DNA-Controlled Synthesis

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Multistep DNA-Templated Reactions for the Synthesis of Functional Sequence Controlled Oligomers**

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Maintaining a high level of control of the order of reactions is a key goal in synthetic chemistry. The most common strategy is to divide the construction of the desired molecule into a sequence of isolated reaction steps, using protecting group chemistry with purification and isolation of intermediate products. In contrast, multistep synthesis of biomolecules is achieved naturally in a single solution by selective catalysis and by controlled modulation of the effective concentrations of particular reactants. Nature's approach avoids the need for complex protecting group chemistries even when multiple reactive species are present simultaneously. Examples of natural templated synthesis include ribosomal and non-ribosomal peptide synthesis and polyketide synthases.

Many advances have been made towards this ideal using the concept of DNA-templated synthesis (DTS). The reactivities of chemical groups attached to oligonucleotide adapters can be controlled by holding them in close proximity by means of DNA hybridization, increasing the effective molarity of the reactive species and thus accelerating the reaction.[1] The rate enhancement can be sufficient to ensure that cross-reactions with other molecules, present in the same solution but not connected by hybridized oligonucleotide adapters, can be neglected.[2] However, the potential of these methods for the synthesis of sequence-controlled functional oligomers of significant length has not yet been fully realized. In this study we investigate a DTS mechanism that has the potential to allow oligomer synthesis without imposing a length restriction.

Multistep DTS can make use of a linear template or DNA multibranched junction to encode the sequence of the desired product.[3–6] With a linear template, sequential reactions can be controlled by stepwise addition of reactive adapters followed by purification (removing spent adapters) at each step,[3,4] and by controlling stepwise changes in the secondary structure of the template by increasing the temperature, bringing successive groups into proximity with the reactive site.[5] A limitation of these methods is that every new DNA adapter is increasingly separated from the reactive end of the template strand, potentially decreasing the reaction efficiency as the number of steps increases.[7] This problem can be reduced by adding a short, constant region, complementary to the end of the template strand, to each adapter such that all oligonucleotide adapters bind both to the end of the template and to their specific binding site (forming a loop).[8] However, the single-stranded portions of the template and DNA adapters can fold into undesired secondary structures, frustrating this designed interaction and reducing the DNA-templated reactivity.[9] DTS has also been implemented using chemically modified DNA strands that self-assemble into each of the arms of multibranched junctions.[6] This method has the advantage that the reactive groups are all located at the center of the junction, providing a constant reaction environment. However, the number of branches in the junction determines the number of building blocks that can be used to build the oligomer, restricting its size.

Here we present a strategy for the synthesis of ordered functional oligomers by means of sequential DNA-templated reactions, using a strand displacement system that provides the same reaction environment at each step. The general mechanism is depicted in Scheme 1. Oligonucleotide adapters have one of two complementary binding sequences and are functionalized at the 5′ or 3′ end such that, when consecutive adapters are annealed, they hybridize to each other to bring their reactive groups into close proximity. Each adapter also includes a unique "toehold" domain used to remove it from the active complex, once its reaction is complete, by addition of a fully complementary "remover" DNA strand. The chemistry used is such that the growing oligomeric product is transferred to the incoming adapter; this stepwise oligomer growth is reminiscent of the natural peptide and polyketide syntheses. After removal of the spent adapter, the active adapter bearing the growing chain can hybridize and react with the next adapter strand, allowing the cycle to be repeated. A drawback to this approach is that the sequence of the product oligomer is determined by the sequence in which reactants are added, not by a template. This excludes potential applications, such as molecular evolution, which rely on retention of a DNA-coded sequence record attached to the product.[1,10] An advantage is that the configuration of the DNA adapters bearing reactants, and the local environment

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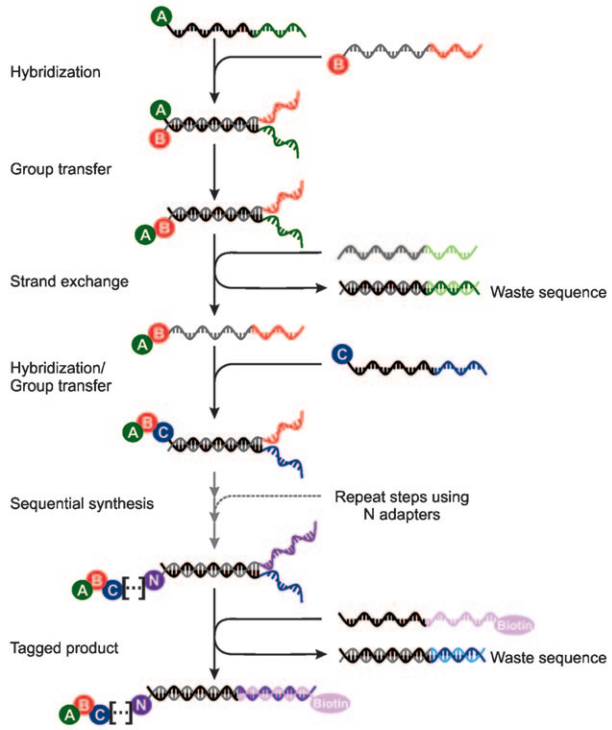
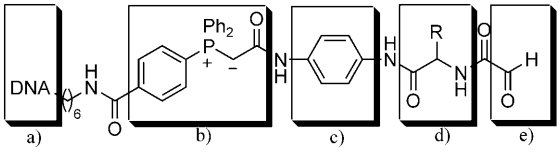


Scheme 1. Strand displacement mechanism for oligomer synthesis. Hybridization brings reactive chemical groups into close proximity allowing chemical group transfer from the first to the second adapter. Subsequent strand exchange using remover strand 1 displaces the first strand as a waste product. This cycle is repeated with different DNA adapters to achieve sequential synthesis. The final product can be isolated from the reaction mixture by using a biotin-tagged remover strand.

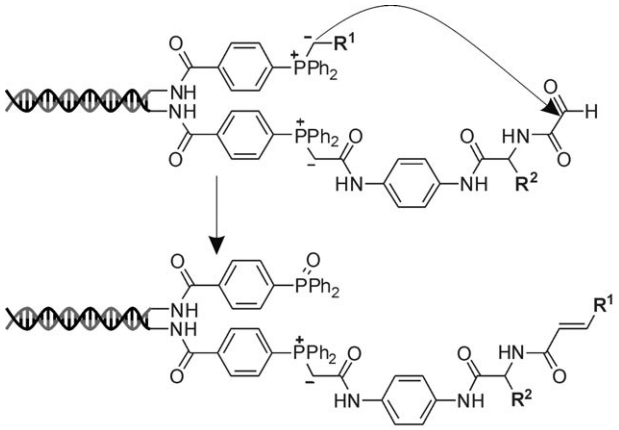
for each reaction, is the same at each step, so the reactivity of an incoming group should be independent of the number of preceding coupling steps and the oligomer length limited only by the intrinsic yield of a single step.

To test this strand displacement system, a series of olefin 4-mers were synthesized using three sequential DNA-templated Wittig reactions. This coupling chemistry was chosen because of its robustness in aqueous solution[11] and because it has been successfully used to synthesize triolefins using phosphine modified oligonucleotides.[5] The design of the DNA adapters used in this work is shown in Scheme 2. All of the reactive oligonucleotides strands are modified, through a terminal amine, with a bifunctional adapter containing both a phosphine ylide (Scheme 2b) and an aldehyde moiety (Scheme 2e).[5] The first and last adapters are exceptions: the first



Scheme 2. Design of functional DNA adapter unit. a) DNA adapter for template control of Wittig reaction, b) triphenylphosphophonium (ylide moiety), c) *para*-phenyl spacing unit (to minimize intramolecular Wittig reaction), d) function unit (amino acid), e) aldehyde.

adapter contains only a phosphine modification whereas the last contains only the aldehyde moiety. This chemistry allows for transfer of a building block attached to the ylide of one DNA adapter to the aldehyde of the next adapter with simultaneous cleavage from the first (Scheme 3).

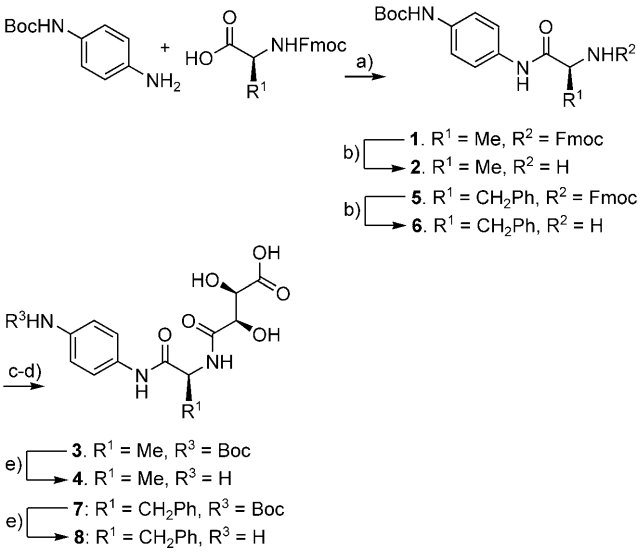


Scheme 3. Transfer of the first building block to the second DNA adapter. In the next step, the unreacted ylide moiety of the second adapter will be used to couple to the aldehyde of the third adapter.[12]

The bifunctionality of the DNA adapter enables ordered multistep transfer and the internal *para*-phenyl spacing unit minimizes intramolecular Wittig reaction (Scheme 2c).[6] There is no need for addition of auxiliary reagents, deprotection, or the purification of intermediates during DTS. The strand displacement system keeps the distance between reacting groups constant, even as the oligomeric chain grows in length. Importantly, the adapter presented here includes a variable side chain, introduced as the side chain of an amino acid during synthesis, which permits the introduction of additional functionality (Scheme 2d).

The synthesis of the bifunctional ylide–aldehyde adapter (Scheme 4) was achieved using a *p*-phenylenediamine as the spacing unit and amino acids to introduce the variable side chain. As proof of principle, L-alanine (Ala) and L-phenylalanine (Phe) were incorporated to demonstrate the preparation of olefin oligomers with a controlled sequence of side chains. Synthesis proceeded by coupling an Fmoc amino acid to *N*-Boc-*p*-phenylenediamine (**1**, **5**),[13] followed by Fmoc deprotection (**2**, **6**).[14] The masked aldehyde moiety (Scheme 2e) was introduced by reacting the amine with diacetyl-L-tartaric anhydride, followed by basic removal of the acetyl protecting groups (**3**, **7**).[15] Unmasking of the aldehyde moiety was later achieved by exposure to 50 mM $NaIO_4$, 500 mM NaOAc, pH 3.5 (see Supporting Information).

Boc deprotection was achieved using TFA to give the free amine (**4**, **8**).[16] Amino modified DNA strands **S1**–**S3** (see Supporting Information) were coupled with diphenylphosphine benzoic acid post-synthetically using standard peptide coupling reagents.[17] To triphenylphosphino strands **S2** and **S3** was added an in situ mix of amine (**4**, **8**) and *N*-succinimidyl iodoacetate to give ylide–aldehyde DNA adapters **S2-ALA**, **S2-PHE**, and **S3-PHE** (Scheme 5). The first monofunctionalized adapter was formed by reacting triphenylphosphino **S1**



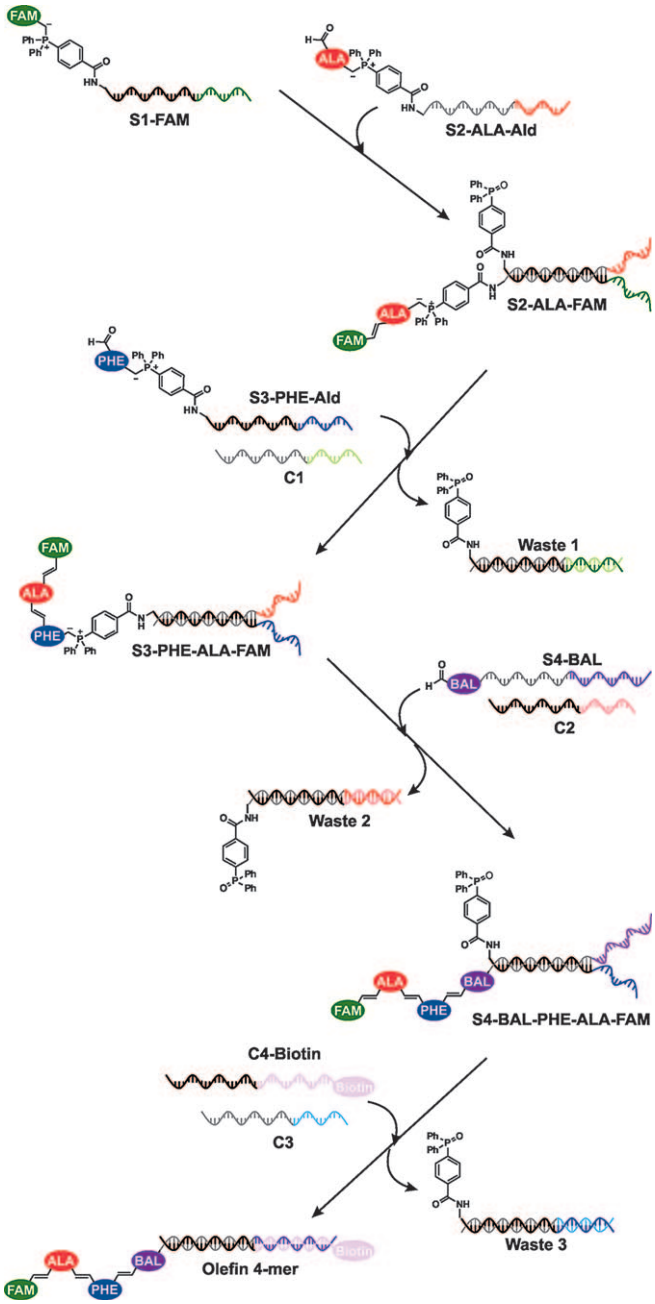
Scheme 4. Synthesis of ylide–aldehyde DNA precusors **4** and **8**. Conditions: a) HBTU, DMF, 98% (**1**), 95% (**5**); b) cyclohexylamine, CH_2Cl_2, DMF, 95% (**2**), 95% (**6**); c) (+)-*O*,*O*-diacetyl-L-tartaric anhydride, CH_2Cl_2, 0°C; d) NaOH, MeOH, 78% over 2 steps (**3**), 74% over 2 steps (**7**); e) TFA, CH_2Cl_2, 100% (**4**), 100% (**8**). HBTU = *O*-(benzotriazol-1-yl)tetramethyluronium hexafluorophosphate; TFA = trifluoroacetic acid; Boc = *tert*-butoxycarbonyl; Fmoc = 9-fluorenylmethoxycarbonyl.

strand with 5-(iodoacetamido) fluorescein to give fluorescent ylide DNA **S1-FAM**; the fluorescent tag was chosen to facilitate monitoring of the transfer reaction. The last adapter was monoaldehyde-functionalized by reacting 4-formylbenzoic acid *N*-hydroxysuccinimide ester with amino-modified strand **S2** or **S4** to give **S2-BAL** or **S4-BAL**, respectively. Table 1 shows characterization of the complete oligonucleotide adapters.

Table 1: ESI-MS characterization of DNA Wittig adapters.

DNA adapter	Calculated mass [Da]	Observed mass [Da]
S1-FAM	10195.9	10193.9
S2-BAL	9487.3	9486.1
S2-ALA	9994.8	9992.6
S2-ALA-Ald	9918.7	9917.7
S2-PHE	10070.9	10068.7
S2-PHE-Ald	9994.8	9993.7
S2-FAM	10032.9	10031.4
S3-PHE	10201.9	10199.7
S3-PHE-Ald	10125.8	10124.7
S4-BAL	12453.2	12452.2

The overall group transfer efficiency between oligonucleotide adapters was initially determined by synthesizing olefin dimers in a single coupling step using oligonucleotides **S1-FAM** and **S2-BAL**, **S2-ALA** or **S2-ALA-Ald** (see Supporting Information). The transfer reactions were carried out in 0.1M TAPS (*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid), 1M NaCl pH 8.5 (2 h incubation at room temperature). The desired products were observed (Table 2)



Scheme 5. Synthesis of olefin 4-mers using strand exchange mechanism.

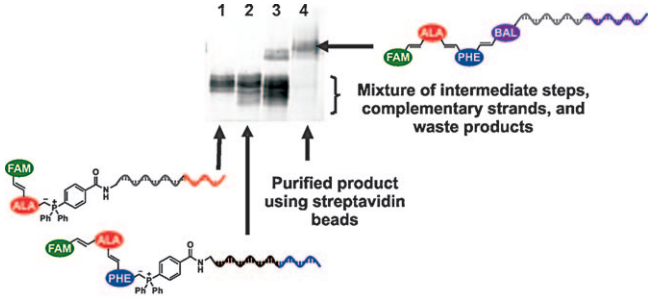
only when the aldehyde moiety of **S2-ALA** was unmasked or when adapter **S2-BAL** was used (yields between 61 to > 95% depending on reaction conditions, Supporting Information). Little or no transfer was observed between adaptors with non-

Table 2: ESI-MS of olefin oligonucleotide products.

Olefin product	Calculated mass [Da]	Observed mass [Da]
S2-BAL-FAM	9858.3	9857.2
S2-ALA-FAM	10290.1	10289.1
S4-BAL-PHE-ALA-FAM	13419.2	13417.9
S4-BAL-PHE-PHE-FAM	13495.3	13493.9

complementary sequences (Supporting Information). The limiting factor of the Wittig transfers is the potential oxidation of the ylide adaptors as a side reaction.[5] This can be minimized by lowering reaction times, which is possible as olefin products were observed in as little as 5 min reaction time (Supporting Information).

Any number of bifunctional oligonucleotide adapters could be used in additional reaction cycles to make longer oligomers. Here, two further coupling steps were introduced using **S2-ALA** or **S2-PHE** and **S3-PHE** for the synthesis of two different olefin 4-mers. After each transfer reaction (2 h incubation per step) the spent adapter bearing an unreactive phosphine oxide was displaced by addition of its complementary remover strand. The reaction was terminated by the addition of the final monoaldehyde-functionalized **S4-BAL** adapter. This strand was designed to be longer than other DNA adapters to facilitate analysis by PAGE. The final product was isolated from waste products by using a biotinylated remover strand, fully complementary to the final adapter, and streptavidin-coated magnetic beads (Scheme 6). Densitometric analysis showed 72% overall



Scheme 6. Resulting olefin 4-mers are easily purified after the final reaction step. 20% Denaturing PAGE gel after Sybr Gold staining. Lane 1: One-step reaction dimers; Lane 2: two-step reaction trimers; Lane 3: three-step reaction 4-mers; Lane 4: 4-mer after streptavidin magnetic bead purification (Supporting Information). In all cases products remain conjugated to the final adapter (30 nucleotides, except in the case of the 4-mer which was attached to a longer, 40-nucleotide adapter to facilitate PAGE analysis).

fluorescent group transfer (see Supporting Information) from the initial adapter through the chain of transfer reactions to the 4-mer olefin product **S4-PHE-PHE-FAM** and 41% for **S4-BAL-PHE-ALA-FAM**. ESI analysis confirmed the formation of the desired oligomers **S4-BAL-PHE-ALA-FAM** and **S4-PHE-PHE-FAM**, respectively (Table 2). The fact that a 4-mer with defined sequence can be obtained in good yields indicates that even longer oligomers could be synthesized by this mechanism.

In summary, we have developed a DNA-templated synthesis mechanism that is designed to facilitate the synthesis of oligomers with controlled sequences of subunits. We have demonstrated selective and robust sequence-controlled formation of two related olefin 4-mer sequences using templated Wittig chemistry with bifunctional (ylide and aldehyde) adapters. Each building block of the polyolefin product incorporates a side chain which can be used to introduce further chemical or physical functionality. Key features of the mechanism are that successive coupling reactions take place in near-identical environments, independent of the number of coupling steps, and that purification is only necessary in the last synthesis step. This mechanism could be applied to other coupling chemistries that effect transfer between adapters (rather than simple ligation), for example, peptide bond formation.[5] This technique provides an opportunity to prepare long, functional oligomers using DNA-templated synthesis.

Experimental Section

All oligonucleotides were supplied by Integrated DNA Technologies Inc. (USA); see Supporting Information for sequences, a detailed description of DNA adapter synthesis, experimental procedures used, and further characterization data.

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