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A Novel Approach to Oligonucleotide Synthesis Using an Imidazolium Ion Tag as a Soluble Support

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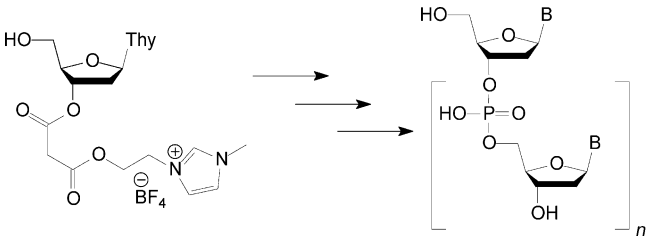

The synthesis of oligonucleotides in solution using a soluble, ionic liquid based support is described. Short oligomers of varying base composition were synthesized using this method in high yields and high purity, requiring no chromatography for purification prior to cleavage from the support. The solution-phase-synthesized oligomers were compared to the same sequences prepared using standard gene machine techniques by LCMS. This methodology may provide a cheaper route for the large-scale synthesis of oligonucleotides.

The demands of the scientific community for synthetic oligonucleotides have grown exponentially over the past decades. The ready availability of DNA oligonucleotide primers has satisfied the tremendous needs of the genome sequencing efforts, research into functional genomics, and polymerase chain reaction (PCR)-based detection methods, but significant advances in structural biology and biochemistry have only been achieved through concomitant advances in DNA and RNA chemical synthesis.[1−5] Oligonucleotides have begun to see widespread use in the development of therapeutics and in diagnostic applications, and large quantities are now required.

Since Merrifield[6] and Letsinger[7,8] et al. introduced the use of polymer supports for the synthesis of oligopeptides and oligonucleotides, the use of insoluble supports has become an important tool for organic synthesis, especially in the synthesis of biopolymers, such as oligonucleotides, peptides, and, more recently, carbohydrates.[9−11] Although extremely successful, because of the heterogeneous nature of the insoluble polymers, solid-phase synthesis has all the problems generally associated with heterogeneous reaction conditions. In addition, the high cost of the supports themselves must be considered, as in the case of the synthesis of oligonucleotides for therapeutic applications where 30−40% of total raw materials cost come from the solid support.[12]

In recent years, the use of *soluble* polymer supports has received considerable attention because such "solution-phase" syntheses retain many of the advantages of conventional solution chemistry, while still permitting facile purification of the product. Thus, soluble poly(ethylene glycol) (PEG) and other polymers have been used for the synthesis of oligopeptides,[9] nucleotides,[10] saccharides,[11] and small molecules.[13,14] Some limitations experienced when using soluble polymer supports include low loading capacity, limited solubility during the synthesis of longer peptides, and often low aqueous solubility,[13,14] as well as an energy intensive cooling step required for purification.[10]

The idea of using ionic liquid based supports for organic synthesis[15,16] has been recently demonstrated for the synthesis of small molecules,[17,18] oligopeptides,[19] and oligosaccharides.[20] We demonstrate here, for the first time, that ionic liquid supported synthesis (ILSS) can be applied to oligonucleotide synthesis, using simple precipitation and phase separation methods without the need for chromatography. This method provides reasonably high product purity at each step and should be much more amenable to large-scale manufacturing.

Solid-phase synthesis of oligonucleotides using the phosphoramidite method[1−5] is an iterative process in which a solid support with an attached nucleoside is deblocked at the terminus by removing an acid labile protecting group, thus liberating a nucleophilic 5′-hydroxyl group. This terminal nucleophile is then allowed to couple to a protected 3′-*O*-phosphoramidite monomer in the presence of an activator. The newly created phosphite triester 3′,5′-linkage is then oxidized to provide the desired and more stable phosphate triester. This process is repeated until an oligomer of the desired length and composition is obtained.

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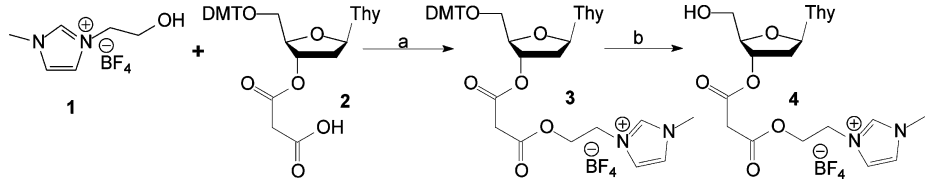
SCHEME 1. Derivatization of Ionic Liquid[a]



[a] Reagents and conditions: (a) DCC, DMAP, CH_3CN, 3 days, rt; (b) 3% TFA in CH_2Cl_2 or CH_3CN.

The same iterative methodology may be used when soluble supports are employed.

The synthesis of the ionic liquid supported oligonucleotide began with the ionic liquid **1** (Scheme 1), which was readily prepared from *N*-methylimidazole and 2-bromoethanol followed by anion exchange in accordance with literature procedures.[19]

The succinylated 5′-DMT-thymidine derivative **2**[21] was then coupled to the ionic liquid **1** using dicyclohexylcarbodiimide (DCC) and catalytic amounts of 4-(dimethylamino)pyridine (DMAP) in acetonitrile (CH_3CN) to give the ionic liquid supported nucleoside **3**. Compound **3** was isolated and purified by precipitation from an ethyl ether−ethyl acetate solution. The resulting precipitate was then taken up in chloroform and extracted with water. The excess ionic liquid **1** was removed with the aqueous phase, while the desired product **3** remained in the organic phase due to the hydrophobicity of the dimethoxytrityl protecting group attached at the 5′-position of thymidine. Exposure of **3** to acidic conditions yielded compound **4** as a light brown foam in 96% yield. Structure and purity of **3** and **4** were verified by ^{1}H NMR and ESI-MS.

The dinucleoside phosphotriesters TpT, ApT, CpT, and GpT (**6a**−**d**)[22] were prepared at the 250 μmol scale by reacting the ionic liquid supported nucleoside **4** with a 1.5-fold excess of the appropriate phosphoramidite derivatives (**5a**−**d**) using 4,5-dicyanoimidazole (DCI) as the activating agent in THF or CH_3CN and stirring for 1−2 h. After the reaction had come to completion, the excess activated phosphoramidite was quenched through the addition of anhydrous ethanol and a further 10 min of stirring. Quenching the phosphoramidites facilitates their removal during purification. The dinucleoside phosphite triester intermediates were then isolated by simply precipitating from 1:9 ethyl acetate:ethyl ether at room temperature, prior to oxidation. Precipitation of the dinucleoside phosphite triester at this stage is critical as the quenched excess mononucleoside 3′-*O*-phosphoramidite is much more soluble in ethyl acetate−ethyl ether than the coupled product, thus making its removal from the dinucleoside phosphite triester possible. Generally, the precipitation was repeated in order to enhance the purity of the desired product. While we recognize that dinucleoside phosphite triesters (P-III) are less stable than the corresponding phosphate triesters (P-V), no decomposition was observed at this step.

To carry out the oxidation of the phosphite triester intermediates, the collected precipitate was again dissolved in a small amount of CH_3CN, a small excess of pyridine, or 2,4,6-collidine, and a large excess (2−5 equiv) of a 0.1 M solution of iodine in 2:1 THF:water was added. Once the persistence of color due to iodine was established (5 min), 5% w/v aqueous sodium bisulfite was added to reduce the excess iodine. This reaction mixture is then diluted with chloroform and extracted with water. The aqueous layer removed the resultant salts (NaI, Na_2SO_4, excess bisulfite, pyridinium iodide, etc.) in addition to any uncoupled ionic liquid supported nucleoside(tide) since it lacked a terminal trityl group, rendering it water soluble. The organic layer contained, in principle, only the desired product. Removal of the organic solvent under reduced pressure yielded the products (**6a**−**d**) as light brown foams in good yields (Table 1) and high purity, eliminating the need for a capping step. Detritylation of **6a**−**d** was achieved by the addition of 3% v/v trifluoroacetic acid (TFA) in dichloromethane or CH_3CN, stirring for 20 min, and precipitation in 1:9 ethyl acetate:ethyl ether. The material obtained at this point may contain 5% or more of tritylated starting material, resulting from the equilibrium established during this step ($ROH + DMT^+ \rightarrow DMT - OR + H^+$). Quenching with ethanol to trap the DMT cation did not seem to eliminate this problem. However, purity is significantly enhanced (no tritylated product observed by ESI-MS in the bulk product) if the TFA treatment is repeated (i.e., redissolution of the precipitate in 3% v/v TFA solution followed with a second precipitation by dropwise addition to 1:9 ethyl acetate:ethyl ether). The purified products are simply filtered off, yielding off white to light yellow powdery solids in high yields (Table 1). The collected solids (**7a**−**d**) were ready for further coupling or, in cases where the desired length of oligo had been achieved, deprotection.

TABLE 1. Recovery and Physical Data for Ionic Liquid Supported Oligomers[a]

compound	sequence	% recovery	^{31}P NMR (ppm)	*m*/*z* (exptl)	*m*/*z* (calcd)
6a	^{DMT}ApT	89	−1.324, −1.477	1223.4	1223.4
6b	^{DMT}CpT	91	−1.545, −1.754	1199.4	1199.4
6c	^{DMT}GpT	90	−1.149, −1.194	1205.6	1205.4
6d	^{DMT}TpT	91	−1.494, −1.584	1110.4	1110.4
7a	^{HO}ApT	93	−1.176, −1.516	921.4	921.3
7b	^{HO}CpT	95	−1.381, −1.613	897.3	897.3
7c	^{HO}GpT	96	−1.047, −1.064	903.4	903.3
7d	^{HO}TpT	78	−1.188, −1.284	808.3	808.3
8	^{DMT}TpTpT	92	−1.081 to −1.477	1467.5	1467.8
9	^{HO}TpTpT	98	−1.157 to −1.531	1165.2	1165.3
10	^{DMT}TpTpTpT	89	−1.169 to −1.859	1824.2	1824.5
11	^{HO}TpTpTpT	100	−1.142 to −1.51	1522.4	1522.4

[a] Ade N6 and Cyt N4 benzoyl protected, Gua N2 isobutyryl protected; 3′-terminal thymidine linked through succinyl ester to **1** as in **3** of Scheme 1.

In addition to the dimers **7a**−**d** described above, a thymidine trimer (**8**, **9**) and a tetramer (**10**, **11**) were also synthesized at the 50−100 μmol scale. The identities of the ionic liquid supported compounds have been confirmed with both low- and high-resolution ESI-MS as well as ^{31}P NMR in conjunction with the use of a ^{31}P CIGAR experiment,[23] allowing the observation of the expected 3′−5′ connectivity through the diastereomeric phosphotriester linkages (Table 1). The yields are expressed as recoveries since traditional solution-phase phosphoramidite chemistry is known to go to 98−100% completion, but for the

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TABLE 2. HPLC/MS Analysis of Deprotected Oligonucleotides[a]

sequence	synthetic support	retention time (min)	% total area	low resolution MS (−ve mode)
ApT	IL	26.3	99.3	554.2
	CPG	26.3	96.1	554.2
CpT	IL	16.7	93.3	530.2
	CPG	16.8	89.5	530.2
GpT	IL	19.0	97.1	570.2
	CPG	18.9	95.1	570.2
TpT	IL	25.3	91.7	545.2
	CPG	25.3	92.5	545.2
TpTpT	IL	29.9	92.1	849.1
	CPG	29.5	94.0	849.2
TpTpTpT	IL	32.4	87.3	1153.2
	CPG	31.9	90.5	1153.2

[a] IL = prepared with ionic liquid support; CPG = prepared with controlled pore glass with gene machine; % total area for IL based oligonucleotides excludes peak area due to IL peaks.

methodology employed, there are small losses associated with the manipulations involved in the purifications of the compounds. These losses are likely fixed, and larger-scale synthesis should show higher proportional recoveries.

The oligonucleotides synthesized above in solution have been compared to the same sequences synthesized on controlled pore glass (CPG; 1 μmol scale), a commonly used solid support, and the two systems have been deprotected in parallel. Complete deprotection of the desired oligonucleotides was achieved by treating them with concentrated ammonium hydroxide/ethanol for 48 h at room temperature or 16 h at 60 °C. These conditions ensure complete cleavage of the cyanoethyl protecting group, the ionic liquid moiety, any protection of the exocyclic amines of the bases (Ade, Cyt, and Gua), and the monosuccinate linker. The oligonucleotides were isolated by removal of the ethanol and ammonium hydroxide solution under vacuum, redissolution in water (the solid support is simply settled by centrifugation for the CPG supported oligomers), and then chromatographic purification by ion-pairing reverse phase HPLC, anion-exchange HPLC, or polyacrylamide gel electrophoresis.

The products of the ILSS procedure have been compared via LCMS to those obtained through automated solid-phase synthesis techniques[1] (Table 2). In all cases, the retention times of the IL generated material correlate well with the CPG generated oligomer. The purities of the oligomers prepared by the two methods are comparable. For the dimers, the IL method gave products of better purities, whereas for the trimers and tetramers, the CPG method gave slightly purer products. The origin of impurities appears to be different in the two methods. In the ILSS generated trimer and tetramer, the impurities are due to the presence of small amounts of "$n - 1$" peaks, which are likely due to the incomplete detritylation at the stage prior to coupling. This tritylated material would not be removed during the extraction and would be deblocked in the detritylation after coupling giving the $n - 1$ oligomer. Thus it is extremely important to ensure complete detritylation at each step. On the other hand, thymidine nucleoside is visible in several of the HPLC traces of oligomers synthesized on CPG. Its presence arises from incomplete coupling or partial detritylation of the solid support prior to the initial capping step.[24] Though this is normally present in materials synthesized on CPG, the post-oxidation extraction largely removes this material from the IL mediated synthesis.

In this study, a new strategy to synthesize oligonucleotides using an ionic liquid as a soluble support has been demonstrated. Oligonucleotides up to tetrameric species have been synthesized and have been shown to be comparable to products generated using standard automated DNA synthesis techniques. Overall, the solution based ILSS method provides a novel route to construct and characterize (by TLC, ^{1}H NMR, ^{31}P NMR, and MS techniques) oligonucleotides during chain assembly as well as facile, precipitation based purifications of the intermediates during chain elongation. At this point, the method has been demonstrated at the 100−250 μmol scale and should be scalable. Compared to the solid phase method, it offers the potential advantage of reduced cost of the relatively expensive solid supports (CPG, OligoPrep, NittoPhase, etc.). Compared to solution-phase synthesis using soluble polymer supports, such as poly(ethylene glycol) (PEG, MW = 5000−20 000), the ILSS method with compound **1** (MW = 214) as the support has a much better loading capacity. Of course, while we have demonstrated that the ILSS approach is compatible with standard oligonucleotide synthesis chemistries, it remains to be determined if the approach is applicable to longer oligomers.

Experimental Section

$^{DMT}T_{Succ-IL}$ (3). Compound **2** (1 g, 1.63 mmol), substituted imidazolium tetrafluoroborate **1** (0.38 g, 1.76 mmol), and DMAP (0.052 g, 0.41 mmol) were placed in a dry, nitrogen-purged 100 mL round-bottom flask. To this mixture was added dicyclohexylcarbodiimide (DCC) (0.68 g, 3.3 mmol), followed by dry CH_3CN (20 mL). The reaction mixture was stirred for 3 days at room temperature. TLC analysis in 9:1 chloroform:methanol showed the formation of a more polar product. When the reaction was stopped, the insoluble DCU byproduct was allowed to settle, and the reaction mixture was filtered and washed with CH_3CN several times. The solvent was evaporated, and the residue was again washed with ether to remove any unreacted DCC and finally collected and dried under vacuum. The product **3** was obtained as a light brown foam (1.1 g, 83% yield). ^{1}H NMR (acetone-d_6): δ 10.03 (1H, s, NH), 9.10 (1H, s, CH), 7.83 (1H, s, CH), 7.71 (1H, s, CH), 7.60 (1H, s, CH), 7.50−6.92 (14H, m, Ar−H), 6.33 (1H, t, J = 8 Hz, CH), 5.50 (1H, d, J = 6 Hz, CH), 4.55−4.51 (2H, m, CH_2), 4.70−4.66 (2H, m, CH_2), 4.15 (1H, br s, CH), 4.05 (3, s, CH_3), 3.80 (3H, s, CH_3), 3.50−3.40 (2H, m, CH_2), 2.70 (4H, br s, 2 CH_2), 2.61−2.40 (2H, m, CH_2), 1.42 (3H, s, CH_3). ESI-MS for $C_{40}H_{43}N_4O_9^+$: calcd 723.3, found 723.4.

$^{HO}T_{Succ-IL}$ (4). To a solution of **3** (2.96 g, 3.65 mmol) in dichloromethane (200 mL) was added 3% v/v TFA in dichloromethane or CH_3CN (100 mL). During the addition of TFA, the solution became reddish−orange, and stirring was maintained for 20 min. The product was precipitated from 1:9 ethyl acetate:ethyl ether, filtered, redissolved in a minimum amount of the acid solution, precipitated again, and filtered. The precipitate was rinsed with 1:9 ethyl acetate:ethyl ether, recovered from the filter by dissolving in CH_3CN, and evaporated under reduced pressure, yielding compound **4** as a light brown foam (1.88 g, 96% yield). ^{1}H NMR (DMSO-d_6): δ 11.40 and 11.39 (total of 1H, 2 s, NH), 9.10 (1H, s, CH), 7.76 (1H, s, CH), 7.72 and 7.45 (1H, s, CH), 7.70 (1H, s, CH), 6.15 (1H, m, CH), 5.27 and 5.19 (1H, m, CH), 4.60 and 3.60 (2H, m, CH_2), 4.45 (2H, br s, CH_2), 4.39 (2H, br s, CH_2), 4.24−3.93 (1H, m, CH), 3.86 (3, s, CH_3), 2.61−2.60 (4H, unresolved m, 2 CH_2), 2.40−2.16 (2H, m, CH_2), 1.77 (3H, s, CH_3). ESI-HRMS for $C_{20}H_{27}N_4O_8^+$: required 451.18289, found 451.18234.

$^{DMT}TpT_{Succ-IL}$ (6a). Compound **4** (0.24 g, 0.45 mmol), thymidine phosphoramidite **5a** (0.57 g, 0.77 mmol), and dicyanoimidazole

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(0.66 g, 5.6 mmol) were transferred to a 50 mL oven-dried, nitrogen-purged round-bottom flask. To the mixture was added dry THF or CH_3CN (5 mL) to the flask, and the resulting solution was stirred at room temperature for 1−2 h. At this point, a small amount (1−2 equiv) of anhydrous ethanol was added and stirring was continued for a further 10 min. The product was then precipitated twice from 1:9 ethyl acetate:ethyl ether. At this point, the precipitate was redissolved in CH_3CN, and 2,4,6-collidine or pyridine (approximately 300 μL) was added followed by addition of an aqueous iodine solution (0.1 M in THF/water 2:1, excess), dropwise until the iodine color persisted, to oxidize the phosphite triester intermediate. After 5 min, the reaction mixture was quenched with an aqueous sodium bisulfite solution (9 mL), diluted with 90 mL of $CHCl_3$, and extracted with 50 mL of water. The product was obtained in high yield as a foam of **6a** (0.492 g, 91%). ^{31}P NMR (acetonitrile-d_3): δ −1.494, −1.584. ESI-MS for $C_{54}H_{61}N_7O_{17}P^+$: calcd 1110.4, found 1110.4.

^{HO}TpT$_{Succ-IL}$ (7a). To a solution of **6a** (0.21 g, 0.18 mmol) in CH_3CN (1−2 mL) was added 3% v/v TFA in CH_3CN (2−3 mL). The reaction mixture was worked up following the same procedure as that for **4** giving **7a** as a foam in good yield (0.123 g, 78%). ^{31}P NMR (acetonitrile-d_3): δ −1.188, −1.284. ESI-HRMS for $C_{33}H_{43}N_7O_{15}P^+$: required 808.25548, found 808.25493.

^{DMT}TpTpT$_{Succ-IL}$ (8). Compound **7a** (0.065 g, 0.073 mmol) was mixed with 3′-phosphoramidite **5a** (0.233 g, 0.31 mmol) and dicyanoimidazole (0.31 g, 2.6 mmol) in dry CH_3CN (5 mL) at room temperature. After being stirred for 2 h and quenched with anhydrous ethanol for 10 min, the product was precipitated from 1:9 ethyl acetate:ethyl ether twice, oxidized, and extracted in the same manner as for compound **6a**, to give compound **8** (104 mg, 92% yield) in high purity. ^{31}P NMR (acetonitrile-d_3): δ −1.081, −1.194, −1.233, −1.262, −1.381, −1.403, −1.448, −1.477. ESI-MS for $C_{67}H_{77}N_{10}O_{24}P_2^+$: calcd 1467.5, found 1467.8.

^{HO}TpTpT$_{Succ-IL}$ (9)b. To a solution of **8** (97 mg, 0.06 mmol) in CH_3CN (1−2 mL) was added 3% v/v TFA in CH_3CN (2−3 mL). The reaction mixture was worked up following the same procedure as that of **4** giving **9** as a glassy solid (77 mg, 98% yield). ^{31}P NMR (acetonitrile-d_3): δ −1.157 to −1.531 (broad overlap of peaks). ESI-HRMS for $C_{46}H_{59}N_{10}O_{22}P_2^+$: required 1165.32807, found 1165.32752.

^{DMT}TpTpTpT$_{Succ-IL}$ (10). Compound **9** (170 mg, 0.136 mmol) was mixed with 3′-phosphoramidite **5a** (0.160 g, 0.215 mmol) and DCI (0.21 g, 1.8 mmol) in dry CH_3CN (5 mL) at room temperature. After being stirred for 2 h and quenched with anhydrous ethanol for 10 min, the product was precipitated from 1:9 ethyl acetate: ethyl ether twice, oxidized, and extracted in the same manner as for compound **6a**, to give compound **10** (231 mg, 89% yield) in high purity. ^{31}P NMR (acetonitrile-d_3) −1.169 to −1.859 (broad overlap of peaks). ESI-MS for $C_{80}H_{93}N_{13}O_{31}P_3^+$: calcd 1824.5, found 1824.2.

^{HO}TpTpTpT$_{Succ-IL}$ (11). To a solution of **10** (190 mg, 0.122 mmol) in CH_3CN (1−2 mL) was added 3% v/v TFA in CH_3CN (2−3 mL). The reaction mixture was worked up as that of **4** giving compound **11** (160 mg, 99.9% yield). ^{31}P NMR (acetonitrile-d_3): δ −1.142 to −1.51 (broad overlap of peaks). ESI-HRMS for $C_{59}H_{75}N_{13}O_{29}P_3^+$: required 1522.40066, found 1522.40011.

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Supporting Information Available: Representative ^{1}H NMR spectra, ^{31}P NMR spectra, comparative HPLC chromatograms of compounds **12a**−**f** (cleaved and deprotected products of compounds **7a**−**d**, **9**, and **11**), and expanded experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

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