Composite Magnetic and Non-magnetic Beads as Efficient Solid Supports for Machine-Aided Oligonucleotide Synthesis

by **Pradeep Kumar**, **Ashwani K. Sharma**, and **Kailash C. Gupta***

Nucleic Acids Research Laboratory, Centre for Biochemical Technology, Mall Road, Delhi University Campus, Delhi-110007, India

and **Shlomo Margel** and **Sigalit Gura**

Department of Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel

and **Hartmut Seliger**

Universität Ulm, Sektion Polymere, Albert Einstein Allee 11, D-89069 Ulm

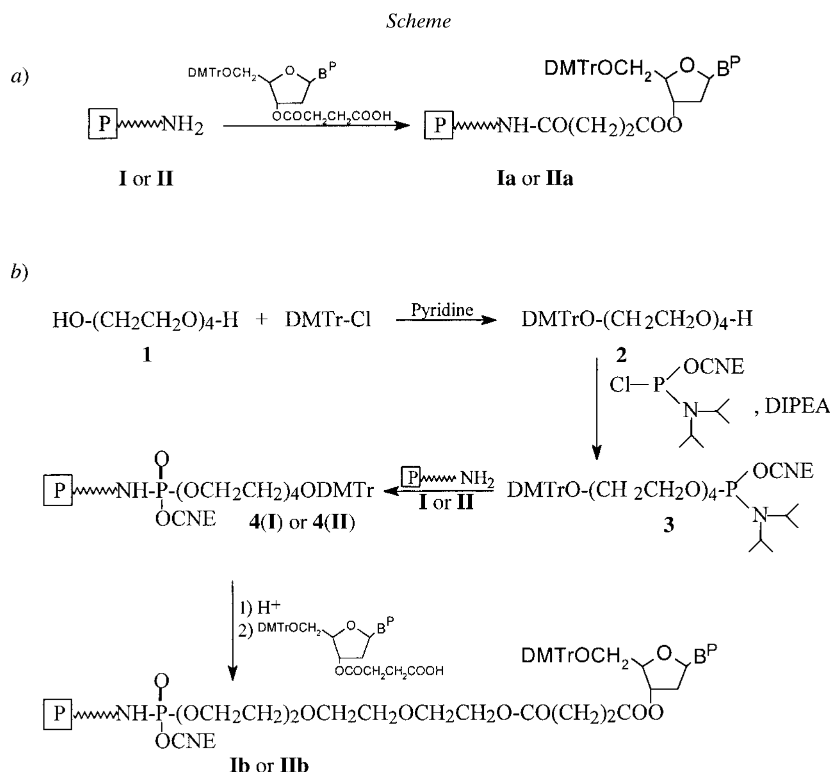
The synthesis of oligodeoxyribonucleotides on composite magnetic and nonmagnetic beads of 4.5 μm with a cleavable and non-cleaveable linker for biochemical applications is described.

Introduction. – A large number of organic and inorganic polymer supports have been described for routine oligonucleotide synthesis [1–4]. These supports are mainly macroporous in nature, which allows easy accessibility of reagents to the growing oligonucleotide chains. However, for certain biological applications where support-bound oligonucleotides are directly used in enzymatic reactions, oligonucleotides on nonporous supports would be preferred to avoid the steric hindrance within pores of the matrix. This can be achieved either by synthesizing the oligonucleotide first on a porous support and then immobilizing it on a nonporous support or by directly synthesizing the oligonucleotide on a nonporous support with a noncleaveable linkage between the oligomer and the support. Some attempts have already been made to synthesize oligonucleotides on nonporous supports [5][6].

In this communication, we wish to describe the use of new composites of magnetic and nonmagnetic beads as effective supports for oligonucleotide synthesis. These composite bead systems are made up of a uniform inner core of polystyrene (4.5 μm) and an outer shell of silica. However, in the case of magnetic composite beads, the inner core of polystyrene is covered first with a magnetic material followed by silica. Both of the composite systems, the nonmagnetic **I** and the magnetic **II**, were further derivatized with a silylating reagent to incorporate an aminoalkyl or mercaptoalkyl functionality. To use these composite systems for oligonucleotide synthesis, they were either first modified with a suitable linker and then attached to a appropriately protected nucleoside or directly loaded with the appropriately protected nucleoside, yielding ready-to-use supports for an automated DNA synthesizer. Supports **I** and **II** were derivatized and found to have nucleoside loadings in the range of 20–27 μmol/g support. Both of the supports were successfully used for oligonucleotide synthesis.

Results and Discussion. – The supports **I** and **II** were allowed to react with appropriately protected nucleoside succinates, giving ready-to-use supports **Ia** and **IIa** as shown in the *Scheme*, *a*, whereas the functionalization of the supports **I** and **II** with a spacer arm is depicted in the *Scheme*, *b*. Commercially available tetraethylene glycol (TEG; **1**) was selected as spacer arm on supports **I** and **II**. Thus, **1** was monotritylated by treatment with 4,4′-dimethoxytrityl chloride (DMTrCl) and the resulting monosubstituted tetraethylene glycol phosphitylated with 2-cyanoethyl diisopropylphosphoramidochloridite to give the desired linker molecule **3**. The latter was subsequently coupled to the aminoalkylated support **I** or **II** in the machine following the nucleoside-phosphoramidite-coupling procedure to furnish the modified polymer supports **4**(**I**) or **4**(**II**). To obtain the ready to use, fully functionalized modified supports **Ib** and **IIb**, the supports **4**(**I**) and **4**(**II**) were subjected to detritylation in the machine itself, followed by coupling with the appropriate nucleoside succinate according to the reported procedure [7].

Scheme



I	= nonmagnetic support		DMTr	= 4,4′-dimethoxytrityl
II	= magnetic support		CNE	= CH_2CH_2CN
B^P	= dA^{bz}, dC^{bz}, dG^{ibu} or dT		DIPEA	= $^iPr_2EtN$
P	= Polymer support			

According to the trityl-cation assay, a loading in the range of 20–22 µmol/g was obtained on the supports **Ib** and **IIb**. The spacer arm was employed anticipating that the proposed spacer might promote better coupling efficiencies during oligonucleotide synthesis. However, no significant difference in the coupling efficiency was noticed during oligomer assembly on supports with or without spacer arm. We successfully synthesized up to 20mer oligonucleotides on both kinds of supports, at a 0.2-µmol scale on the *Pharmacia-LKB Gene Assembler Plus* following the standard protocol of the manufacturer [8]. Attempts for the synthesis of longer oligonucleotides were, however, not very encouraging. Therefore, these supports are being further modified to make them suitable for the synthesis of longer oligomers. The coupling yields with the supports **Ia**, **b** and **IIa**, **b** were comparable to those obtained with the commercially available standard supports which are used for routine oligonucleotide synthesis.

The quality of the oligonucleotides synthesized on the supports **Ia**,**b** and **IIa**,**b** was ascertained by HPLC analysis with a reversed-phase *C-18* column. *Figs. 1* and *2* show the HPLC profiles of crude d(TTT TTT TTT TTT TTT TTT TT) synthesized on supports **Ib** and **IIb**, respectively. The identity of these oligomers was further confirmed by co-injecting them with the corresponding standard d(TTT TTT TTT TTT TTT TTT TT). We thus established that the composite supports **Ia**,**b** and **IIa**,**b** are suitble for oligonucleotide synthesis.

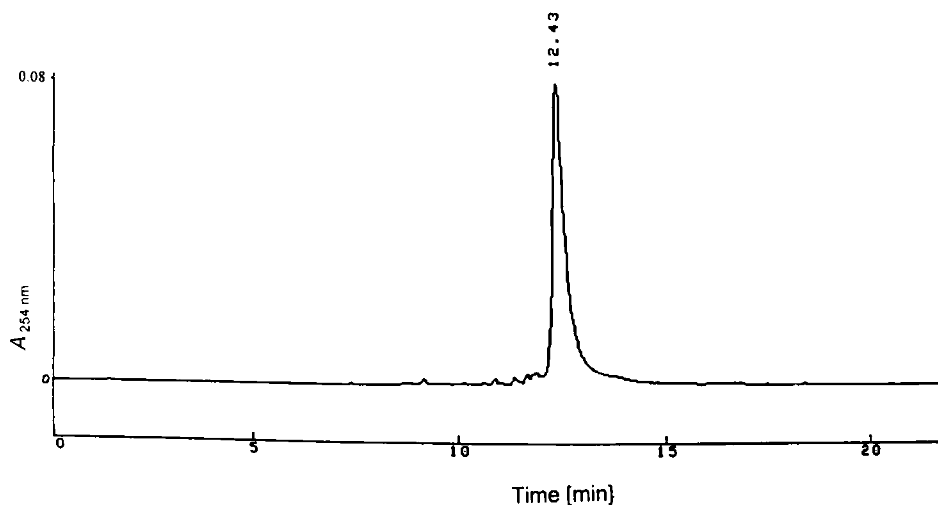


Fig. 1. *Reversed-phase HPLC profile of crude d(TTT TTT TTT TTT TTT TTT TT), synthesized on non-magnetic support* **Ib**. Column: *Lichrosphere C-18RP*; gradient: 0–50% *B* in 25 min; solvent *A*, 0.1M NH_4OAc, pH 7.1, solvent *B*, MeCN; Resolution 0.08.

To prepare oligonucleotides still anchored at the support after aqueous ammonia treatment, the magnetic support **4(II)** was directly employed for oligonucleotide synthesis in the automated DNA synthesizer. The usual aqueous ammonia treatment removed the internucleoside phosphate protecting groups, resulting in a support-bound d(TTT TTT TTT TTT TTT T), which was then used in hybridization studies with a complementary oligomer d(AAA AAA AAA AAA AAA A), as described in the

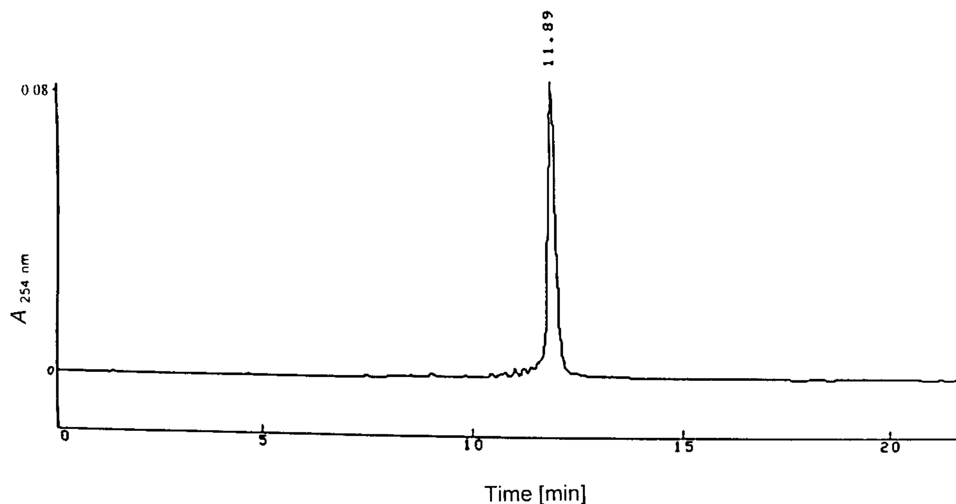


Fig. 2. *Reversed-phase HPLC profiles of crude d(TTT TTT TTT TTT TTT TTT TT), synthesized on magnetic support* **IIb**. Column: *Lichrosphere C-18RP*, gradient: 0–50% *B* in 25 min; solvent *A*, 0.1M NH_4OAc, pH 7.1, solvent *B*, MeCN. Resolution 0.08.

Exper. Part. A loading of 111.6 A_{254} units of oligo d(TTT TTT TTT TTT TTT T)/g of support was found, which seems to be moderate for carrying out biochemical studies.

The financial assistance from the Department of Science and Technology (DST), New Delhi, India is gratefully acknowledged. The authors thank Mr. *Sunil Agrawal* and Mr. *V. K. Chauhan* for their technical support.

Experimental Part

1. *General.* Solvents and reagents were purified just prior to their use. Styrene monomer, polyvinylpyrrolidone, triphenylphosphine, bromotrichloromethane, 4-(dimethylamino)pyridine (= *N,N*-dimethylpyridinamine), $^iPr_2EtN$, silicon tetraethoxide ($Si(OEt)_4$), and tetraethylene glycol (**1**) were purchased from *Aldrich Chemical Co.*, USA. Oligonucleotide synthesis was performed at a 0.2-μmol scale with a *Pharmacia-LKB Gene Assembler Plus* following the standard protocol. HPLC: reversed-phase *C18* column, *Shimadzu-LC-4A* system, variable-wavelength UV detector set at 254 nm (*Shimadzu Corp.*, Japan); *C-R7A Chromatopac* recorder. Compounds were characterized by ^1H-NMR and MALDI-TOF. NMR Spectra: *Bruker Avance DPX-300* operating at 300 MHz; δ in ppm. Mass spectra (negative mode): *Kompact SEQ* (*Kratos*, UK), norharmane as matrix material.

2. *Preparation of Composite Particles and Their Functionalization.* The composites, made of core-shell particles of 4.5 μm with a core of polystyrene microspheres and either a shell of silica (non-magnetic) or a shell of first a magnetic-material and then silica (magnetic), were prepared following the procedure published earlier [9][10]. The polystyrene-core particles were prepared by dispersion polymerization of styrene in EtOH in the presence of polyvinylpyrrolidone as stabilizer. The silica coating was performed by seed polymerization of $Si(OEt)_4$ onto the polystyrene-core particles by *Stober*'s method [11]. Magnetic particles were prepared by seeded polymerization of iron salts on the polystyrene-core particles. A further coating of silica was carried out to prevent leaching of iron ions. Aminoalkyl and mercaptoalkyl functionalities were introduced by reacting the silica-coated particles with $Si(OEt)_3(CH_2CH_2X)$ ($X = NH_2$ or SH), according to the procedure in [12].

3. *Functionalization of Supports for Oligonucleotide Synthesis.* 3.1. *Preparation of a Linker Molecule.* Tetraethylene glycol (**1**; 20 mmol) was dried by co-evaporation with anh. pyridine (2 × 25 ml) and finally taken up in pyridine (25 ml). Then 4,4′-dimethoxytrityl chloride (15 mmol) was added at once, and the mixture was

allowed to stir at r.t. for 8 h. Then the solvent was evaporated, the syrupy residue dissolved in AcOEt (200 ml), the org. phase sequentially washed with aq. sat. $NaHCO_3$ soln. and brine (2×50 ml each), dried, and evaporated: *2-{2-{2-{2-[bis(4-methoxyphenyl)phenylmethoxy]ethoxy}ethoxy}ethoxy}ethanol* (**2**).

^{1}H-NMR ($CDCl_3$): 3.3 (*s*, 16 H); 3.6 (*s*, 6 H); 6.7 – 7.2 (*m*, 13 H). MALDI-TOF-MS: 495 (496).

Monotritylated tetraethylene glycol **2** (2 mmol) was co-evaporated with MeCN (2×25 ml) and finally taken up in dry 1,2-dichloroethane (20 ml). $^iPr_2EtN$ (10 mmol) was added and the soln. cooled in an ice bath. Then, 2-cyanoethyl diisopropylphosphoramidochloridite (2.5 mmol) was injected dropwise with continuous stirring. After 30 min (TLC monitoring) the reaction was quenched by addition of anh. MeOH (200 µl). The mixture was diluted with CH_2Cl_2 (25 ml), the soln. washed with 10% aq. $NaHCO_3$ soln. (2×20 ml) and brine (2×20 ml), dried (Na_2SO_4), and evaporated, and the residue submitted to column chromatography (silica gel, CH_2ClCH_2Cl/AcOEt/Et_3N 5:4.5:0.5 *v*/*v*/*v*): *2-cyanoethyl 2-{2-{2-{2-bis[(4-methoxyphenyl)phenylmethoxy]-ethoxy}ethoxy}ethoxy}ethyl diisopropylphosphoramidite* (**3**) Syrupy material.

3.2. *Attachment of a Linker Molecule to the Nonmagnetic or Magnetic Support* **I** *and* **II**, *respectively.* The support to which the linker molecule was to be attached was taken in the DNA synthesizer cassette and allowed to react with **3** after appropriate washing steps as per the standard protocol [8]. After the cycle was complete, the 4,4′-dimethoxytrityl group was removed and the exposed OH group coupled to 5′-*O*-(dimethoxytrityl)thymidine 3′-(hydrogen succinate) by PPh_3/$CBrCl_3$/*N*,*N*-dimethylpyridinamine (oxidation-reduction condensation) [7] for 3 min, following the procedure reported earlier from this laboratory with minor modifications: supports **Ib** and **IIb**, resp. The loading on the supports **Ib** and **IIb** was 20 – 22 µmol/g support.

4. *Oligonucleotide Synthesis, Deprotection, and Purification*. To demonstrate the usefulness of the modified supports **Ia**, **IIa**, **Ib**, and **IIb**, a number of oligonucleotides, *viz*., d(CAC ATC AAG GAA-CAT TTC TT), d(TTT TTT TTT TTT TTT TTT TT), d(GAT GCT TTA T), d(TTT TTT TTT TTT TTT), d(TTT TTT TTT TTT TTT T), and d(GAA GTC ACC TCT TCT), were assembled at a 0.2-µmol scale (see *General*). The same oligomers were assembled on the standard polymer supports for comparison purposes.

The cleavage of oligonucleotides from the supports and removal of protecting groups from the exocyclic amino and internucleotidic phosphate groups were performed following the standard procedure [13][14]. The fully deprotected oligomers were desalted on *Sephadex G-25* and eluted with 0.1M (Et_3NH)OAc buffer, pH 7.1. Desalted oligomers were then analyzed by HPLC (*Lichrosphere RP-18* (*Merck*, Germany); mobile phases: 100 mM NH_4OAc (pH 7.1) (*A*) and MeCN (*B*)).

5. *Synthesis of Oligonucleotides on Support* **4(II)**. Support **4(II)** was subjected to the oligonucleotide synthesis at a 0.2-µmol scale, as described above for normal oligonucleotide synthesis. An oligomer, d(TTT TTT TTT TTT TTT T) was synthesized, and after the usual workup and deprotection steps [13][14], the support-bound oligomer was used for hybridization studies.

6. *Hybridization Studies of Support-Bound Oligonucleotide*. To the magnetic support **4(II)** bound oligomer d(TTT TTT TTT TTT TTT T), complementary oligomer d(AAA AAA AAA AAA AAA A) (2.04 *O.D.*) was added in phosphate buffer of pH 7.1 containing 0.5M NaCl. The suspension was heated to 60° followed by slow cooling under gentle agitation and finally kept at 4°. After 2 h, the tube was kept on a magnetic base and the supernatant recovered; then the beads were resuspended in phosphate buffer (0.5 ml). These steps were repeated twice and the supernatants pooled and subjected to absorption measurement at 254 nm. A total of 0.57 *O.D.* of oligomer was found in the supernatant. The non-oligonucleotide carrying support **4(II)** was found to retain 0.12 *O.D.* of d(AAA AAA AAA AAA AAA A) under identical conditions.

REFERENCES

[1] N. D. Sinha, in 'Protocols for Oligonucleotides and Analogs: Synthesis and Properties', Ed. S. Agrawal, Humana Press, Totowa, 1993, p. 437.
[2] P. K. Ghosh, P. Kumar, K. C. Gupta, *J. Ind. Chem. Soc*. **1998**, *75*, 206.
[3] R. T. Pon, K. K. Ogilvie, *Tetrahedron Lett*. **1984**, *25*, 713.
[4] E. Bayer, *Angew. Chem., Int. Ed.*. **1991**, *30*, 113.
[5] H. Seliger, *J. Chromatogr.* **1989**, *476*, 49.
[6] E. Birch-Hirschfeld, Z. Foldes-Papp, K.-H. Guhrs, H. Seliger, *Nucleic Acids Res.* **1994**, *22*, 1760.
[7] P. Kumar, A. K. Sharma, P. Sharma, B. S. Garg, K. C. Gupta, *Nucleosides Nucleotides* **1996**, *15*, 879.
[8] 'Pharmacia-LKB Gene Assembler Plus Manual', Upsala, Sweden.
[9] H. Bamnolker, B. Nitzen, S. Margel, S. Gura, *J. Mat. Sci. Lett*. **1997**, *16*, 1412.

[10] S. Margel, H. Bamnolker, Patent Application WO96/11054.
[11] W. Stober, A. Fink, E. Bohn, *J. Colloid Interface Sci*. **1968**, *26*, 62.
[12] P. Wikstrom, C. F. Mandenius, P. O. Larsson, *J. Chromatogr.* **1988**, *455*, 105.
[13] M. J. Gait, in 'Oligonucleotide Synthesis: A Practical Approach', Ed. M. J. Gait, IRL Press, Oxford 1984, p. 1.
[14] N. D. Sinha, J. Biernat, H. Koster, *Nucleic Acids Res.* **1984**, *12*, 4539.

Received September 1, 2000