrile, pH 7.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 295 nm. After HPLC purification oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare), aliquoted into eppendorf tubes and stored at −20 °C.

Denaturing Anion-Exchange HPLC Analysis of Click Ligation Reactions and Purification of ODN-6. Click ligation reactions were analyzed on a Gilson HPLC system using a Resource Q anion-exchange column (6 mL volume, GE Healthcare). The HPLC system was controlled by Gilson 7.12 software, and the following protocol was used: run time, 30 min; flow rate, 6 mL per min; binary system. Gradient (time in mins (% buffer B)): 0 (0), 3 (0), 4 (5), 23 (100), 26 (100), 27 (0), 30 (0). Elution buffers: (A) 0.01 M aqueous NaOH, 0.05 M aqueous NaCl, pH 12.0; (B) 0.01 M aqueous NaOH, 1 M aqueous NaCl, pH 12.0. Elution of oligonucleotides was monitored by ultraviolet absorption at 270 nm. ODN-6 was purified by this method monitoring at 295 nm. After HPLC purification it was desalted using a NAP-10 Sephadex column (GE Healthcare), aliquoted into eppendorf tubes and stored at −20 °C.

Enzymatic Digestion of Linear and Circular Oligonucleotides. A solution of linear ODN-11 (0.47 nmol) and ODN-16 (0.7 nmol) in sterile water (20 μL) was heated to 90 °C then cooled slowly to 20 °C. This was repeated three times to prepare the double-strand DNA. The DNA solution was added to a microcentrifuge tube containing 10.6 μL of water, 4 μL of 10X buffer [0.06 M Tris-HCl (pH 7.5), 0.5 M aqueous NaCl, 0.06 M $MgCl_2$, and 0.01 M DTT], and 0.4 μL of acetylated BSA (0.1 mg/mL). To this solution 8 μL of the restriction enzyme Mbo11, (Promega), (6u/μL) was added and the mixture was gently mixed by pipetting. The tube was closed and centrifuged for 10 s. The sample was then incubated at 37 °C for 2.5 h.

Purified circular oligonucleotide ODN-14 was digested following the same procedure. Purification of oligonucleotide (ODN-14) from the circularization reaction mixture was done by denaturing 8% polyacrylamide gel electrophoresis (as described above).

Fluorescence Melting Experiments. The experiments were performed in a total volume of 10 μL with a concentration of 0.5 μM of each oligonucleotide at pH 7.0 in 0.01 M sodium phosphate buffer with 0.2 M aqueous NaCl and 0.001 M EDTA-Na_2; SYBR Green I (Roche, 1 μL) was added. Each sample was prepared in triplicate and the average T_m is given.

Roche Lightcycler Method. Following initial rapid denaturation from 30 to 95 °C at 20 °C/s, fluorescence was measured in step mode from 95 to 35−95 °C at 0.5 °C intervals, with a 30 s equilibration interval at each step.

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Supporting Information Available: Cyclic and linear alignment of ODN-9 and ODN-11; fluorescence melting curve of ODN-7/ODN-8 and ODN-7/ODN-6 duplexes; ^{13}C NMR spectra of compounds **1**, **2**, **4**, and **5**; MALDI TOF MS of ODN-2, ODN-4, ODN-6, and ODN-15; ES^- MS of ODN-15. This material is available free of charge via the Internet at http://pubs.acs.org.

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