High-Loading Supports for Oligonucleotide Synthesis

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A simple protocol for the preparation of high-loading supports, suitable for large-scale synthesis of oligonucleotides, has been developed. The method involves the use of inexpensive reagents and is amenable to large-scale preparation of supports. The derivatized supports were successfully employed in an automated DNA synthesizer without any difficulty. The quality of the synthesized oligonucleotides was found to be comparable to that of the corresponding oligomers prepared with commercially available standard supports.

Introduction. – Synthetic oligonucleotides and their modified analogs have become vital tools for various biological studies. They are finding widespread use in the development of oligonucleotide-based therapeutic and diagnostic tools [1–4]. Generally, oligonucleotides, in the size range of 15–30 residues, are required in abundance, but in tiny quantities for studies like DNA sequencing, *etc.* However, in case of developing DNA based diagnostics and therapeutics (for use as antisense oligomers), relatively larger quantities of short oligomers are required. To synthesize these molecules in large quantities, the present day methodologies [5–7] have proven to be somewhat expensive. Hence, there is a need to develop economically viable routes or protocols for the synthesis of oligonucleotides to meet this exponentially growing demand for these molecules. In the last several years, tremendous improvements have been reported in the area of oligonucleotide synthesis [8][9]. The introduction of new sets of labile protecting groups, fast coupling reagents, and improved polymer supports have revolutionized the entire field so that even a non-chemist can synthesize these molecules quite easily. Since most of the syntheses are currently carried out on solid supports, which play a significant role in deciding the cost of these molecules, there is a need to develop supports having a relatively higher density of the functional groups, which could ultimately reduce the cost of these molecules without compromising the quality of the final product. During the last few years, however, although several polymer supports have been developed and tested, LCAA-CPG (long-chain alkylamine/controlled-pore glass) is still the standard polymer support [10] for routine synthesis of oligonucleotides. Recently, we published an express protocol for the functionalization of polymer supports useful for oligonucleotide synthesis [12], in which we reported the loading of LCAA-CPG in the range of 45–50 μmol/g of support.

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We describe herein a simple and economical protocol for the preparation of high-loading polymer supports (*ca.* 82–90 μmol/g LCAA-CPG) with commonly available reagents. The functionalized support has been successfully used for oligonucleotide synthesis in an automated DNA synthesizer. The quality of the synthesized oligonucleotides was compared with that of corresponding oligomers synthesized on standard LCAA-CPG support (leader nucleoside loading 45–50 μmol/g support).

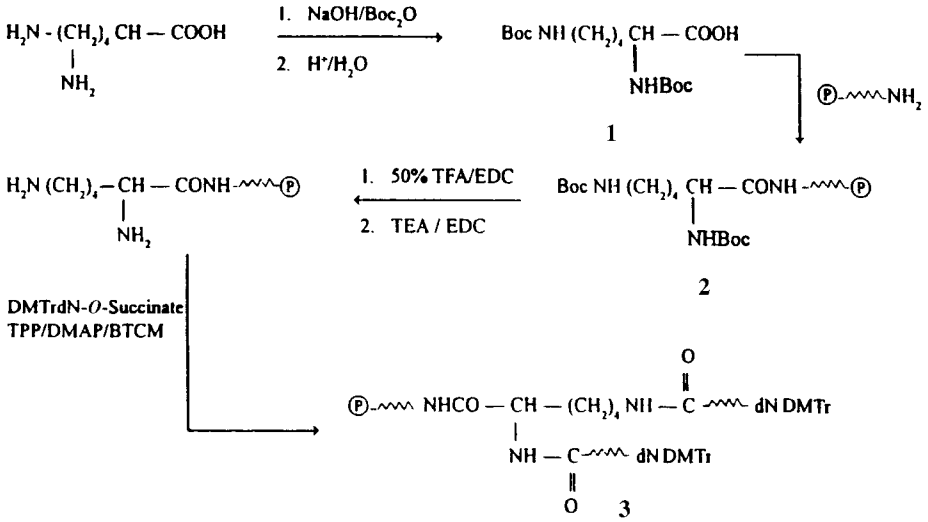
Results and Discussion. – In view of future demand for oligomers in larger quantities for various applications, we decided to develop a simple, versatile, and economical protocol for the preparation of high-loading supports that could ultimately be used to produce oligomers in larger quantities without affecting the quality of the product. The method involves the use of commonly available reagents. Recently, several groups, including our own, have reported protocols for the functionalization of polymer supports with improved nucleoside loading up to a moderate level of 45–50 μmol/g of LCAA-CPG.

In an attempt to increase the density of the functional groups on the polymer support, we employed a linker molecule (lysine) with two amino groups. The preparation of the linker molecule begins with the protection of both the α- and ε-NH_2 groups of lysine with *tert*-butyloxycarbonyl (Boc) group. The final product, $(Boc)_2$Lys OH, was obtained in 85% yield. The purity of the product was checked by thin-layer chromatography (TLC), and the product was further characterized by ^{1}H-NMR. In the subsequent step, the linker molecule was attached to LCAA-CPG by the oxidation-reduction condensation procedure. After the coupling reaction, the Boc-protecting groups were removed with 50% trifluoroacetic acid (TFA) in 1,2-dichloroethane. The increase in the density of the amino groups on LCAA-CPG was measured after reaction with *S*-(4,4′-dimethoxytrityl)-3-mercaptopropionic acid [13] by oxidation-reduction-condensation followed by acid treatment. The released dimethoxytrityl cation in the solution was measured spectrophotometrically at 498 nm. Amino-group loading was obtained in the range of 110–114 μmol/g of LCAA-CPG (average of three experiments).

To demonstrate the utility of this method to produce oligomers in large quantities, the above-described support was reacted with nucleoside-succinates by the oxidation-reduction condensation process [10]. Nucleoside loading in the range of 82–90 μmol/g of LCAA-CPG was obtained. Functionalization of support is depicted in the *Scheme*. The residual amino functionalities on the support were capped and used for oligomer synthesis.

The efficiency of these supports for oligonucleotide synthesis was established by synthesizing a number of oligonucleotides, *viz.*, **i**–**vii** (varying in length from 10–40 residues). All of these oligomers were synthesized on the 0.2 μmol scale by means of phosphoramidite chemistry in a *Pharmacia-LKB Gene Assembler Plus* by a standard protocol [14]. The corresponding oligomers were also synthesized on standard supports for comparison purposes. The coupling efficiencies, as determined by assaying the released 4,4′-dimethoxytrityl cation, were found to be $>99\%$ in each case and identical to the oligomers synthesized on the standard support, which indicates that the higher loading on the supports does not interfere with the synthesis.

Scheme



Boc = (*tert*-butoxy)carbonyl, TEA = Et_3N, DMAP = *N*,*N*-dimethylaminopyridine, TFA = trifluoroacetic acid, DMTr = 4,4′-dimethoxytrityl, BTCM = bromotrichloromethane, EDC = 1,2-dichloroethane, TPP = triphenylphosphine, dN-*O*-succinate = deoxynucleoside-*O*-succinate

The synthesized oligomers were cleaved from the support and fully deprotected under standard deprotection conditions (*i.e.* aq. ammonia at 55° for 16 h), followed by the usual work-up. The quality of the oligomers was evaluated by RP-HPLC. The base composition of the purified oligomers was determined by treating the oligomer first with snake-venom phosphodiesterase, followed by alkaline phosphatase and HPLC analysis, and was found to be comparable with the oligomers assembled on the standard supports.

The elution profile of crude d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT) is shown in *Fig. 1*. *Fig. 2* shows the HPLC profile of the crude oligomer d(CCA GAG GCA AGA GCT CCC CTT GTG GCA GCT TAT CCG). The HPLC profile of the crude oligomer d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-TTT TTT T) is shown in *Fig. 3*. The identity of these oligomers was further established by co-injection with the corresponding oligomers prepared on standard supports. The yields of HPLC-purified oligomers are summarized in the *Table*.

In conclusion, a simple, convenient, and economical protocol has been developed for the preparation of high-loading support for large-scale synthesis of quality oligonucleotides, which can be directly used for most biological applications without purification.

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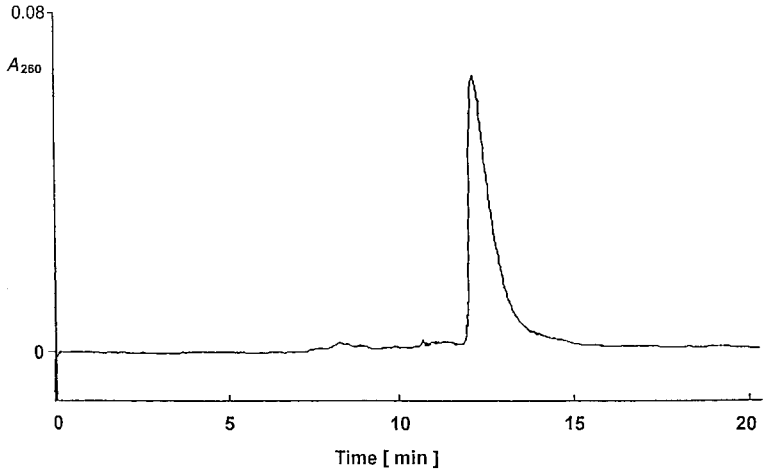

Fig. 1. *RP-HPLC Profile of crude d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT) synthesized on lysinylated LCAA-CPG* (dT-Lys-LCAA-CPG). Column: *Lichrosphere C-18RP*; gradient: 0–50% B in 25 min; $A = 0.1$M ammonium acetate (pH 7.1), $B =$ MeCN.

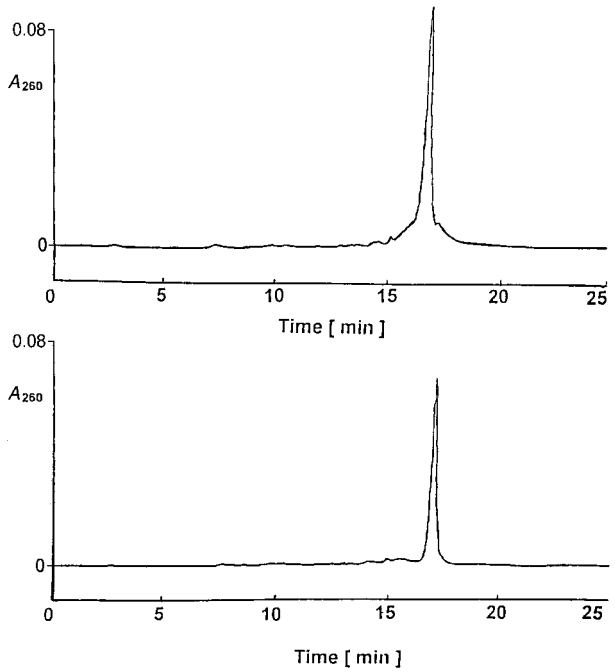

Fig. 2. *RP-HPLC Profiles of crude* a) *d(CCA GAG GCA AGA GCT CCC CTT GTG GCA GCT TAT CCG) synthesized on the standard dG-polymer support and* b) *d(CCA GAG GCA AGA GCT CCC CTT GTG-GCA GCT TAT CCG) synthesized on lysinylated-LCAA-CPG* (dG-Lys-LCAA-CPG). Column: *Lichrosphere C-18RP*; gradient: 0–50% B in 25 min; $A = 0.1$M ammonium acetate (pH 7.1); $B =$ MeCN.

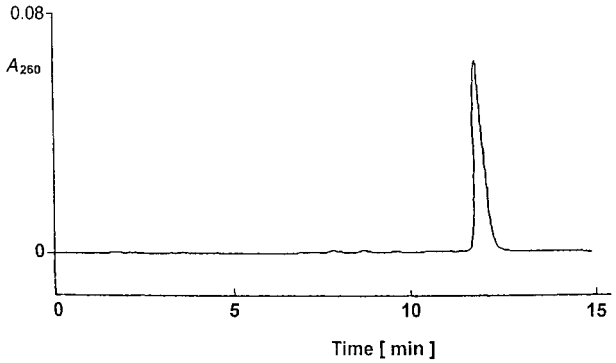

Fig. 3. *RP-HPLC Profile of crude d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T) synthesized on lysinylated-LCAA-CPG* (dT-Lys-LCAA-CPG). Column: *Lichrosphere C-18RP*; gradient: 0–50% B in 25 min; $A = 0.1$M ammonium acetate (pH 7.1), $B =$ MeCN.

Table. *Purity of Oligonucleotides Synthesized on the Derivatized Polymer Supports*

Oligomer		Purity [%]
I	d(TTT TTT TTT T)	98.5
II	d(TCA ATG CAA C)	97.8
III	d(TCA ATG CAA G)	97.5
IV	d(TCA ATG CAA A)	97.6
V	d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT)	96.9
VI	d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T)	96.5
VII	d(CCA GAG GCA AGA GCT CCC CTT GTG GCA GCT TAT CCG)	94.7

Experimental Part

General. All solvents and reagents used in the present study were purified prior to use. Ph_3P, 4-dimethylaminopyridine (DMAP), bromotrichloromethane ($MeBrCl_3$), 2,4,6-trimethylpyridine, and *tert*-butyl pyrocarbonate were purchased from *Fluka Chemie AG* (Switzerland). Long-chain alkylamine-controlled-pore glass (LCAA-CPG, 500 Å), TFA and 3-mercaptopropionic acid were procured from *Sigma Chemical Co.* (USA). Other reagents and chemicals were obtained from local suppliers.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a *Shimadzu LC 4A* system fitted with a variable-wavelength UV detector operating at 254 nm. Chromatograms were recorded on a *Shimadzu C-R7A chromatopac*.

Oligonucleotide synthesis was carried out on a 0.2-μmol scale on a *Pharmacia-LKB Gene Assembler Plus* by a standard protocol.

Preparation of (Boc)$_2$–Lys–OH (**1**). Lysine (5 mmol) was dissolved in aq. NaOH (1M, 20 ml), and *tert*-butyl pyrocarbonate (15 mmol) in dioxane (20 ml) was added dropwise. The mixture was stirred at r.t. for 5 h. Completion of the reaction was determined by TLC, whereupon the solvents were removed and the residue was taken up in CH_2Cl_2 (100 ml). The org. phase was sequentially washed with H_2O, 10% aq. citric acid, and brine (50 ml of each). The org. phases were combined, concentrated, and triturated with petroleum ether (40–60°) to give **1** (85%). ^{1}H-NMR ($CDCl_3$): 1.40 (*s*, 18 H); 1.51–1.86 (*m*, 6 H); 3.02 (br., 2 H); 4.3 (br., 1 H).

Preparation of Lysinylated-LCAA-CPG (**2**). To a soln. of **1** (0.25 mmol) in anh. DMF was added DMAP (0.5 mmol) and $MeBrCl_3$ (0.5 mmol). Ph_3P (0.25 mmol) in dry DMF (0.5 ml) was added to the above soln., followed by LCAA-CPG (50 mg). The suspension was agitated for 30 min, then excess reagents were removed by washing with DMF and Et_2O (20 ml of each) and dried under vacuum. The dried support was treated with 50% TFA in dry 1,2-dichloroethane for 30 min at r.t., followed by washing with 1,2-dichloroethane, 10% Et_3N in 1,2-dichloroethane and Et_2O (2×25 ml each). After drying the NH_2-group density on LCAA-CPG was

determined with *S*-(4,4′-dimethoxytrityl)-3-mercaptopropionic acid, Ph_3P, DMAP, and $MeBrCl_3$ as discussed above, followed by acid treatment for measurement of the released dimethoxytrityl cation at 498 nm.

Coupling of 5′-O-DMTr-2′-Deoxynucleoside-3′-O-succinate to Lysinylated-LCAA-CPG (**3**). 5′-O-DMTr-2′-deoxyribonucleoside-3′-O-succinate (18 μmol), DMAP (36 μmol), and $MeBrCl_3$ (36 μmol) were mixed in dry MeCN (0.5 ml) followed by the addition of TPP (18 μmol). To the mixture, after brief agitation, was added **2** (40 mg), and the suspension was agitated for 30 min at r.t. Then it was washed with MeCN and Et_2O. The residual amino groups were capped with Ac_2O/DMAP/2,4,6-trimethylpyridine/MeCN (1:0.5:0.5:6, *v/v*) for 30 min at r.t., followed by washing with MeCN and Et_2O (20 ml of each). After filtration, the support **3** was dried under vacuum and subjected to loading capacity determination by a standard protocol [11]. Attempts to prepare supports with amino-alkyl loading higher than 114 μmol/g by lysine coupling were unsuccessful, therefore the support with 114 μmol/g was used in oligonucleotide synthesis.

Oligonucleotide Synthesis, Deprotection, and Purification. To demonstrate the utility of the support and the quality of the final product, a number of oligonucleotide sequences were synthesized, *viz.*, d(TTT TTT TTT T) (**I**), d(TCA ATG CAA C) (**II**), d(TCA ATG CAA G) (**III**), d(TCA ATG CAA A) (**IV**), d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT) (**V**), d(TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T) (**VI**), d(CCA GAG GCA AGA GCT CCC CTT GTG GCA GCT TAT CCGI) (**VII**). These same oligomers were assembled on the standard supports for comparison purposes. The coupling efficiencies in each case were found to be > 99% based on the monitoring of the released dimethoxytrityl cation.

After synthesis, the oligomers were cleaved from the support and the protecting groups were removed from nucleic bases and internucleotide phosphates by the treatment with aq. ammonia (30%) at 55° for 16 h. The fully deprotected oligomers were concentrated and subjected to desalting on a *Sephadex G-25* column. The oligomers were eluted with 0.1M triethylammonium acetate (pH 7.2) and concentrated in a speed vac. The desalted oligonucleotides were then analyzed by RP-HPLC on *Lichrosphere RP-18* supplied by *E. Merck* (Germany). The mobile phases were 100 mM ammonium acetate (pH 7.1) (*A*) and MeCN (*B*).

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