Preparation of High-Loading Polymer Supports by Polymerization Reaction Useful for Oligonucleotide Synthesis

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A simple protocol based on polymerization reactions has been developed for the preparation of high-loading polymer supports, useful for large-scale synthesis of oligonucleotides. Polymer supports of different pore sizes have been employed in the present investigation to improve the functional-group density on them. A ten- to twelvefold increase in the loading of the functional groups, after the polymerization reaction, has been observed. The support was then used in the subsequent reaction to attach the leader nucleoside to obtain fully functionalized supports **6a**–**6c** by oligonucleotide synthesis in an automated DNA synthesizer. The amino-alkylated-supports **5a**–**5c** were directly employed for the synthesis of oligonucleotide 3′-phosphates. The oligonucleotides and oligonucleotide 3′-phosphates synthesized on these supports were compared with the corresponding standard oligomers with respect to their retention time on HPLC. These were further characterized on MALDI-TOF mass spectrometry.

Introduction. – Oligonucleotides are finding a wide variety of applications in the area of genomics. To develop DNA-based diagnostics and therapeutics, oligomers in the size range of 15–20mer are required in relatively large quantities [1–4]. To meet exponentially growing demands for these molecules, the present-day methodologies have proved to be quite inadequate in terms of speed and cost. Hence, there is a need to develop a cost-effective and rapid method for their synthesis. Since most of the syntheses are carried out by solid-phase methodology, the loading of leader nucleoside/nucleotide on the polymer supports plays a major role in deciding the cost of these molecules [5–7]. To reduce the production cost of these molecules, one way to address this problem is to develop polymer supports with a higher density of functional groups (nucleoside/nucleotide). Therefore, the search for newer methods for the preparation of high-loading supports is still on [8][9]. Though a number of protocols, including one from our laboratory, have been proposed for this purpose [10], there is still a need to develop economical methods that use commonly available chemicals. The main difficulty in the preparation of high-loading silica/glass-based supports is primarily the smaller surface area available for functionalization. This problem can be circumvented either by designing organic polymer-based supports or devising new protocols for functionalization of the existing supports to allow higher loading of functional groups on them.

Here, we report a novel method for the preparation of high-loading supports. The method is based on the polymerization reaction. The method involves the co-polymerization of a reagent, *N*-(6-aminohexyl)prop-2-enamide (**3**), with acryloylated polymer supports in the presence of an initiator and a catalyst. The degree of polymerization can be controlled by adjusting the concentration of the reagents as well as the reaction time for polymerization, which enables one to have the supports of desired loading. The strategy has also been tested on a number of supports varying in porosity. A ten- to twelvefold increase in the loading of the functional groups has been observed. A number of oligonucleotide sequences were assembled on these supports and were compared with the oligomers synthesized on standard supports.

Results and Discussion. – Preparation of high-loading polymer supports is of immense importance for antisense-oligonucleotide research, where large quantities of these molecules are required. Moreover, it is difficult to obtain high loading of leader nucleosides on high-porosity supports, because of the smaller surface area available for functionalization. Some attempts have already been made in this direction. We have tried to address this problem in the present investigation by designing a unique strategy. The present methodology has been designed keeping in mind that the method should be simple, rapid, and economical. It should not involve the use of specialized chemicals or reagents, nor should it involve a multistep-synthesis scheme. Following these points, we have been able to develop a simple protocol for the preparation of high-loading polymer supports, suitable for large-scale synthesis of oligonucleotides.

The support functionalization strategy is depicted in the *Scheme*. The method involves the generation of a polymerizable group (acryloyl) on one end of a linker molecule, hexane-1,6-diamine (**1**), by a simple chemical reaction of prop-2-enoyl chloride (**2**) with hexane-1,6-diamine. The desired molecule, *N*-(6-aminohexyl)prop-2-enamide (**3**)**,** was obtained in *ca*. 75% yield. Commercially available controlled-pore glass (CPG) of different porosities was modified with 3-(trimethoxysilyl)propyl methacrylate according to the procedure reported in [11]. The loading of methacryloyl groups on these supports were deliberately kept low (*Table 1*) to demonstrate the utility of the proposed method. The residual silanol functionalities were capped with Me_3SiCl in pyridine. The polymerization reaction was initiated by mixing linker molecule **3** with one of the supports **4a**–**4c** in the presence of *N,N,N′,N′*-tetramethyl-ethylenediamine (TMEDA), ferrous ammonium sulfate (FAS), and ammonium persulfate (APS) in aqueous solution under Ar for 7 h at room temperature. The resultant polymer support bearing aminoalkyl functionalities was recovered and subjected to washing with H_2O, MeOH, and Et_2O, and dried first in air and then under vacuum. The density of the aminoalkyl groups on the derivatized supports was determined according to the protocol published by our group [12]. The same procedure was followed to generate aminoalkyl functionalities on polymer supports having different porosity. The results are summarized in *Table 1*. A ten- to twelvefold increase in amino-group loading was observed in each case.

The optimum time required for the polymerization reaction was determined by a kinetic study. A small amount of polymer support (CPG 500 Å, *ca.* 25 mg) was taken in each of fifteen sample tubes and treated with the polymerizing mixture (**3**, TMEDA, FAS, and APS) as described in *Exper. Part*. Tubes were withdrawn initially at an

Scheme. *Preparation of High-Loading Polymer Supports*

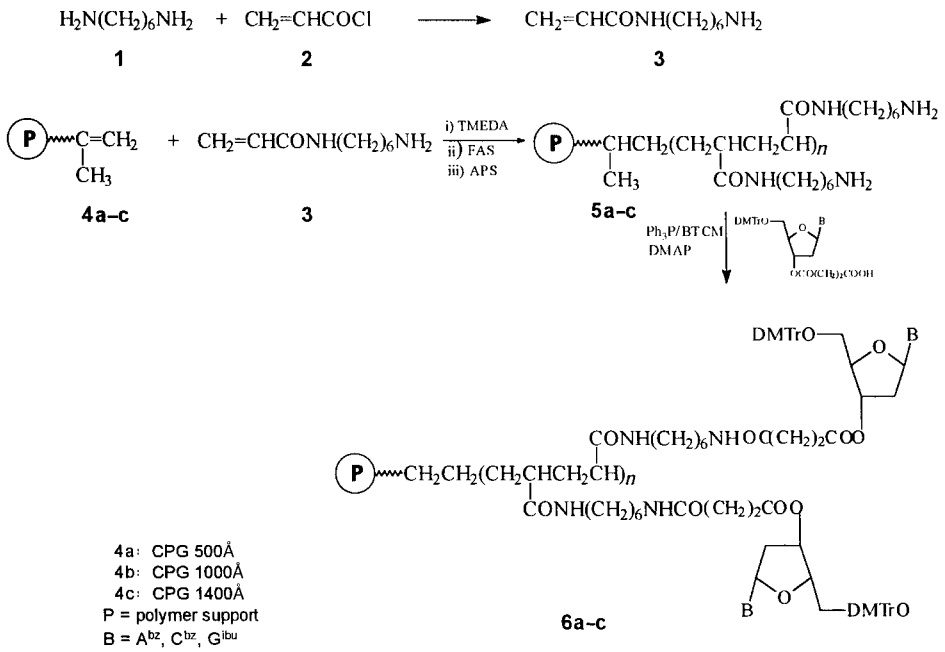

Table 1. *Estimation of Polymer-Supported Functional Groups/Leader Nucleoside*

Entry	Polymer Support	Loading μmol/g of support		
		Acryloyl groups	Amino groups	Leader nucleoside
1	CPG 500 Å	11	110	56–61
2	CPG 1000 Å	8	89	39–43
3	CPG 1400 Å	6	66	27–31

interval of 30 min (30 min–5 h) and then at an interval of 1 h (5–9 h). The polymer support was recovered on a sintered-glass funnel, where it was washed successively with H_2O, MeOH, and Et_2O, and dried as described above. At the end of the kinetic studies, supports were subjected to estimation of amino-group density according to the method reported in [12]. Amino-group loading *vs.* time (h) is plotted in *Fig. 1*. From the plot, it was found out that the polymerization reaction was essentially complete in 7 h. Similarly, to arrive at the optimum concentration (equiv.) required to obtain the maximum density of functional groups on the polymer supports, a plot relating concentration of polymerizing reagent, **3**, to loading on the polymer support was drawn. *Fig. 2* shows the results of a study carried out by taking a fixed amount of one of the polymer supports **4a**–**4c** with varying concentrations of the polymerizing reagent. It can be seen from this study that a proportional increase in functional-group loading on polymer support during polymerization reaction was found for up to *ca.* a 60-fold excess (concentration) of the reagent **3**, and, afterwards, there was no further change.

Therefore, we deduced that *ca.* a 60-fold excess of the reagent **3** was an optimum concentration for the polymerization reaction under the given conditions and, in the present study, this concentration was used for all reactions.

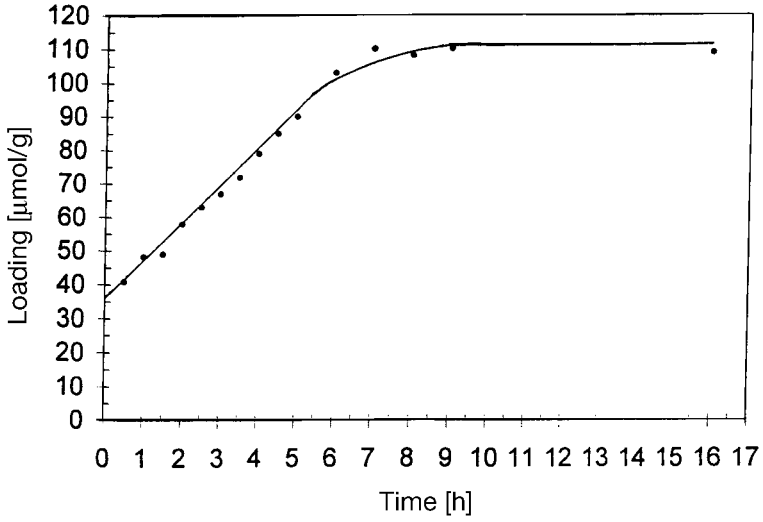

Fig. 1. *Time course of the polymerization reaction*

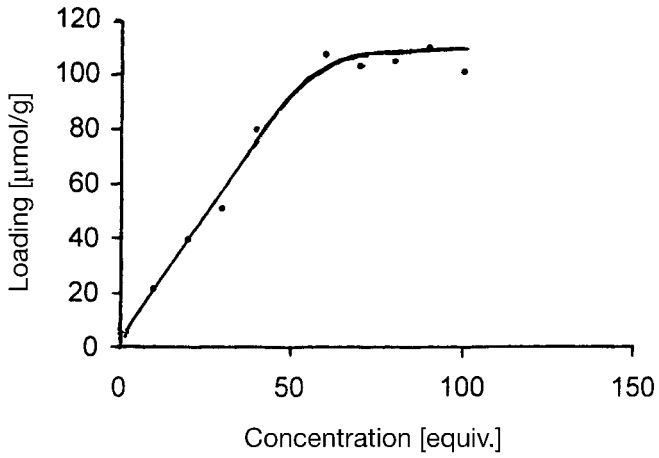

Fig. 2. *Concentration dependence of the polymerization reaction*

To demonstrate the usefulness of the modified polymer supports **5a**–**5c** and **6a**–**6c**, a number of oligonucleotide sequences (*Table 2*) were assembled. The same oligomers were synthesized on standard supports for comparison purposes. The oligomers were synthesized at 0.2-µmol scale in the *Gene Assembler Plus* according to the manufacturer's standard protocol [13]. The coupling efficiencies in each case were found to be >99% based on monitoring the release of 4,4′-dimethoxytrityl cation.

Table 2. *Oligonucleotide Sequences Synthesized, Their Retention Times, and Mass Values on MALDI-TOF*[a])

Entry	Oligonucleotide sequence	Retention time [min]	MALDI-TOF	
			Observed	Expected
1	d(TTT TT-PO_4^{-3}) 500 Å **5a**	20.48	1536.8	1538
2	d(TTT TT-PO_4^{-3}) 1000 Å **5b**	20.52	1538.2	1538
3	d(TTT TT-PO_4^{-3}) 1400 Å **5c**	20.74	1540.2	1538
4	d(TTT TTT TTT T) 1000 Å **6b**	19.98	2979.3	2980
5	d(TTT TTT TTT T) 1400 Å **6c**	19.97	2978.8	2980
6	d(CCT TGA TAC CAA CCT GC-PO_4^{-3}) 1000 Å **5b**	10.86	5167	5168.4
7	d(CCT TGA TAC CAA CCT GT) 500 Å **6a**	10.94	5104	5105.4

[a]) *Entries 1–3:* HPLC conditions: flow rate 1 ml/min; 0–15% *B* in 25 min; column, *Lichrosphere RP-18*; buffer *A*, 0.1M ammonium acetate, pH 7.1; solvent *B*, MeCN; Auf, 0.01. *Entries 4* and *5:* HPLC conditions: flow rate 1 ml/min; 0–20% *B* in 25 min; column, *Lichrosphere RP-18*; buffer *A*, 0.1M ammonium acetate, pH 7.1; solvent *B*, MeCN; Auf, 0.01. *Entries 6* and *7:* HPLC conditions: flow rate 1 ml/min; 0–50% *B* in 25 min; column, *Lichrosphere RP-18*; buffer *A*, 0.1M ammonium acetate, pH 7.1; solvent *B*, MeCN; Auf, 0.01.

Supports **5a–5c** were used for the synthesis of oligomer 3′-phosphates. The oligonucleatides were assembled according to the standard protocol and deprotected in a two-step process as described previously in [14]. The cleaved oligomer 3′-phosphates were analyzed on a reverse-phase (*RP-18*) column and characterized by co-injection with the corresponding standard oligomers. *Fig. 3* shows the HPLC profiles of crude d(TTT TT-PO_4^{-3}) synthesized on supports **5b** and **5c**, while the inset shows the MALDI-TOF mass spectrum of the same oligomer.

Supports **5a–5c** were further functionalized with appropriately protected nucleoside 3′-*O*-succinates to obtain supports **6a–6c**, by a slight modification of a procedure published by this laboratory [10]. These supports were used for normal oligonucleotide synthesis at 0.2-μmol scale. The oligomers were cleaved from the supports **6a–6c** with aqueous ammonia (29%) and, after concentration and desalting, were analyzed on reversed-phase HPLC. *Fig. 4* shows a typical HPLC profile of crude d(CCT TGA TAC CAA CCT GT) synthesized on support **6a** and its MALDI-TOF spectrum as an inset. Oligomers synthesized on the supports **5a–5c** and **6a–6c**, their retention times, and mass obtained on MALDI-TOF are given in *Table 2*. To compare the yields on the standard as well as the proposed high-loading polymer supports, an oligomer d(TTT TTT TTT TTT TTT) was assembled on both the supports. The overall coupling yields based on 4,4′-dimethoxytrityl release in the machine were found to be *ca.* 71 and 77% on the standard and proposed supports, respectively. The oligomers were deprotected and concentrated in a *Speed Vac.* The crude oligomers were obtained in *ca.* 29 A_{260} (standard support) and *ca.* 32 A_{260} units (proposed support).

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Experimental Part

1. *General.* All solvents and reagents employed were purified prior to their use. LCAA-CPG, hexane-1,6-diamine, *N,N,N′,N′*-tetramethylethylenediamine (TMEDA), ammonium persulfate (APS), and *sym*-collidine were procured from *Sigma Chemical Co.*, USA. 3-(Trimethoxysilyl)propyl methacrylate, 4-(dimethylamino)-

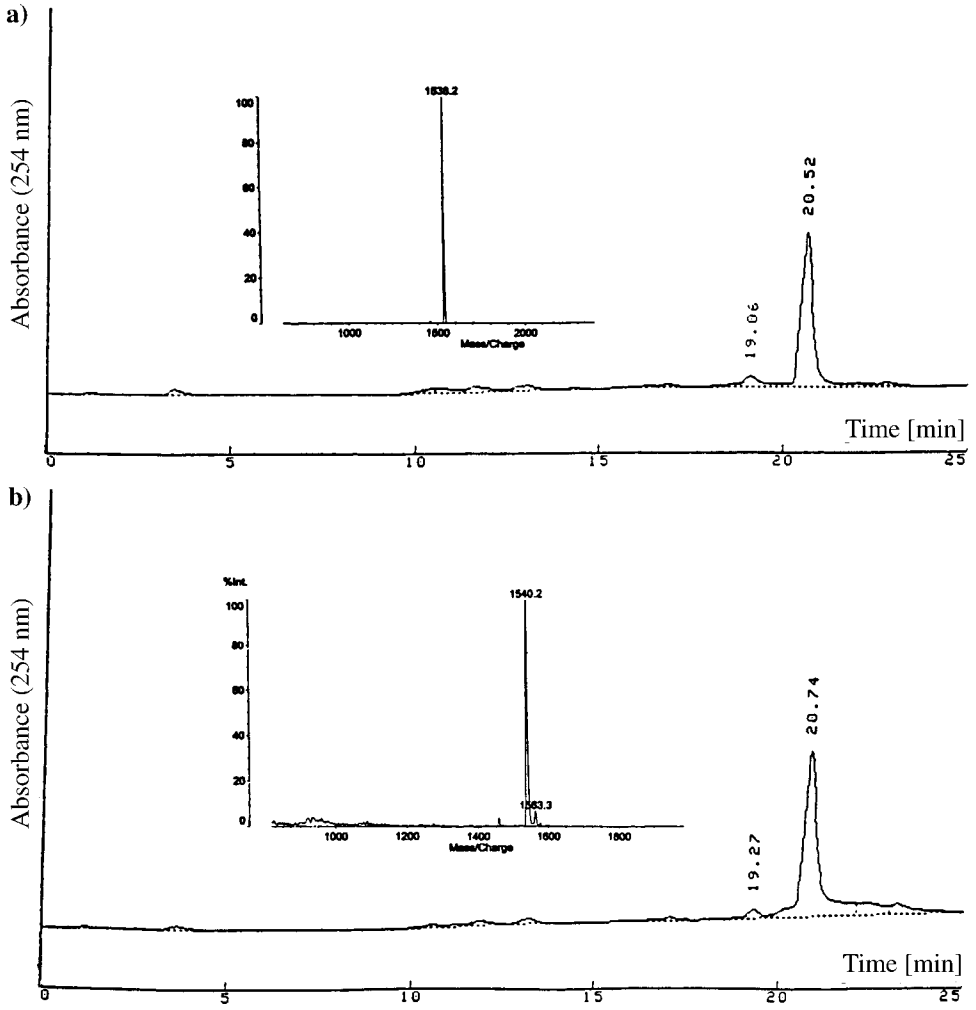

Fig. 3. *RP-HPLC Profiles of a crude d(TTT TT-PO$_4^{3-}$) synthesized on* a) *polymer support* **5b**; inset: *MALDI spectrum, and* b) *polymer support* **5c**; inset: *MALDI spectrum*

pyridine (DMAP), and Ph_3P (TPP) were purchased from *Fluka Chemical Co.*, Switzerland. Other chemicals and reagents were obtained from local suppliers and were purified before use. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on *Shimadzu LC-4A* and *LC-10A* systems fitted with a variable wavelength UV detector operating at 254 nm. Chromatograms were recorded on a *Shimadzu C-R7A* chromatopac. Oligonucleotide synthesis was performed at 0.2-μmol scale according to the manufacturer's protocol with modified as well as standard supports on *a Pharmacia LKB Gene Assembler Plus.* Characterization of oligonucleotides was carried out by MALDI-TOF (*SEQ IV, Kratos Inc., UK*) with tetrahydroxyacetophenone and 3-hydroxypicolinic acid as matrices for small (up to 10mer) and longer (>10mer) oligonucleotides, resp.

Reagents used: polymerizing reagent (0.5M), 1% aq. ferrous ammonium sulfate (FAS), 1% aq. APS, TMEDA.

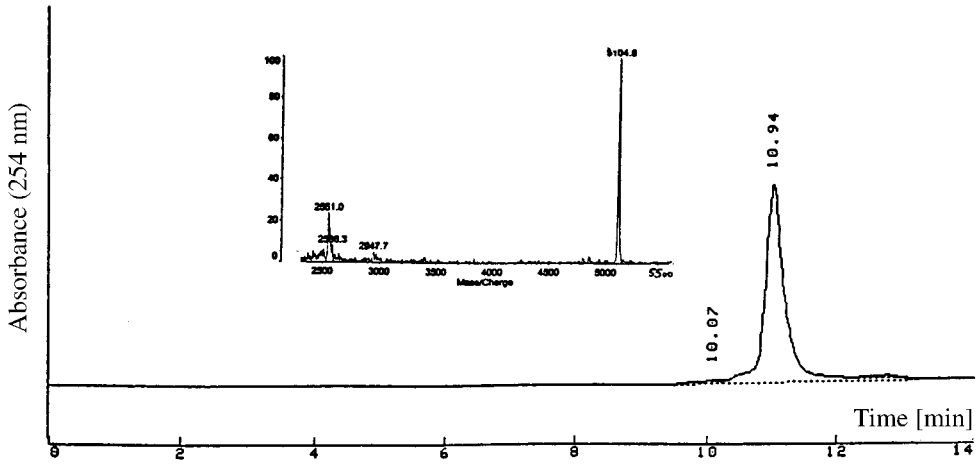

Fig. 4. *RP-HPLC Profile of a crude d(CCT TGA TAC CAA CCT GT) synthesized on polymer support* **6a**; inset: *MALDI spectrum*

2. *Synthesis of 5-Amino-*O*-(4,4′-dimethoxytrityl)pentan-1-ol.* 5-Aminopentan-1-ol (20 mmol, 2.06 g) and Na_2CO_3 (12 mmol, 1.27 g) were dissolved in H_2O (15 ml) and added to THF (10 ml). (9*H*-Fluoren-9-yl)methyl chloroformate (21 mmol, 5.4 g) dissolved in THF (25 ml) was added dropwise to the above mixture during 10 min at 4°. The mixture was stirred at r.t. for 4 h. The reaction was monitored by TLC and, the mixture was concentrated *in vacuo*. The residue was dissolved in AcOEt (100 ml) and washed sequentially with H_2O, 10% aq. citric acid, and H_2O (2 × 30 ml of each). The org. layer was collected and concentrated to dryness to obtain 5-amino-*N*-[(9*H*-fluoren-9-yl)methoxycarbonyl]pentan-1-ol in almost quant. yield.

To the above compound (10 mmol, 3.24 g) dissolved in dry pyridine, 4,4′-dimethoxytrityl chloride (12 mmol, 4.05 g) was added, and the mixture was allowed to stir at r.t. for 6 h. Then the mixture was concentrated on a rotary evaporator and partitioned between AcOEt (150 ml) and 10% aq. $NaHCO_3$ soln. The org. layer was washed with $NaHCO_3$ soln. (3 × 50 ml). The upper layer was collected and concentrated to a syrupy mass. To this syrupy mass, morpholine (100 ml) was added, and the soln. was allowed to stir overnight at r.t. After completion of the reaction, the mixture was concentrated and partitioned between AcOEt (150 ml) and sat. NaCl soln. The org. phase was collected and concentrated. The compound was purified on a silica-gel column with increasing gradient of MeOH in ethylene dichloride containing Et_3N (1%). Pure fractions were pooled and concentrated *in vacuo* to yield 5-amino-*O*-(4,4′-dimethoxytrityl)pentan-1-ol in 65% yield.

3. *Preparation of* N*-(6-Aminohexyl)prop-2-enamide.* Hexane-1,6-diamine (10 mmol, 1.162 g) was placed in a round-bottomed flask and dried by co-evaporation with pyridine (15 ml). Finally, it was taken up in pyridine (15 ml), and acryloyl chloride (2 mmol, 163 µl) was added dropwise. The mixture was stirred at r.t. for 2 h. The reaction was monitored by TLC, and the solvent was removed under reduced pressure. The residual mass was partitioned between BuOH (50 ml) and sat. NaCl soln. (2 × 10 ml). The org. phase was dried (Na_2SO_4), filtered, and concentrated *in vacuo* to obtain the title compound as a syrupy material (209 mg, *ca.* 75%). The compound was characterized by MALDI-TOF-MS: 168 ($[M-2]^+$; calc. 170; matrix: mesotetrakis(pentafluorophenyl)-porphyrin). The syrupy material was redissolved in distilled H_2O (2.5 ml) to a final concentration of 0.5M.

4. *Support Functionalization to Generate Acryloyl Function.* Unmodified CPG (500 mg) was derivatized with 3-(trimethoxysilyl)propyl methacrylate according to the procedure described in [11]. The residual silanol groups were capped with Me_3SiCl in anh. pyridine.

5. *Estimation of Acryloyl Functionalities on Supports.* Methacryloylated CPG (100 mg) was suspended in a 0.5M soln. of 5-amino-*O*-(4,4′-dimethoxytrityl)pentan-1-ol (1 ml) in DMF, and $EtN(i\text{-}Pr)_2$ (50 µl) was added. The suspension was agitated at 50° for 48 h, and aliquots were withdrawn at regular time interval. The support withdrawn was washed with DMF and Et_2O (15 ml of each). After drying the support, the loading of

methacryloyl groups was determined by measuring the absorbance of the released 4,4′-dimethoxytrityl cation at 498 nm.

6. *Polymerization Reaction.* Methacryloylated support (*ca.* 25 mg) was mixed with compound **3** (28 µl) and TMEDA (10 µl). The suspension was agitated for a while and purged with Ar. Then, FAS (5 µl of 1% aq. soln.) and APS (5 µl of 1% aq. soln.) were added, and the mixture was agitated at r.t. After the stipulated time, the support was washed with H_2O, MeOH and Et_2O (20 ml of each) and dried in a desiccator under vacuum. Similarly, CPG supports (1000 Å and 1400 Å) were used for polymerization reaction. The amino-group density on the polymer supports was determined according to the method described in [12].

7. *Time Kinetics of Polymerization Reaction on Methacryloylated Polymer Supports.* For kinetic studies, 15 reaction vials, purged with Ar, were charged with a weighed amount of methacryloylated polymer support (*ca.* 25 mg). To each vial, polymerizing reagent **3** (28 µl), TMEDA (10 µl), FAS (5 µl) and APS (5 µl) were added. The vials were kept agitating at r.t. for up to 16 h. Vials were withdrawn at appropriate time intervals, and the support was washed sequentially with H_2O, MeOH, and Et_2O (20 ml of each). The supports were dried under vacuum, and the amino-group density was calculated according to the standard method [12].

8. *Concentration Kinetics of Reaction of Polymerizing Reagent with Methacryloylated Polymer Supports.* Ten reaction vials, purged with Ar, were charged with a weighed amount of methacryloylated polymer support (*ca.* 25 mg). To each vial, appropriate amounts of polymerizing reagent **3**, TMEDA (10 µl), FAS (5 µl), and APS (5 µl) were added. The vials were kept for agitation at r.t. for 16 h. Then, polymer support was recovered on sintered glass funnel and washed sequentially with H_2O, MeOH, and Et_2O (20 ml of each). The supports were dried under vacuum, and the amino group density was calculated as described above.

9. *Coupling of* N*-Protected 2′-Deoxy-5′-*O*-DMTr-ribonucleoside 3′-*O*-Succinates to CPGs. N*-Protected 2′-Deoxy-5′-*O*-DMTr-ribonucleoside 3′-*O*-succinate (250 µmol), DMAP (61 mg, 500 µmol) and $BrCCl_3$ (25 µl, 250 µmol) were mixed together in dry DMF (2.0 ml), followed by the addition of a soln. of Ph_3P (65.5 mg, 250 µmol) dissolved in DMF (0.5 ml) to the above mixture. After brief agitation, CPG (500 Å, 250 mg, *ca.* 25 µmol amino groups) was added, and the suspension was agitated for 30 min. at r.t. Then, it was washed with DMF, MeCN, and Et_2O (20 ml of each). The residual amino groups were capped by the standard protocol before determination of loading [15].

10. *Oligonucleotide Synthesis, Deprotection, and Purification.* A number of oligonucleotides (*Table 2*) were synthesized on the modified polymer supports **5a**–**5c** and **6a**–**6c**. The same oligomers were assembled on standard supports for comparison purposes. The oligomers were synthesized at 0.2-µmol scale in the *Gene Assembler Plus* according to manufacturer's standard protocol [13]. The coupling efficiencies in each case were found to be >99% based on the monitoring of the released 4,4′-dimethoxytrityl cation.

The deprotection of oligonucleotides (removal of protecting groups and cleavage from the supports) was achieved according to the standard protocol [14][15]. The deprotected oligonucleotides were desalted on a RP silica gel with 30% MeCN in H_2O as an eluent. After concentration in a *Speed Vac*, the oligomers were resuspended in ammonium acetate buffer and analyzed on RP-HPLC. Further characterization was carried out by MALDI-TOF-MS.

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