

Universal Reusable Polymer Support for Oligonucleotide Synthesis

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Abstract: A new efficient reusable universal polymer support for oligonucleotide synthesis, based on a non-ammoniacal cleavable linker, is described. Twenty six cycles of oligonucleotide synthesis have been carried out without compromising the quality of the fully deprotected oligonucleotides.

For constituting an assembly of oligonucleotides of defined sequence, current protocols center around the use of suitable polymer supports.^{1,2} The conventional methods are based mainly on aminoalkylated controlled pore glass (CPG), where the leader nucleoside is pre-attached via its 3'-hemisuccinate moiety, and the labile linkage is cleaved from the support with aqueous ammonium hydroxide treatment. This methodology suffers from the disadvantage that the polymer support, after a single use, is rendered unsuitable for refunctionalization for the next cycle of oligonucleotide synthesis. This problem has partly been addressed in recent reports, including those from the authors' laboratory,³⁻⁷ wherein universal supports based on nucleosidic and non-nucleosidic linkages have been employed in the synthetic protocol. However, a universal polymer support that would permit repetitive syntheses (of the order of 25 or more) leading to multiple oligonucleotides is still elusive.

Considering the wide potential applications of such supports, some efforts have been made in this direction, viz., development of new linker phosphoramidite reagents for the synthesis of two oligonucleotides per synthesis (TOPS),⁸ useful for synthesis of PCR primers. The other approach, tandem oligonucleotide synthesis,⁹ is simplified in a way that it allows the synthesis of two different isolable oligonucleotides. In this approach, the first oligonucleotide is synthesized on a succinylated support and 5'-hydroxyl of the first oligonucleotide is used as the starting point for the synthesis of second oligonucleotide after introducing a base-labile hydroquinone-*O,O*-diacetyl (Q) linker at 5'-hydroxyl group. The oligonucleotides can

be cleaved from the support in sequential fashion, if required. However, these approaches are limited to two oligonucleotide syntheses on a single support. In recent communications,^{10,11} Pon et al. have reported (using LCAA-CPG support with generated hydroxyl groups on the surface and with the leader nucleoside molecule attached through a labile Q-linker) carrying out six cycles of oligonucleotide synthesis, *albeit* support functionalization with appropriately protected nucleosides has been claimed for 25 cycles. However, this system inherently admits of a limitation insofar as CPG is vulnerable to alkaline conditions and that repeated exposures to ammonium hydroxide/methylamine could affect the morphology of the glass support surface. The above methodology^{10,11} also makes use of a non-conventional capping reagent (chloroacetic anhydride/methoxyacetic anhydride/*p*-tert-butylphenoxyacetic anhydride) for blocking residual hydroxyls on the polymer support. Moreover, at least four different pre-derivatized supports (dA, dC, dG, dT) would be required for all kinds of oligodeoxyribonucleotide synthesis. Recently, in another attempt, Pon and Yu¹² have demonstrated the use of a new class of linker phosphoramidites (containing a cleavable 3'-ester linkage), for functionalization of supports in the machine itself. However, the preparation of four nucleoside-linker phosphoramidites and the requirement of four additional ports in the machine for coupling of these linker molecules are the main limitations of this strategy.

In the context of designing a new support for multiple oligonucleotide synthesis, our investigations have focused on two aspects, i.e., looking for a non-nucleosidic universal linker to avoid the preparation of a large number of pre-derivatized supports, and second, the search for a non-ammoniacal cleavable linkage for attachment of the universal linker to the support (CPG) to avoid surface erosion during cleavage of oligonucleotide chains from the support in aqueous ammonium hydroxide.

We now report on a new fully functionalized reusable universal polymer support with a disulfide moiety and a universal linker (Scheme 1). A rationale for incorporating disulfide linkage in the support system rests on the fact that the synthesized oligonucleotide can be cleaved from the support under non-ammoniacal conditions without affecting the glass surface. Again, a universal linker has been incorporated for which a wide range of fast deprotection conditions have been reported, including some from this laboratory.^{4,5,7} The projected polymer support was successfully and repeatedly used over more than 25 cycles of oligonucleotide synthesis without compromising the quality of fully deprotected oligonucleotides (HPLC profiles and mass, MALDI). The loading of the universal linker was found to be almost the same on regenerated universal polymer support after each cycle of synthesis (Table 1). This is the first reusable universal polymer support where 26 cycles of oligonucleotide synthesis have been realized.

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TABLE 1. Observed Loading on the Support for the Decameric Oligonucleotide d(TTT TTT TTT T) at Commencement of Cycles and the Yield of the Crude Oligomers

cycle no.	loading ($\mu\text{mol/g}$) on the support based on DMTr cation release	yield of oligomer A_{260} units
1	20.3	19.4
5	19.8	18.9
10	19.5	18.6
15	19.1	18.4
20	18.8	18.1
25	18.4	17.9
26	18.3	31.7

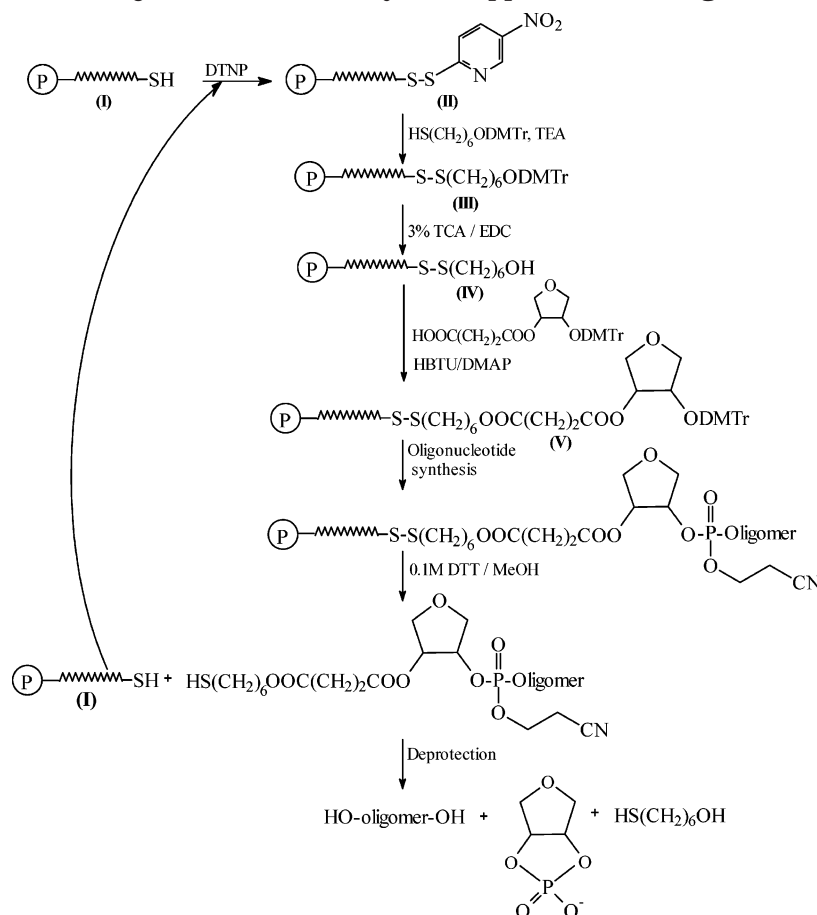
The reusable polymer support was prepared beginning with 3-mercaptopropyl-CPG (**I**), which was reacted successively with the thiol-specific reagent, 2,2'-dithiobis(5-nitropyridine) (DTNP) and *O*-(4,4'-dimethoxytrityl)-6-mercaptohexan-1-ol followed by treatment with 3% trichloroacetic acid (TCA) in 1,2-dichloroethane to obtain the support (**IV**) (containing free generated hydroxyl groups). Later, the universal linker, [2(3)-*O*-(4,4'-dimethoxytrityl)-anhydroerythritol-3(2)-hemisuccinate], was coupled to the support (**IV**) via ester linkage using *O*-benzotriazol-1-yl-*N,N,N,N*-tetramethyluroniumhexafluorophosphate (HBTU) and 4-(dimethylamino)pyridine (DMAP) to obtain the fully functionalized reusable polymer support (**V**).

To demonstrate the utility of the projected support (**V**) for oligonucleotide synthesis, a decamer sequence, d(TTT

TTT TTT T) was synthesized on (**V**) in an automated DNA synthesizer using phosphoramidite chemistry.^{2,13} Cleavage was achieved through treatment of the support-bound oligonucleotide chains in the cassette itself with 0.1 M dithiothreitol (DTT) in methanol. The solution containing the cleaved oligonucleotide was removed by centrifugation at low speed (3000 rpm), and the support (**I**) was washed with methanol (2×2 mL). Both fractions (filtrate and washings) were pooled together and concentrated under vacuum. Fully deprotected oligonucleotide was obtained after treatment of the residue with aqueous ammonium hydroxide (30%) containing spermine (1 M) and lithium chloride (0.5 M) at 60 °C for 8 h (cf. 4). The ammoniacal solution was concentrated in a speed vac. The residue obtained was resuspended in double-distilled (dd) water, desalted on RP-18 silica gel column, and subjected to HPLC and MALDI-TOF analysis for characterization.

The 3-mercaptopropyl-CPG (**I**), obtained after cleavage reaction with DTT, was refunctionalized according to Scheme 1 and used for another cycle of oligonucleotide synthesis. Likewise, 24 additional cycles of oligonucleotide synthesis were carried out without significant change in the quality of the fully deprotected oligonucleotides. Figure 1 shows HPLC elution pattern of the crude oligomer, d(TTT TTT TTT T), synthesized on support (**V**) after cycles 5, 10, 15, 20, and 25. The identity of the eluted oligomer was further confirmed by co-injecting it with standard d(TTT TTT TTT T). The results reveal that

SCHEME 1. Preparation of Projected Reusable Polymer Support (V) and Oligonucleotide Synthesis.^a



^a Deprotection conditions: aq ammonium hydroxide (30%), containing spermine (1 M), and lithium chloride (0.5 M) at 60°C for 8 h.

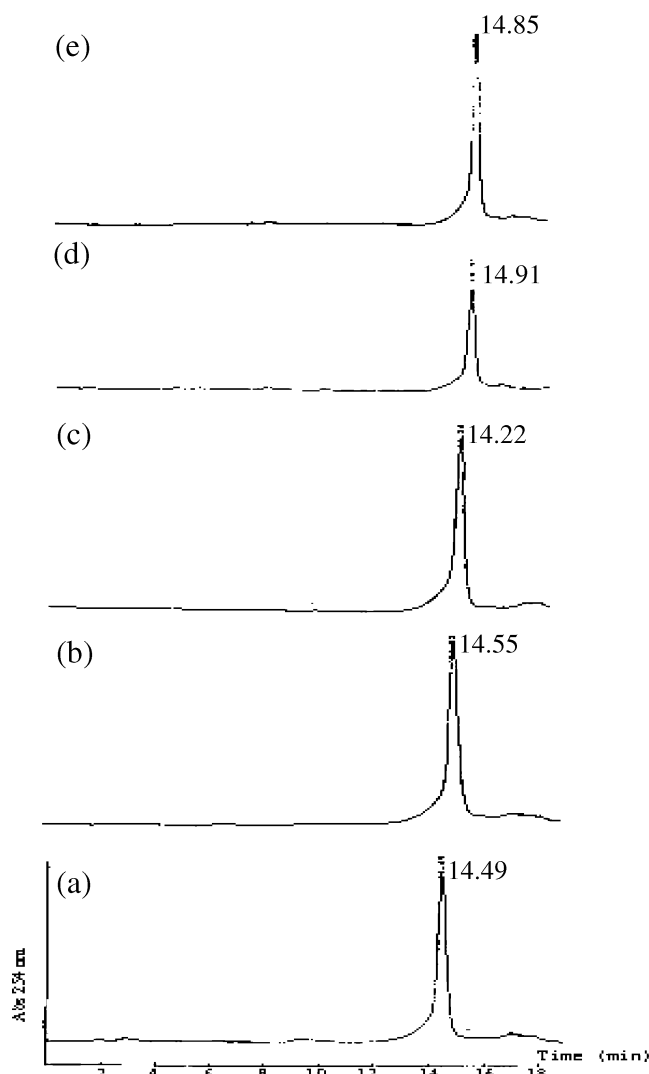


FIGURE 1. HPLC profiles of fully deprotected crude d(TTT TTT T), synthesized on support (V) after cycles (a) 5, (b) 10, (c) 15, (d) 20, and (e) 25. HPLC conditions: column, Lichrosphere RP-18; buffer A, 0.1 M NH_4OAc ; solvent B, acetonitrile; gradient, 0–50% B in 25 min; flow rate, 1 mL/min; Auf, 0.005; UV detector set at 254 nm.

the quality and the quantity of the synthetic oligomer remains undiminished, after 25 cycles of synthesis. This was substantiated through determination of the loading-on-support prior to commencement of synthesis and by measuring the absorbance of the released oligomer in the solution spectrophotometrically at 260 nm (Table 1). The 26th cycle was performed by synthesizing a primer sequence, d(TTC GTA CGT AGC GTC ATT TTC CCC), for PCR amplification experiment. Deprotection was carried out in the manner described above. Figure 2 sketches results of the PCR amplification experiment, and mass analysis data of the same primers is provided in Supporting Information.

Further, to ensure the complete cleavage of d(TTT TTT TTT T) from the support, the primer sequence, d(TTC GTA CGT AGC GTC ATT TTC CCC), after cleavage was analyzed on RP-HPLC. From the elution pattern, shown

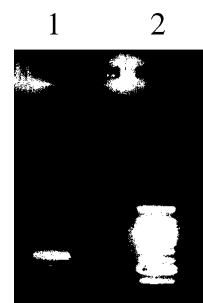


FIGURE 2. PCR-amplified product of Mus Musculus Nebulin-related anchoring protein (Nrap). Lane 1: Nebulin-related anchoring protein fragment of 634 bp amplified using d(TGG CAC CAC ACC TTC TAC ATT) (m/z , 6300) (forward primer), synthesized on support (V), and d(CAA GAA GGA AGG CTG GAA AAG) (m/z , 6584) (reverse primer), synthesized on standard dG support. Lane 2: 100 bp DNA ladder from New England Biolab.

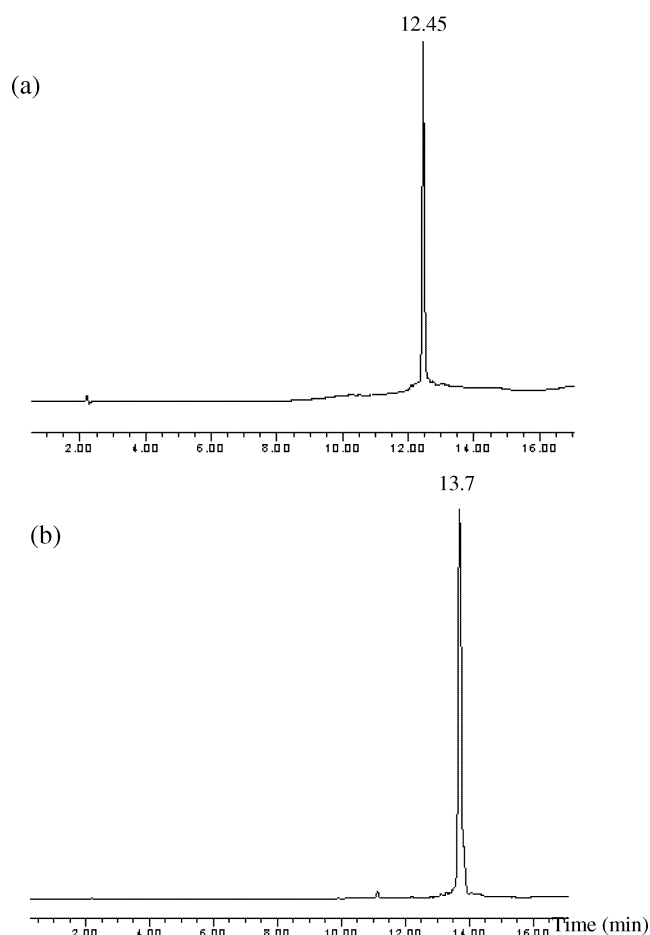


FIGURE 3. HPLC elution profiles of fully deprotected crude (a) d(TTT TTT TTT T) and (b) d(TGG CAC CAC ACC TTC TAC ATT), synthesized on support (V) after cycles 25 and 26, respectively. HPLC conditions: column, Hypersil Gold RP-18; buffer A, 0.1 M NH_4OAc ; solvent B, acetonitrile; gradient, 0–60% B in 25 min; Flow rate, 1 mL/min; PDA detector (254 nm).

in Figure 3, it was concluded that complete cleavage of disulfide linkage occurred during DTT treatment.

In conclusion, a reusable support has been prepared for carrying out multiple cycles of oligonucleotide syntheses without affecting the surface morphology of the support material. The method may also be utilized for

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synthesis of phosphorothioate oligonucleotides, an important class of antisense molecules.

Experimental Section

2(3)-(4,4'-Dimethoxytrityl) anhydroerythritol-3(2)-hemisuccinate was prepared according to the published protocol.⁵ Mercaptoalkylation of controlled pore glass was also carried out following the published procedure.¹⁴ Thin-layer chromatography was carried out on fluorescent silica gel plate (F₂₅₄). Spots were visualized under short-wavelength UV light, and DMTr-containing compounds were further visualized by spraying detritylating reagent (1% trichloroacetic acid in 1,2-dichloroethane). New compounds prepared in this communication were characterized by ¹H NMR and elemental analysis. Oligonucleotide sequences were assembled at 0.2 μmol scale on a Pharmacia LKB Gene Assembler Plus following standard phosphoramidite coupling cycle. DMTrdC(acetyl)-3'-phosphoramidite was used instead of DMTrdC(benzoyl)-3'-phosphoramidite in the present work. RP-HPLC was carried out on a Shimadzu LC-10A system fitted with a variable UV detector and a Waters Delta 600 system attached with a PDA 996 detector. Oligomers, d(TTT TTT TTT T), were analyzed on a Shimadzu HPLC system (Figure 1), whereas the primer sequence (cycle 26) and d(TTT TTT TTT T) (cycle 25) (Figure 3) were analyzed on a Waters Delta 600 system. MALDI-TOF mass spectra were recorded using a 3-hydroxypicolinic acid precoated chip using a Sequenom mass spectrometer in negative ion mode.

(i) Preparation of *O*-(4,4'-Dimethoxytrityl)-6-mercaptohexanol. 6-Mercaptohexanol (5 mmol) was co-evaporated twice with anhydrous pyridine (20 mL) and finally taken up in dry pyridine (25 mL). 4,4'-Dimethoxytrityl chloride (12 mmol) and 4-(dimethylamino)pyridine (1.0 mmol) were added, and the reaction mixture was agitated at room temperature for 8 h. After completion of the reaction, methanol (1 mL) was added and the reaction mixture was concentrated in a vacuum. Traces of pyridine were removed by co-evaporation with toluene. The residual syrupy mass was dissolved in ethyl acetate (100 mL) and washed with saturated sodium chloride solution (2 × 25 mL). The organic phase was collected and concentrated in a vacuum. The resulting syrupy material was redissolved in chloroform (30 mL) containing 0.1% triethylamine and treated with a saturated solution of silver nitrate (5 equiv) in ethyl alcohol at room temperature for 1 h. Then, a solution of DTT (7 equiv) in ethyl alcohol was added and the reaction mixture was agitated for 30 min. The precipitated material was filtered out, and the residue washed with ethyl alcohol (2 × 20 mL). The filtrate was concentrated *in vacuo* and the syrupy material dissolved in ethyl acetate. The organic phase was washed with water (5 × 20 mL). The organic phase was collected, concentrated *in vacuo*, and then purified by silica gel chromatography using an increasing gradient of methanol in 1,2-dichloroethane containing triethylamine (0.5%, v/v). The fractions containing the desired material were pooled and concentrated to obtain the title compound in ~76% yield. The material was further characterized by ¹H NMR and elemental analysis. ¹H NMR (CDCl₃) δ: 1.15–1.25 (m, 8H, 4 × –CH₂–), 2.68 (t, 2H, –SCH₂–), 3.45 (t, 2H, –OCH₂–), 3.65 (s, 6H, 2 × –OCH₃), 6.8–7.45 (m, 13H, Ar–H). Anal. Calcd for C₂₇H₃₂O₃S: C, 74.31; H, 7.32; S, 7.32. Found: C, 72.83; H, 7.43; S, 7.34.

(ii) Preparation of Reusable Polymer Support (V). 3-Mercaptopropylated CPG (I) (1.0 g) was activated with 2,2'-dithiobis(5-nitropyridine) (DTNP) (1.0 mmol) in chloroform containing triethylamine (1.0 mmol) for 2 h with occasional agitation at room temperature. The support was then recovered on a sintered funnel and washed with chloroform (5 × 25 mL) extensively to remove excess reagent, 5-nitropyridine-2-thione, and finally dried under vacuum to obtain activated polymer support (II).

Activated polymer support (II) (500 mg) was subsequently treated with *O*-(4,4'-dimethoxytrityl)-6-mercaptohexanol (1.0

mmol) in 1,2-dichloroethane containing triethylamine (1%, v/v). The suspension was agitated for 2 h at room temperature. Then, the excess reagent was removed by filtration on a sintered glass funnel and the support (III) washed with 1,2-dichloroethane (3 × 15 mL) containing triethylamine (1%, v/v). The support was dried under vacuum, and the loading on the support was determined using perchloric acid method.¹

The polymer support (III) (400 mg) was treated with 3% trichloroacetic acid in 1,2-dichloroethane (15 mL) for 5 min at room temperature with continuous shaking and then filtered through a sintered disk glass funnel. The recovered support (IV) was washed with 1,2-dichloroethane (2 × 15 mL). After drying, 2(3)-(4,4'-dimethoxytrityl)-anhydroerythritol-3(2)-hemisuccinate (1.0 mmol) was coupled to the support (IV) in the presence of a condensing reagent HBTU (1.0 mmol) in DMF (5 mL) containing DMAP (1.0 mmol). After 30 min, the support was filtered, washed with DMF (3 × 15 mL) and diethyl ether (2 × 15 mL), and then dried under vacuum to obtain support (V). The capping of the residual hydroxyl groups was performed with acetic anhydride using standard protocol.¹ After the usual workup, the support (V) was dried and kept at 4 °C until used for oligonucleotide synthesis.

(iii) Oligonucleotide Synthesis and Deprotection. The oligonucleotide sequences were synthesized on the support (V) at 0.2 μmol scale following standard phosphoramidite coupling. Coupling efficiency based on released 4,4'-dimethoxytrityl cation was found to be >98%.

In a typical experiment, an oligomer d(TTT TTT TTT T) was synthesized on the support (V). The complete deprotection of support-bound oligonucleotide was achieved in two steps. In the first one, the supported oligonucleotide chains, in the cassette itself, were treated with 0.1 M dithiothreitol (DTT) in methanol (1 mL) containing triethylamine (0.5%) at room temperature. After 1 h, the supernatant was recovered by low-speed (3000 rpm) centrifugation and the support in the cassette was again washed with methanol (2 × 1 mL). The fractions were combined together and concentrated *in vacuo*. The solid residue obtained was treated with aqueous ammonium hydroxide (30%, 1 mL) containing spermine (1 M), and lithium chloride (0.5 M) and kept at 60 °C for 8 h to get rid of protecting groups from nucleic bases and internucleotidic phosphates. Then, the supernatant was removed and concentrated *in vacuo*.

The polymer support (I), in the cassette, was washed with methanol and diethyl ether (2 mL of each), dried, reactivated with DTNP, and refunctionalized as discussed above. It was then subjected to another cycle of synthesis.

(iv) Desalting, Analysis, and Characterization of Deprotected Oligonucleotides. The residue containing the crude oligonucleotide mixture, obtained in the above step, was redissolved in dd water and desalted on a reversed-phase silica gel column. The oligomer was eluted from the matrix by using 30% acetonitrile in water. The solution was concentrated and analyzed on RP-HPLC using a gradient of acetonitrile in ammonium acetate buffer, pH 7.1. The primers synthesized on the support (V) as well as standard supports were characterized by MALDI-TOF using 3-hydroxypicolinic acid as a matrix.

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Supporting Information Available: MALDI-TOF spectra for primers synthesized on polymer support (V) and standard dG support. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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