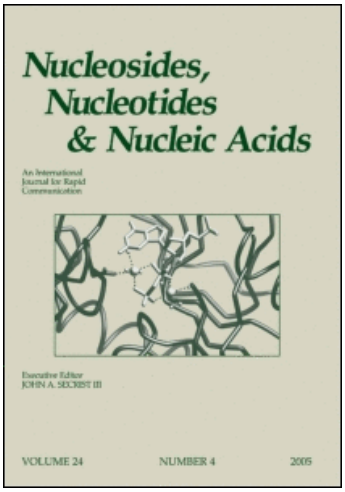

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Very Stable End-Sealed Double Stranded DNA by Click Chemistry

Afaf H. El-Sagheer[ab]
[a] School of Chemistry, University of Southampton, Highfield Southampton, United Kingdom [b] Chemistry Branch, Department of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez Canal University, Suez, Egypt

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VERY STABLE END-SEALED DOUBLE STRANDED DNA BY CLICK CHEMISTRY

Afaf H. El-Sagheer

School of Chemistry, University of Southampton, Highfield Southampton, United Kingdom and Chemistry Branch, Department of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez Canal University, Suez, Egypt

□ *An efficient and simple method has been established for the intermolecular click ligation of two complementary DNA strands to produce an end-sealed duplex with a triazole linkage at each end. The resultant end-sealed duplex is thermally very stable ($\Delta T_m \sim 30^\circ$ C relative to a normal duplex) and a fluorescent version remained intact for up to 3 days in Fetal Bovine serum. In contrast a single strand was completely degraded in 2 hours. These favorable properties suggest that such cyclic DNA duplexes might have potential for in vivo applications and nanotechnology.*

Keywords DNA ligation; click chemistry; CuAAC reaction; nucleic acid; end sealed; DNA duplex; serum stability

INTRODUCTION

The copper-catalyzed alkyne-azide cycloaddition reaction (CuAAC), the best example of click chemistry, was recently discovered by Meldal[1] and Sharpless.[2] Cu(I) catalysis increases the rate of the reaction dramatically to give the 1,4-regioisomer of the 1,2,3-triazole. The reaction is clean, fast, and tolerant of other functional groups and requires minimal work-up and purification. It can be performed in a variety of solvents including water, which is ideal for DNA chemistry.

Cyclic duplexes have potential biological uses.[3] For example, oligonucleotide aptamers are used in vivo as decoys to sequester DNA-binding proteins.[4] It is important that these oligonucleotides remain double

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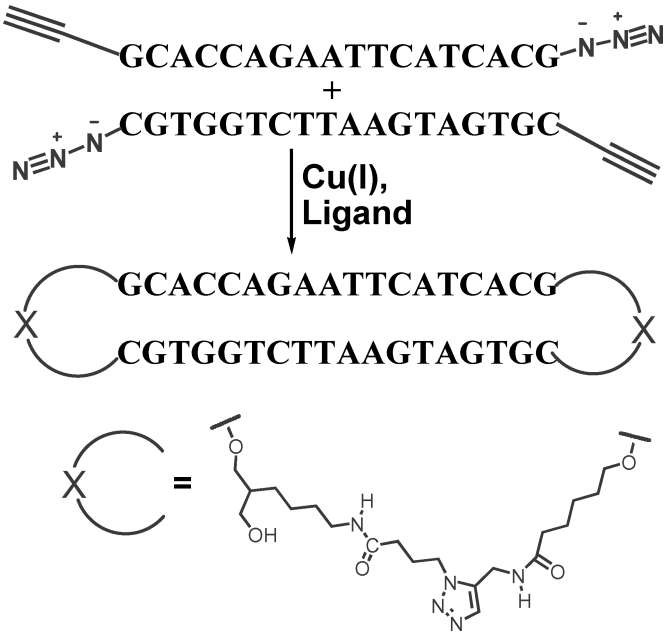
Address correspondence to Afaf H. El-Sagheer, School of Chemistry, University of Southampton, Highfield SouthamptonSO17 1BJ, UK. E-mail: ahes@soton.ac.uk or afafelsagheer@hotmail.com

stranded in the cell and are stable to DNases that occur in biological media. Enzymatic degradation occurs predominantly from the 3′-end of single stranded DNA and at the frayed ends of duplexes. Therefore cyclic DNA duplexes are more resistant, and there is evidence that they are more readily taken up by cells.[5] Cyclic oligonucleotide constructs with unique topologies are also of interest in nanotechnology.[6–11] Conventional DNA nanostructures which are held together only by Watson-Crick base pairing can be disassembled by heat or various denaturing agents. Consequently they have to be analyzed under nondenaturing conditions. This complicates their characterization and purification[12] and also reduces their value as building blocks that can be stored for subsequent use in the assembly of larger complex nanostructures. Chemical methods for the synthesis of DNA constructs in which the strands are covalently interlocked offer a potential solution to this instability problem. For all the above applications efficient and simple methods for the synthesis of oligonucleotides constructs with optimum properties are essential.

We recently used the CuAAC reaction for the intramolecular cyclization of oligonucleotides containing alkyne and azide substituents at the termini.[13–15] Now an efficient method of intermolecular ligation of two complementary oligonucleotide strands is reported. This simple method facilitates the synthesis of very stable end-sealed duplexes with triazole linkages at each end. The resultant constructs are thermally very stable and remain intact in Fetal Bovine Serum, a readily available representative mammalian serum, up to 3 days while the single strand is completely degraded in 2 hours.

RESULTS AND DISCUSSION

Chemical ligation of two modified single stranded oligonucleotides was carried out in order to produce a very stable end-sealed duplex with a triazole linkage at the termini. Each single strand had a 5′-terminal alkyne and 3′-azide. The CuAAC reaction was used to link the two strands by covalently joining the 5′- alkyne to the 3′-azide (Scheme 1). The alkyne, which was derived from 6-propargylamido hexane **1**,[13] (Figure 1) was incorporated into the oligonucleotide as phosphoramidite monomer during solid phase synthesis, and 4-azidobutyrate was added as succinimidyl ester **2**[13] (Figure 1) to the 3′-aminoalkyl modified oligonucleotide after solid-phase synthesis. The click ligation was carried out in presence of Cu(I) and, the water soluble ligand, tris-hydroxypropyl triazole.[16] The reaction proceeded essentially to completion as indicated from polyacrylamide gel electrophoresis (PAGE) analysis (Figure 2). As each of the two complement single strands has both alkyne and azide function groups, the click ligation could yield many possible products. For example, reaction between the



SCHEME 1 Click reaction of oligonucleotides ODN-1 and ODN-2 to give the end-sealed duplex EsD-1 with a triazole linkage at each end.

alkyne and azide moiety in the same strand would give a cyclic single stranded oligonucleotide. Alternatively the azide of one strand might react with the alkyne of a similar strand to yield a cyclic or linear dimer, trimer or polymer instead of reacting in the desired manner with its complement. To prevent these unwanted reactions the two oligonucleotides have to be mixed and annealed slowly before Cu(I) catalysis.

Evidence for formation of the required end-sealed duplex was provided by PAGE, capillary electrophoresis (CE) and mass spectrometry. MALDI-TOF mass spectrometry gave the following results: ODN-1 (calc. 6002, found

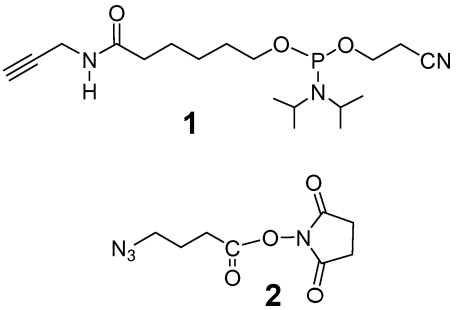


FIGURE 1 Chemical structure of the alkyne phosphoramidite monomer and the azide active ester used in this study.

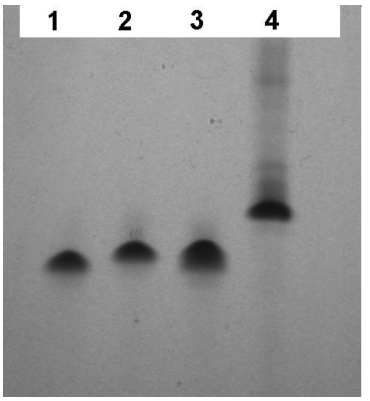


FIGURE 2 Gel analysis of crude reaction mixture to form EsD-1. Lane 1: ODN-1, lane 2: ODN-2, lane 3: ODN-1 + ODN-2 [without Cu(I)], lane 4: crude reaction mixture of EsD-1. 8% polyacrylamide/7 M urea gel at constant power of 20 watt for 2 hours, using 0.09 M Tris-borate-EDTA buffer (pH 8.0).

6006), ODN-2 (calc. 6095, found 6097) and the end-sealed duplex EsD-1 (calc. 12097, found 12102). Extra proof for the formation of the end-sealed duplex was obtained by *Eco*RI restriction enzyme digestion and CE analysis. The normal (control) duplex showed four cleavage peaks whereas the end-sealed duplex gave the predicted two cleavage peaks around 22 minutes (Figures 3a and 3b). In addition to these two digestion peaks there was a single peak at 26 minutes for the undigested EsD-1 and another peak at 27 minutes. This is probably an intermediate structure produced from cutting one strand of the end-sealed duplex which then quickly opens up with the consequence that the other strand is not cut (the restriction enzyme

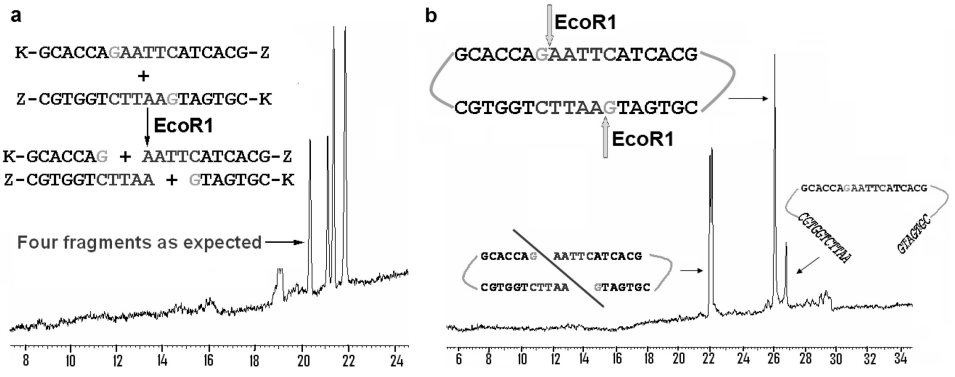


FIGURE 3 Capillary electrophoresis (CE) analysis of: a) digestion of open-ended duplex (ODN-1 + ODN-2), b) digestion of end-sealed duplex (EsD-1). A standard ssDNA 100-R Gel, Tris-Borate-7 M Urea gel was used (Kit No 477480) on a Beckman Coulter P/ACE MDQ Capillary Electrophoresis System using 32 Karat software. UV-254 nm, inject-voltage 10.0 kv and separation-voltage 9.0 kv (45.0 minutes duration). X-axis is time (minutes), Y-axis is UV absorbance at 254 nm.

TABLE 1 Single and double stranded DNA sequences

Entry	Sequences (5′-3′)
ODN-1	***K***GCACCAGAATTCATCACG***Z***
ODN-2	***K***CGTGATGAATTCTGGTGC***Z***
ODN-3	***K***CGTGA***F***GAATTCTGGTGC***Z***
EsD-1	GCACTACTTAAGACCACG
	Tz ***Tz***
	CGTGATGAATTCTGGTGC
EsD-2	GCACTACTTAAGACCACG
	Tz ***Tz***
	CGTGA***F***GAATTCTGGTGC

K = alkyne, ***Z*** = azide, ***Tz*** = 1,2,3-triazole linker, ***F*** = fluorescein dT.

only cleaves double stranded DNA). These results were confirmed by PAGE analysis.

Having established that the end-sealed duplex is more stable than the normal open-ended duplex towards the restriction enzyme digestion (Figures 3a and 3b), the stability of the end-sealed duplex in serum was investigated. For this purpose, one of the single strands was labelled with fluorescein by replacing one thymidine nucleotide in the original sequence of ODN-2 with fluorescein dT to give ODN-3 (Table 1). Click ligation of ODN-1 and fluorescent ODN-3 gave fluorescein labelled end-sealed duplex EsD-2. Both the end-sealed duplex EsD-2 and the single strand ODN-3 were incubated in 50% Fetal Bovine Serum at 37°C and the products were analyzed by denaturing gel electrophoresis. The end-sealed duplex EsD-2 was found to be stable up to 3 days while the single stranded ODN-3 was digested in 2 hours (Figure 4). Similar results were obtained using 10% and 90% serum. The open-ended duplex (unsealed) showed degradation after 1 day, however the main disadvantage of the open-ended duplex that in vivo the two strands will come apart, leading to rapid degradation of the single strands. Obviously this can't occur with the end-sealed duplex.

The thermal stability of the end-sealed and open-ended duplexes was then investigated. An ultraviolet melting study showed that the end-sealed duplex is very stable with a T_m ~30°C higher than that of the open-ended duplex. As the T_m of the end-sealed duplex was very high, formamide (denaturant) had to be added to bring it into the measurable range (Figure 5, Table 2). These results were confirmed by fluorescence melting (Table 2).

CONCLUSIONS

Very stable end-sealed duplexes with triazole linkage at each end have been synthesized in high yield using the copper-catalyzed alkyne-azide cycloaddition reaction (CuAAC). The T_m of the end-sealed construct was

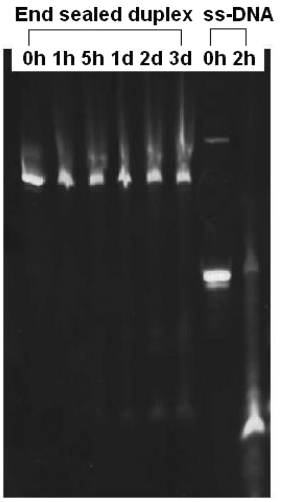


FIGURE 4 Gel analysis of serum stability of end-sealed duplex (EsD-2) (stable up to 3 days) and ss-DNA (ODN-3) (completely digested in 2 hours) in 50% fetal bovine serum. 20% Polyacrylamide/7 M urea gel at a constant power of 20 watt for 3 hours, using 0.09 M Tris-borate-EDTA buffer (pH 8.0).

higher by ~30°C than the open-ended duplex and the end-sealed duplex was stable up to 3 days in 50% serum, whereas in contrast the single strand was completely degraded in 2 hours. These favorable properties suggest that cyclic DNA duplexes of this type could be used in biological applications as decoys for proteins that control gene expression and in nanotechnology.

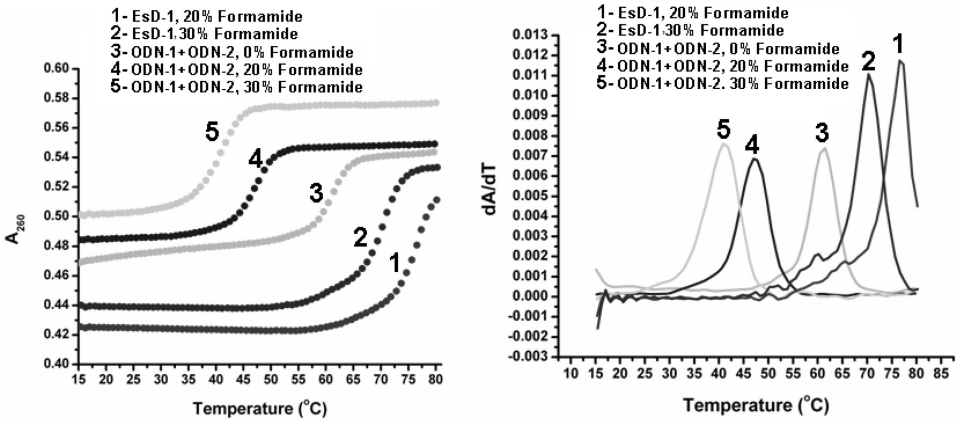


FIGURE 5 UV melting curves and derivatives measured on a Cary 400 Scan UV-Visible Spectrophotometer (Varian) at 1.0 μM concentration of oligonucleotide in 10 mM phosphate buffer and 200 mM NaCl at pH 7.0 to which was added formamide. Spectra were recorded at 260 nm and T_m values were calculated using Cary Win UV Thermal Application Software.

TABLE 2 Ultraviolet (UV) and fluorescence melting (f) of open-ended (normal) duplex (ODN-1+ODN-2) and end-sealed duplex EsD-1 with 0-50% formamide

Formamide (%)	ODN-1+ODN-2 T_m(°C)	EsD-1 T_m (°C)	ΔT_m
0	64.9^{f}, 61.1^{UV}	$>91^{f}$	$>26.1^{f}$
10	55.8^{f}	83.7^{f}	27.9^{f}
20	48.2^{f}, 47.3^{UV}	78.8^{f}, 78.0^{UV}	30.6^{f}, 30.7^{UV}
30	41.3^{f}, 41.0^{UV}	69.2^{f}, 70.0^{UV}	27.9^{f}, 29^{UV}
40	33.7^{f}	62.0^{f}	28.3^{f}
50	$<33^{f}$	58.3^{f}	25.3^{f}

EXPERIMENTAL

Oligonucleotide Synthesis and Purification

Standard DNA phosphoramidites, solid supports and additional reagents including C7-aminoalkyl CPG and Fluorescein dT phosphoramidite were purchased from Link Technologies (Scotland) or Applied Biosystems (United Kingdom) Ltd. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 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 on the DNA synthesizer 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 times were 25 seconds for normal (A,G,C,T) monomers and 10 minutes for the alkyne and Fluorescein dT phosphoramidites. Cleavage of oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 minutes at room temperature followed by heating in a sealed tube for 5 hours at 55°C. To label the oligonucleotide with the azide function, the NHS ester of 4-azidobutyric acid **2** (4 mg) was added post-synthetically to the 3′-amino-modified oligonucleotides (freeze dried) in 120 μL of DMSO:0.5 M Na_2CO_3/$NaHCO_3$ buffer 1:2 at pH 8.75 and the reaction was left to proceed for 4 hours at room temperature. The fully labelled oligonucleotides were gel-filtered using disposable NAP-10 columns (GE Healthcare) according to the manufacturer's instructions then purified by reversed-phase HPLC (C8) in a gradient of acetonitrile in 0.1 M ammonium acetate and desalted by NAP-10 gel-filtration.[14] HPLC showed one peak indicating that the yield of the labelling reactions is >90%.

Synthesis and Reversed-Phase HPLC Purification of End-Sealed Duplexes

To a solution of tris-hydroxypropyl triazole ligand[16] (3.5 μmol in 2.0 mL 200 mM NaCl) under argon was added sodium ascorbate (5.0 μmol in

10.0 μL 200 mM NaCl) followed by $CuSO_4.5H_2O$ (0.5 μmol in 5.0 μL 200 mM NaCl). The two oligonucleotides (10.0 nmol of each oligonucleotide in 500.0 μL 200 mM NaCl) were heated at 80°C for 5 minutes, cooled down slowly to allow annealing to occur and added to the above solution. The reaction mixture was kept under argon at room temperature for 2 hours and a disposable NAP-25 gel-filtration column was then used to remove reagents.

Purification of the end-sealed duplex was carried out by reversed-phase HPLC (C8) in a gradient of acetonitrile in 0.1 M ammonium acetate over 20 minutes then desalted by gel-filtration using NAP-10 Sephadex columns to give the purified product in 45% isolated yield after HPLC while the reaction conversion was quantitative as shown by PAGE (Figure 2).

*Eco*RI Restriction Enzyme Digestions

The end-sealed or the open-ended duplex (8 μg, 0.7 nmoles) in 20 μl NaCl (0.2 M) was heated at 80°C for 5 minutes and cooled down slowly to allow annealing to occur. Water (18.4 μL), 10 X buffer H (6 μL) (Buffer H was provided with the enzyme) and bovine serum albumin, acetylated (BSA) (0.6 μL) (10 μg/μL) were added to the above solution followed by *Eco*RI restriction enzyme (15 μL) (12 units/μL). The mixture was vortexed and incubated at 37°C for 3 hours, then analyzed by CE and PAGE.

Buffer H, 900mM Tris-HCl, pH 7.5, 500 mM NaCl and 100 mM $MgCl_2$, was supplied with the *Eco*RI restriction enzyme.

Serum Stability of ssDNA and End-Sealed dsDNA

A mixture of DNA (1.0 μg) in phosphate buffer (15 μL) (10 mM phosphate, pH 7, 200 mM NaCl) and Fetal Bovine Serum (15 μL) was vortexed and incubated at 37°C for the desired time. The mixture was stored in the freezer until all the samples were collected. Formamide (15 μL) was added to each sample, samples were then vortexed, heated at 80°C for 5 minutes and cooled in ice before analysis by denaturing 20% polyacrylamide gel electrophoresis.

Ultraviolet Melting

UV melting curves were measured on Cary 400 Scan UV-Visible Spectrophotometer (Varian) at 1.0 μM concentration of oligonucleotide in 10 mM phosphate buffer and 200 mM NaCl at pH 7.0. Spectra were recorded at 260 nm. The samples were initially denatured by heating to 80°C at 10°C/min then cooled to 15°C at 1°C.min^{-1} and re-heated to 80°C at 1°C.min^{-1}. Three successive melting curves were measured and T_m values were calculated using Cary Win UV Thermal application Software.

Fluorescence Melting

The experiments were performed on a Roche LightCycler 1.5. Each capillary had a volume of 20 μL. Concentration of end-sealed duplex and open-ended duplex was 0.25 μM. Melting studies were carried out at pH 7.0 in 10 mM sodium phosphate buffer with 200 mM aqueous NaCl; SYBR Green I fluorescent DNA-binding dye (Roche, 2 μL, 1:10000 dilution) was added to each capillary. Following initial rapid denaturation from 30 to 95°C at 20°C.s^{-1}, fluorescence was measured in step mode cooling to 35°C then melting from 35°C to 95°C at 0.5°C intervals with a 30 seconds equilibration interval at each step. Each sample was measured in triplicate and the average T_m is given.

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