Progress toward "Click"-based Small-molecule DNA Hybrids Part II: Di- and Tri-functionalized Core

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Abstract

This paper reports the syntheses of acetylene-terminated small-molecule cores. Also reported are successful attempts at "click" coupling the core molecules with azide-terminated oligonucleotides to construct stable small-molecule DNA hybrids (SMDH). Once these click-based small-molecule DNA hybrids are formed, melting studies will be done that study how the geometry of SMDH aggregate systems affects the suspected sharp melting transitions.

Introduction

As mentioned previously,[1] efficient diagnosis of genetic diseases requires the development of detection assays that are both highly selective and sensitive. While several DNA detection systems recently have been developed based on the sharpened melting profile (compared with that of free DNA) exhibited by aggregated DNA hybrids (Figure 1), a complete description of the parameters that govern such sharp melting behavior has not been achieved.[2] To this end, this study has designed small-molecule DNA hybrids (SMDHs) possessing fixed geometries and a number of DNA strands that can serve as an ideal model system for deciphering structure–property relationships in hybrid DNA materials. As the structure of SMDHs can be tuned in a modular fashion, the parameters that affect the observed sharp melting transitions can be unraveled systematically, enabling superior detection methods.

Stepp and coworkers recently synthesized a three-armed SMDH with a rigid phenylacetylene core that is ideal for maintaining a fixed geometry between parallel DNA arms.[3] While this SMDH exhibits sharp melting when treated with its complementary SMDH at a very dilute concentration, its low stability — a consequence of the labile benzylic phosphate-ester linkage between the core[4] and the DNA arms — prohibited further studies. In addition, synthesizing these SMDHs is less than ideal for creating a diversity of core molecules; any variation of the intended core structure would require a significant reworking of the synthetic route. Hence, the researchers proposed the synthesis of triazole-linked SMDHs through the connection of azide-terminated DNA strands to acetylene-terminated small molecules using "click" chemistry (Figure 2).[1] Stronger and more stable SMDHs can be produced by attaching DNA to rigid phenylacetylene-based core structures through a stable triazole linkage. In addition, the modular synthetic approach will ease diversification, thereby permitting a wide range of SMDH materials to be synthesized and studied — leading to the development of improved DNA detection systems.

Previously, the authors reported the syntheses of two silyl-protected acetylene core molecules.[1] This project extends this chemistry to two other cores and focuses on the click chemistry of coupling the acetylene-terminated cores with azide functionalized DNA.

Background

Commercially available acetylene-modified phosphoramadites (building blocks of DNA) have been shown to undergo click chemistry with 5'-azide functionalized DNA.[5] Manual and automated procedures have also been developed to convert oligonucleotides to 5'-iodo derivatives and finally to 5'-azide forms.[6,7]

Approach

To use click chemistry, acetylene-terminated cores must be obtained (Figure 3). It is important to obtain these molecules in pure form because any impurity could affect their subsequent click coupling

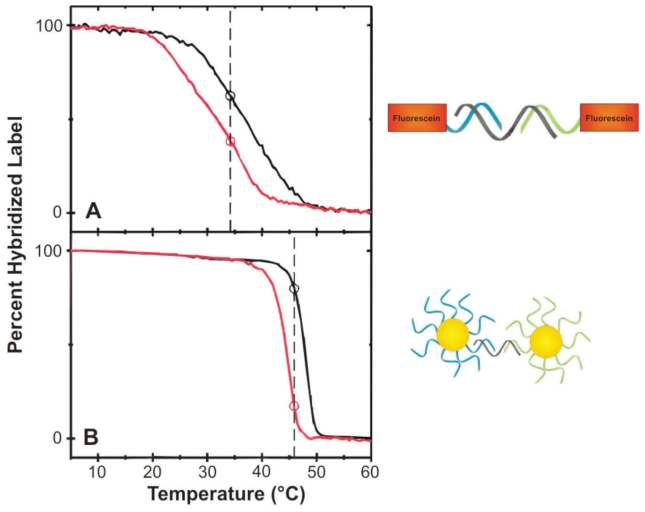

Figure 1. The difference in melting profiles for free DNA (a) and aggregate DNA (b).[2] The graphs indicate the percent of intact duplexes for perfectly complementary DNA pairs (black) and DNA pairs having a single base-pair mismatch (red). The free-DNA system, where each individual unit structure has only a single DNA strand attached, is represented in the top picture by fluorescein-labeled DNA probes. The aggregate system, where each individual unit structure has multiple DNA strands attached, is represented in the bottom picture by the DNA-gold nanoparticle hybrids.

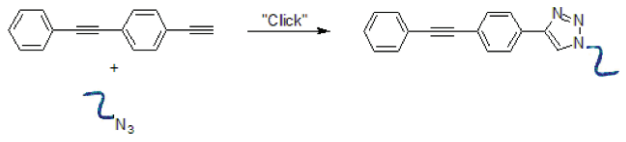

Figure 2. Proposed strategy for linking DNA to a small molecule using click chemistry.

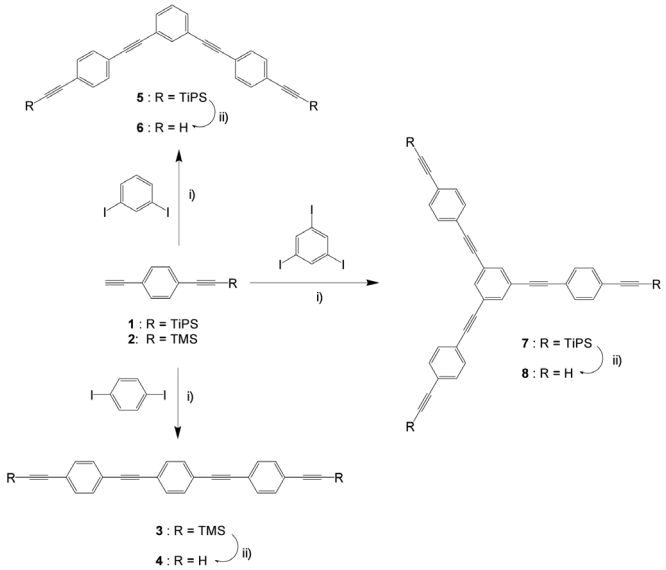

Figure 3. Synthesis of di- and tri-substituted small molecules. i) $(PPh_3)_2PdCl_2$, CuI, NEt_3, 10 hr, RT; ii) TBAF, THF, 3 hr, RT.

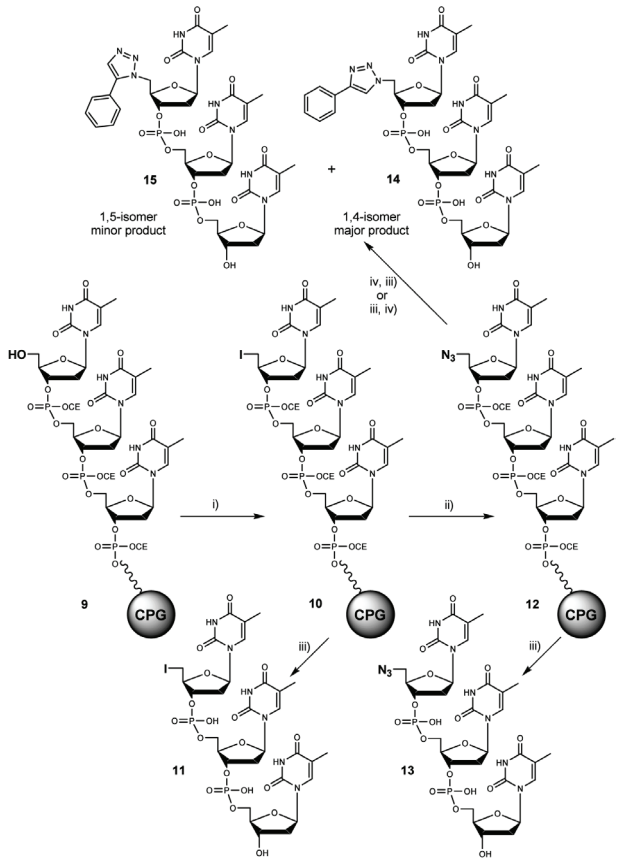

Figure 4. Synthesis of 3'-TTT-OH, 3'-TTT-I, 3'-TTT-N_3 and subsequent click reaction of 3'-TTT-N_3 with phenylacetylene.[8] i) $(PhO)_3PCH_3I$, DMF, 10 min; ii) NaN_3, DMF, 2 h, 50° C; iii) NH_4OH, 10 hr; iv) phenylacetylene, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DMF:H_2O (4:1 v/v), microwave or Eppendorf shaker, 55° C.

with oligonucleotide. The other component for click coupling is the azide-terminated oligonucleotide (Figure 2), which can be synthesized from normal oligonucleotides (Figure 4).

Results and Discussion

General Procedure for Pd(0) Coupling (Figure 3)
In an inert atmosphere glovebox, precursor 1 was added to a 50-mL Schlenk flask containing di- or tri-iodobenzene, CuI (5 mol% per site), $(PPh_3)_2PdCl_2$ (5 mol% per site), and triethylamine (NEt_3). A white precipitate formed in solution immediately upon addition of precursor 1. The resulting reaction mixture was then left stirring under nitrogen overnight before being analyzed by GC-MS. When the reaction had reached completion, the reaction mixture was diluted with excess ethyl ether (20 mL), washed with aqueous HCl (20 mL of a 1 M solution), dried over $MgSO_4$, and gravity filtered. The collected organic layer was then evaporated to dryness, redissolved in toluene, and evaporated to dryness to ensure the removal of all solvents and reactants. The crude product was then redissolved in a minimum amount of methylene chloride and passed through a silica gel column using methylene chloride as an eluent. The product was isolated in vacuo upon evaporation of the solvent.

Synthesis of Bis(trimethylsilylate-protected)-1,4-substituted Small Molecule 3
Product 4 was isolated as white crystals (0.11 g, 33%), using the aforementioned general coupling procedure, from a combination of 2 (0.31 g, 1.56 mmol), 1,4-diiodobenzene (0.23 g, 0.7 mmol), $(PPh_3)_2PdCl_2$ (0.06 g, 0.08 mmol), CuI (0.008 g, 0.08 mmol) and NEt_3 (5 mL). ^1H NMR (400 MHz, $CDCl_3$): 7.486 (d, 4H), 7.451 (s, 4H), 7.393 (d, 4H), 7.26 (s, 1H), 0.262 (s, 18H).

Synthesis of 1,3-disubstituted Small Molecule 5
Product 5 was isolated as white low-melting solid (0.051 g, 18%), using the aforementioned general coupling procedure, from a combination of 1 (0.28 g, 0.992 mmol), 1,3-diiodobenzene (0.148 g, 0.451 mmol), CuI (0.016 g, 22.5 µmol), $(PPh_3)_2PdCl_2$ (0.002 g, 22.5 µmol), and NEt_3 (20 mL). ^1H NMR (500 MHz, $CDCl_3$): δ 7.729 (s, 1H), 7.513 (d, 1H), 7.49 (d, 1H), 7.48 (s, 8H), 7.35 (t, 1H), 7.26 (s, 1H), 1.15 (s, 42H).

Synthesis of 1,3,5-trisubstituted Small Molecule 7
Product 7 was isolated as white crystals (0.17 g, 16%), using the aforementioned general coupling procedure, from a combination of 2 (0.31 g, 1.56 mmol), 1,3,5-triiodobenzene (0.108 g, 0.238 mmol), CuI

(0.011 g, 0.0119 mmol), $(PPh_3)_2PdCl_2$ (0.0083 g, 0.0119 mmol), and NEt_3 (20 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.482 (d, 4H), 7.465 (d, 4H), 7.453 (d, 4H), 7.382 (s, 3H), 0.262 (s, 63H).

General Procedure for Deprotection
To a stirred solution of di- or tri-cores 3, 5, and 7 in THF, excess tetra-n-butylammonium fluoride (TBAF) was added, and the mixture was stirred at room temperature (RT) for 3 hr. The solution was concentrated in vacuo to give a residue that was column-chromatographed over silica using CH_2Cl_2 eluent to afford the product.

Synthesis of 1,4-substituted Core 4
Product 4 was isolated as a yellow solid (9 mg, 93%), using the aforementioned general deprotection procedure, from a combination of 3 (14 mg, 0.0298 mmol), TBAF (47 mg, 0.149 mmol), and THF (2 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.486 (d, 4H), 7.451 (s, 4H), 7.393 (d, 4H), 7.26 (s, 1H), 3.19 (s, 2H).

Synthesis of 1,3-substituted Core 6
Product 6 was isolated as a yellow solid (39 mg, 92%), using the aforementioned general deprotection procedure, from a combination of 5 (85 mg, 0.133 mmol), TBAF (210 mg, 0.666 mmol), and THF (2 mL). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.729 (s, 1H), 7.513 (d, 1H), 7.49 (d, 1H), 7.48 (s, 8H), 7.35 (t, 1H), 7.26 (s, 1H), 3.195 (s, 2H).

Synthesis of 1,3,5-substituted Core 8
Product 8 was isolated as a yellow solid (19 mg, 93%), using the aforementioned general deprotection procedure, from a combination of 7 (29 mg, 0.0316 mmol), TBAF (75 mg, 0.237 mmol), and THF (2 mL). ^{1}H NMR (500 MHz, CDCl3): δ 7.482 (d, 4H), 7.465 (d, 4H), 7.453 (d, 4H), 7.382 (s, 3H), 3.2 (s, 3H).

Synthesis of TTT-OH and TTT-I Single-stranded DNA (9-11, Figure 4)[6,7]
3′-TTT-OH-5′ was synthesized on an Expedite 8909 DNA synthesizer using standard procedures (T = thymine). A new protocol was written in Expedite 8909 according to Miller et al.[6,7] to convert the 3′-TTT-OH-5′ to 3′-TTT-I-5′ via addition of 0.5 M $(PhO)_3PCH_3I$ in DMF. The calcium pectinate gel (CPG) beads were then cleaved from the DNA by suspending the beads overnight in concentrated NH_4OH (1 mL) at 45° C. After the excess ammonia was removed by a stream of N_2, the remaining mixture was filtered through a 0.45 μm Nylon membrane.

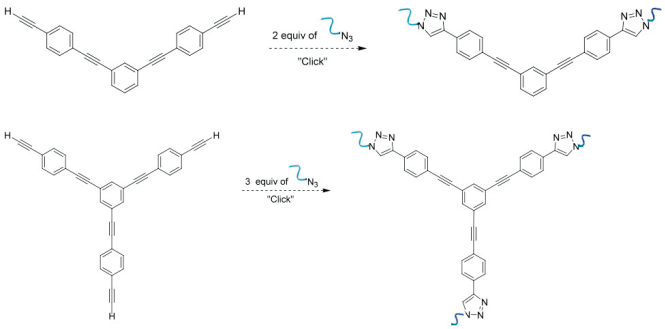

Figure 5. Proposed synthesis of 1,3-disubstituted and 1,3,5-trisubstituted click-based r-SMDH.

The filtrate was lyophilized, affording the product 3′-TTT-I-5′ (11) as a white powder. Analysis of the product using ^{1}H-NMR, 13C-NMR, and IR spectroscopies confirmed its identity.

Synthesis of TTT-N_3 Single-stranded DNA (13, Figure 4)
The CPG-3'-TTT-I-5' (12) beads were removed from the synthesis column (1 μmol DNA) and placed in a 1-mL Eppendorf tube with saturated NaN_3 in DMF (0.6 mL). The tube was then placed in an Eppendorf shaker for 2 hr at 60° C. The excess NaN_3 was rinsed away with nanopure H_2O (5 mL) and MeCN (5 mL). The CPG beads were then cleaved from the DNA by suspending the beads overnight in concentrated NH_4OH (1 mL) at 45° C. After the excess ammonia was removed by a stream of N_2, the remaining mixture was filtered through a 0.45 μm Nylon membrane. The filtrate was lyophilized, affording the product 3'-TTT-N_3-5' (13) as white powder. Analysis of the product using ^{1}H-NMR, ^{13}C-NMR, and IR spectroscopies confirmed its identity.[6,7]

Synthesis of Phenyl SMDHs (14, 15, Figure 4)
Two similar click reactions were carried out in parallel, one in a polypropylene Eppendorf tube and the other in a microwavable glass reaction tube. $CuSO_4.5H_2O$ (0.0125 mg, 0.05 μmol), sodium ascorbate (0.0198 mg, 0.1 μmol), phenylacetylene (3.0mg, 30 μmol), and methanol (400 μL) were combined using stock solutions with CPG-3'-TTT-N3-5' (1 μmol) in each of the tubes. One reaction was placed in a Biotage microwave reactor at 65° C for 15 min; the other was placed on an Eppendorf shaker at 65° C for 24 hr. After this was completed, the CPG beads were then cleaved from the DNA by suspending the beads overnight in concentrated NH_4OH (1 mL) at 45° C. The excess ammonia was removed by a stream of N_2, and the remaining mixtures were filtered through a 0.45 μM nylon membrane. The filtrates were lyophilized, affording the products as white powders.

Analysis of both products was carried out by HPLC along with 3'-TTT-N_3, and 3'-TTT-OH as standards. The two solvents used in the HPLC method were 50 mM TEAA in H_2O (A) and CH_3CN (B). The method consisted of 100% A (0–20 min), [75% A, 25% B] (20–25 min), 100% B (25–30 min), and 100% A (30–35 min). The elution order consisted of the 5'-alcohol DNA, the 5'-azide DNA, and the click product. That the products for the click reactions eluted at a later time than did the more polar 3'-TTT-N_3 and 3'-TTT-OH suggested that the click reactions occurred.

^{1}H-NMR analysis of the products from both reactions showed the presence of a singlet at 8.3 ppm, indicative that the desired 1,4-substituted click product was made. However, while the ^{1}H-NMR spectrum of the product from the Eppendorf shaker reaction showed 99% selectivity for the 1,4-product, that from the microwave reaction showed an additional 10% of the undesirable 1,5-isomer. Thus, future click reactions with azide-substituted oligonucleotides should not be carried out under microwave conditions.

Conclusions

1,3-disubstituted and 1,3,5-trisubstituted acetylene-terminated small molecules (4, 6, and 8, respectively) have been synthesized as the first building blocks in a plan to synthesize small-molecule DNA hybrids. In addition, an azide-functionalized oligonucleotide, the second key

precursor, was successfully synthesized in a two-step approach and coupled to phenylacetylene to demonstrate the feasibility of click chemistry in the synthesis of SMDH.

For future work, click coupling with other cores 4, 6 and 8 will be attempted (Figure 5). When these click-based SMDHs are finally synthesized, subsequent studies on the melting behaviors of SMDH:SMDH hybrids should shed light on the parameters that control sharp melting in cooperative DNA-based materials. Such knowledge will allow for the development of more accurate and selective DNA detection systems.

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