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POLYMER SUPPORT SYNTHESIS

XV[a]. BEHAVIOUR OF NON-POROUS SURFACE-COATED SILICA GEL MICROBEADS IN OLIGONUCLEOTIDE SYNTHESIS

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SUMMARY

Non-porous silica gel microbeads of diameter 1.5 μm have been investigated as supports for oligonucleotide synthesis. In the preparation of oligothymidylates of chain length up to 150 bases, with 5′-di-*p*-anisylphenylmethyl-3′-phosphoramidite as an intermediate, the average yields per chain elongation were up to 99%. Lower overall yields were observed in the case of a support which developed a strong tendency towards aggregation after the build up of an oligonucleotide coating.

INTRODUCTION

For the automated solid-phase synthesis of oligonucleotides, non-swellable porous inorganic carriers, like silica gel or controlled-pore glass (CPG), are currently the most widely used support materials. The growth of oligonucleotide chains on such supports can be seen as the controlled build up of an organic coating on the surface of the inorganic matrix. During the preparation of very long oligonucleotide chains (more than 100 bases) on a support with an average pore diameter 750 Å, the chain growth was found to come to nearly a complete stop after the attainment of a certain length[1]. This was attributed to steric problems created by the filling up of the pores. As a consequence of this observation and of experiments on yield optimization carried out in our laboratory[2], carriers with wide pores of narrow size distribution, *e.g.*,

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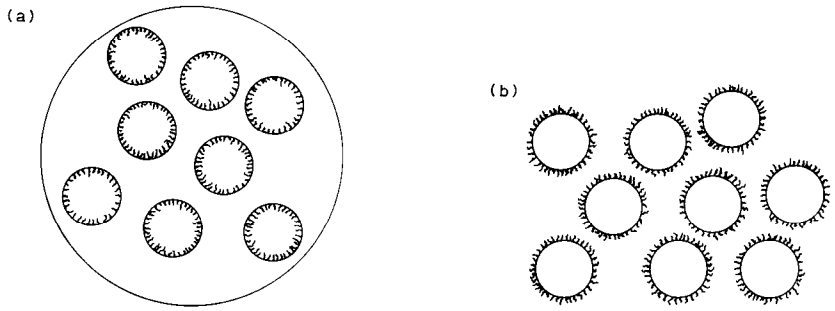

Fig. 1. Schematic representation of (a) a macroporous polymer support with internal organic coating of the pores, and (b) a non-porous microbead support of similiar surface area with external organic coating.

CPG 3000 Å, were found to be suitable for oligonucleotide preparations. Additionally, we have recently found that oligonucleotides grafted onto CPG 3000 also become available for reactions catalyzed by a variety of enzymes, such as polynucleotide kinase, DNA or RNA ligase, DNA polymerase, restriction enzymes or exonucleases[3].

Yet, if one wishes to construct very long polynucleotide chains, it can be envisaged that steric problems will arise, even with these wide pore materials. Therefore, if the accessibility of oligonucleotides inside the pores of the support is a limitation of further chain growth, an advantageous alternative should be found in non-porous microbeads, where the organic coating is on the outer surface only, the oligonucleotide chains being able to extend into the void volume of the resin bed (Fig. 1). The idea of improving the accessibility of support-bound oligonucleotide chains by using microreticular support materials was first suggested by Köster[4,5] about 16 years ago. However, the microgels described in his papers did not find application to oligonucleotide preparations. We have now exploited this concept and tested the performance of non-porous silica gel microbeads, originally prepared as stationary phases for high-performance liquid chromatography (HPLC)[6], in the automated solid-phase synthesis of oligonucleotides.

EXPERIMENTAL

Chemicals

Nucleoside phosphoramidites were products of Applied Biosystems (Foster City, CA, U.S.A.). 3-Aminopropyltriethoxysilane, benzoyl chloride, dichloroacetic acid, 4-(N,N-dimethyl)aminopyridine, acetic anhydride, 1-methylimidazole and trimethylchlorosilane were obtained from Fluka (Buchs, Switzerland). Silica gel 60 H for column chromatography as well as most solvents were obtained from E. Merck (Darmstadt, F.R.G.). Dichloromethane was obtained from Hedinger (Stuttgart, F.R.G.), 4,4'-Dimethoxytrityl chloride from Aldrich (Steinheim, F.R.G.) and [γ-^{32}P]ATP from Amersham, Buchler (Braunschweig, F.R.G.). Acetonitrile (HPLC grade) was supplied by J. T. Baker (Phillipsburgh, NJ, U.S.A.). Tetrazole was prepared from 5-aminotetrazole by a literature procedure[7]. Most solvents were purified and dried according to the literature.

Apparatus

Oligonucleotide synthesis was carried out using automated synthesizers 381 A (Applied Biosystems) and SAM I (Biosearch, San Rafael, CA, U.S.A.). The reaction column of the latter synthesizer was replaced by the Autofix System (E. Merck) as described previously[2,8]. HPLC was performed with a solvent delivery system 1400 A (Applied Biosystems). Polyacrylamide gel electrophoresis (PAGE) was carried out on an LKB Macrophor (LKB, Bromma, Sweden). For scanning electron microscopy a Philips SEM 500 (Eindhoven, The Netherlands) was used. For this purpose, the dry samples were coated by sputtering with gold or palladium.

Supports

Two species of monodisperse silica gel microbeads, prepared as described previously[9–11], were used (supports I and II). Each had a diameter of 1.5 μm (surface area 2.5 m^2/g). Support I in contrast to II had a residual content of very small pores (average diameter *ca.* 4 Å).

Loading of supports

The supports were functionalized by reaction with (1) aminopropyltriethoxysilane and (2) 5′-di-*p*-anisylphenylmethylthymidine-3′-*p*-nitrophenylsuccinate, as described in the literature[12,13]. The major part of the material was treated further with 50% aqueous methanol for 16 h at room temperature[2]. This removes more loosely bonded organic material and reduces the load to roughly one-half. However, the stability of the immobilized oligonucleotides is greatly increased.

Oligonucleotide synthesis

The oligonucleotide synthesis started with immobilized thymidine, the protected monomer for further elongation was di-*p*-anisylphenylmethylthymidine-3′-β-cyanoethoxyphosphoramidite[14]. Cycle times were approximately 10.3 min. After completion of all cycles, small samples of support material were withdrawn from the columns and their contents of target oligonucleotide estimated by spectroscopy at 498 nm in a 0.1 *M* toluenesulphonic acid solution in acetonitrile. The further work-up included standard deblocking and release from the support[12,13], followed by reversed-phase (RP) HPLC and/or PAGE.

RESULTS

The first reaction, which was performed on the surface of the silica gel microbeads, was the aminopropylation with aminopropyltriethoxysilane, followed by addition of 5′-di-*p*-anisylphenylmethylthymidine as 3′-terminal nucleoside via its *p*-nitrophenylsuccinate. The nucleoside load capacity was 2–3 μmol/g (see Table I).

Several test syntheses of oligothymidylates of chain lengths up to 100 bases were done with support I, using automated nucleotide synthesizers. Appropriately protected nucleoside phosphoramidites were used as intermediates. The reaction cycle proceeded mainly as described[12,13]. We found, however, that good results in the preparation of long oligonucleotides required a prolongation of the detritylation step. The reactions and conditions for chain elongation are listed in Table II.

The results, summarized in Table III, show that yields obtained with this sup-

TABLE I

CONDITIONS FOR AND RESULTS OF NUCLEOSIDE LOADING OF DIFFERENT MICROBEAD AND MACROPOROUS SUPPORTS

Support	*Surface area* (m^2/g)	*Capacity, NH_2*				*Capacity, DMTrdT*			
		$\mu mol/g$		$\mu mol/m^2$		$\mu mol/g$		$\mu mol/m^2$	
		a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Microbeads I	2.5	11.0	6.1	4.4	2.4	4.2	3.1	1.7	1.2
Microbeads II	2.5	8.0	3.7	3.2	1.5	3.1	2.1	1.2	0.8
CPG 1400	17.3	87.0	46.0	5.0	2.6	14.1	15.6	0.8	0.9
CPG 3007	6.9[c]	34.0	16.0	4.9	2.3	7.7	5.8	1.1	0.8
Fractosil 2500	8.0	29.0	8.0	3.6	1.0	7.5	6.5	0.9	0.8

[a] Untreated.

[b] Treated with 50% aqueous methanol.

[c] Value obtained from W. Haller (Chevy Chase, MD, U.S.A.).

TABLE II

REACTION CYCLE FOR OLIGONUCLEOTIDE CHAIN ELONGATION USING MICROBEAD SUPPORTS

Function	*Time (s)*
Acetonitrile to column	240
Phosphoramidite and tetrazole to column	27
Capping reagent to column	13
Iodine to column	17
Dichloroacetic acid to column	160

TABLE III

RESULTS OF OLIGONUCLEOTIDE SYNTHESES PERFORMED WITH MICROBEAD SUPPORTS I AND II

Oligonucleotide	*Support*	*Capacity ($\mu mol/g$)*		*Yield after completion of cycles (OD_{260})*[b]	*Average yield per condensation (%)*
		Initial	*Terminal*		
$(dT)_{30}$	Microbeads I	3.1	1.60	1.5	97.8
$(dT)_{50}$	Microbeads I	3.1	0.82	4.3	98.6
$(dT)_{100}$	Microbeads I	3.1	0.64	2.7	98.4
$(dT)_{50}$	Microbeads I[a]	2.1	1.00	4.4	97.8
$(dT)_{50}$	CPG 1400[a]	23.1	18.40	32.6	99.5
$(dT)_{50}$	Microbeads II	2.1	1.30	11.9	99.0
$(dT)_{150}$	Microbeads II	2.1	0.55	7.1	99.1

[a] Syntheses performed simultaneously in the SAM I/Autofix system[2,15]. All other syntheses were performed in the Synthesizer 381 A (Applied Biosystems).

[b] One OD_{260} unit is $1/\varepsilon$ mmol/mol of a dissolved chromophore, measured at 260 nm (ε = absorption coefficient).

port were somewhat lower than usual, being 97–98% per cycle. This was obviously independent of the batch of nucleoside phosphoramidite, since different preparations were used. In order to exclude the possibility that this yield decrease might be due to machine failure or inadequate purity of solvents or reagents, we compared support I with CPG, using a set up of two stacked cartridges in the SAM I/Autofix system described earlier[2,15]. The result of this comparison is also shown in Table III. Obviously, under stringently identical conditions, the performance of the CPG support in the preparation of $(dT)_{50}$ was significantly better, the latter giving average yields of *ca.* 99% per elongation. Incomplete yields in the case of support I were also reflected in the appearance of a significant ladder of truncated sequences on gel electrophoresis of the solid-phase products (Fig. 2) and in a tailing observed on separation of DMTr-$(dT)_{30}$ [DMTr = di-(*p*-anisyl)phenylmethyl-] in RP-HPLC[14].

Recently, a new type of microbead support has become available in which the residual content of micropores is completely eliminated (support II). A first test synthesis of $(dT)_{50}$ with this new support (Table III) indicated a significant improvement in yields. This stimulated us to prepare $(dT)_{150}$, as an example of a very long oligonucleotide. This synthesis was accomplished with an average yield per chain elongation of 99.1%, a result which compares well to previous syntheses of oligonucleotides of 140–175 bases, performed with CPG of average pore diameter 1000–2000 Å (refs. 1, 15 and 17). The products, after release from the support, were subjected to PAGE (Fig. 3). The correct lengths of the various oligothymidylates were checked by the simultaneous electrophoresis of authentic polynucleotide samples of known sizes.

In order to explore possible reasons for the relatively low yields with support I, scanning electron microscopy of supports I and II was performed prior to synthesis, after loading with di-*p*-anisylphenylmethylthymidine and after completion of an oligonucleotide synthesis. From these electron microscopic images it appears that considerable agglomeration of microbeads occurs when the organic coating builds up on support I. This agglomeration effect is not seen in the case of support II where the appearance of the material in scanning electron microscopy is similar before synthesis and after 150 cycles.

DISCUSSION

Non-porous silica gel microbeads can be surface-loaded with nucleosides according to standard procedures to produce polymer supports useful for machine-aided oligonucleotide synthesis. Loads are in the range of 2–3 μmol nucleoside per g support, *i.e.*, 0.8–1.2 μmol nucleoside per m^2 of surface area. Although this is lower than the capacity usually used in solid-phase oligonucleotide synthesis, we do not see this as a disadvantage, since the amount of material needed for biological experiments is generally very small, and both the microbeads and wide-pore CPG will produce enough material for biological experiments if yields are sufficiently high. The capacity of these microbead supports is compared in Table I with those of two species of wide-pore CPG[2]. Although the nucleoside load, measured in μmol/g support, is higher in the case of CPG supports owing to the higher specific surface area, the capacity, calculated in μmol/m^2 surface area, is the same. From this and the following results of oligonucleotide syntheses it is evident that the microbead supports, in accordance with Fig. 1, behave very much like CPG pores "turned inside out".

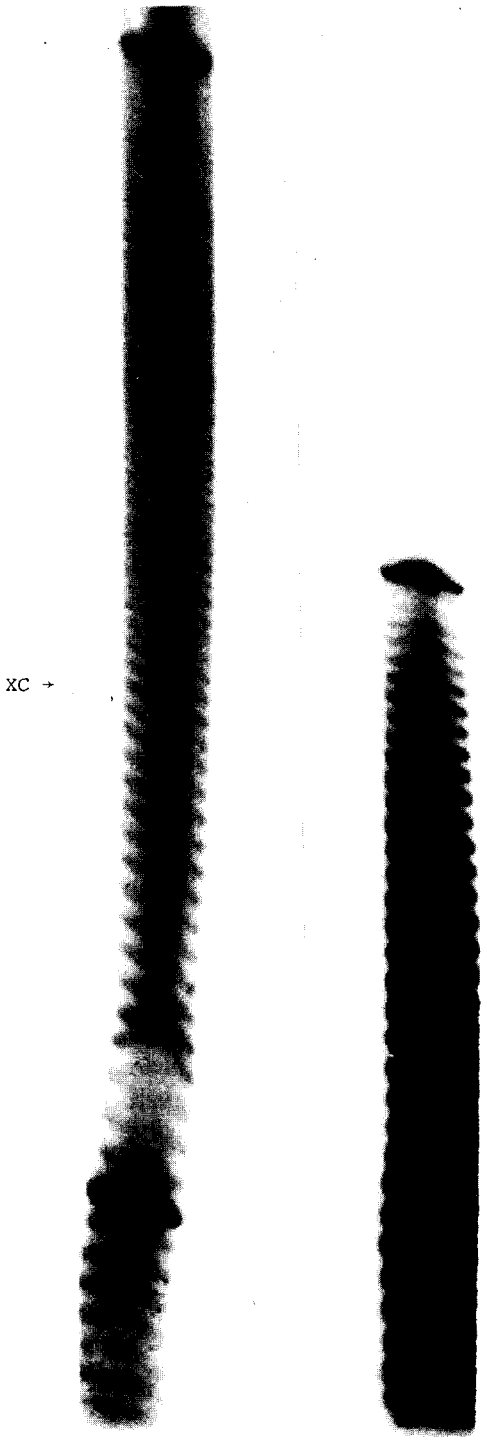

Fig. 2. Autoradiogram of the PAGE separations of crude $(dT)_{100}$ (left lane) and $(dT)_{50}$ (right lane) prepared on support I (12% denaturing polyacrylamide gel; XC = xylene cyanol marker corresponds to the length of *ca.* 48 bases[16].

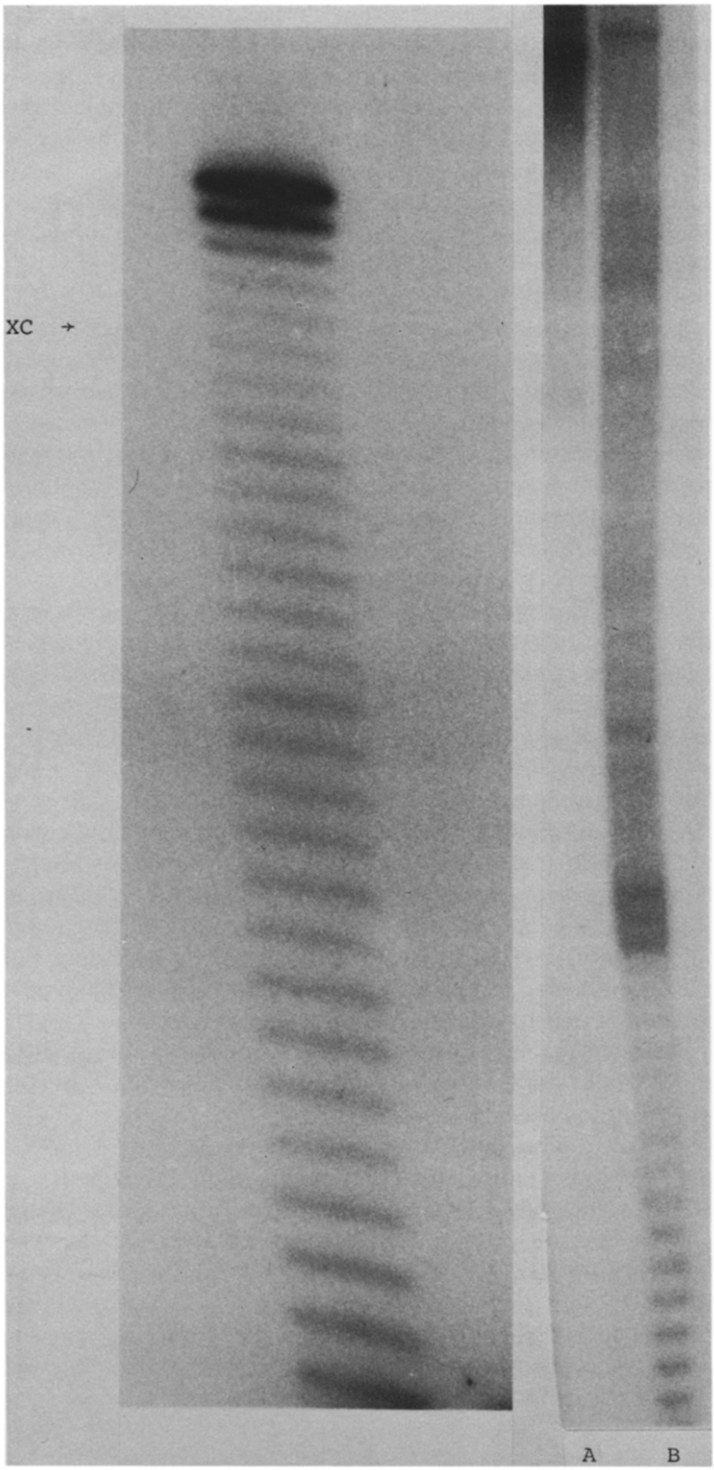

Fig. 3. Autoradiogram of the PAGE separations of crude $(dT)_{50}$ (left panel) and $(dT)_{150}$ (right panel), prepared on support II and 12% denaturing polyacrylamide gels. The left panel contains xylene cyanol (XC) as a marker. The right panel contains as a marker in lane A an authentic sequenced gene fragment of 146 bases[17]. The sample of $(dT)_{150}$ is shown in lane B.

The yields of the oligonucleotide chain elongations in the case of support II may be raised to levels comparable to those obtained with the best macroporous support systems. Thus, microbead supports are suitable for the preparation of long oligonucleotides up to 150 bases. This chain length is not the limit for chemical elongation[1], however it is near the maximum length for which a purification can be achieved by HPLC[15,17,a]. The full advantage of the microbead supports over macroporous resins is expected to be revealed on further enzyme-catalyzed lengthening of the chemically synthesized oligonucleotides. Studies along these lines are now being performed in parallel with CPG[6] and support II in our laboratory.

The slightly inferior yields with support I compared to support II and CPG supports still remain to be explained. From our studies two yield-decreasing factors may be envisaged. First, support I has an high tendency to agglomerate. Inside the chunks that are thus formed the growing oligonucleotide chains are less readily accessible to reagents and washing solutions. Secondly, Ogilvie and co-workers[18] recently observed that small pores on the surface may be opened up during repeated acid detritylations. If this observation applies also to microbead materials, one can imagine that the very small pores of support I might be widened, thus allowing water molecules to be adsorbed. This adsorbed water may then be a source of reagent hydrolysis and yield decrease.

The exposure of the surface layer of oligonucleotide to shearing during the flow of reagent and washing solutions through the column packing was also considered as a possible problem. However, although long polynucleotides are known to be susceptible to chain disruption by mechanical forces, this does not seem to be a significant yield-decreasing process in our case, particularly in view of the highly satisfactory results obtained with support II.

The handling of such microbead supports of very small particle size in automated oligonucleotide synthesizers requires some precautions, although it does not create major difficulties. Standard reaction columns as parts of commercial synthesizers, as well as the reaction cartridges we described earlier for simultaneous syntheses of multiple oligonucleotides[2,15], can be used. However, it is recommended that filters with appropriately small pore size or a double filter layer be used. We have observed that, with suitable reaction columns, the loss of support material was less than 5%, even during the 149 cycles required to prepare $(dT)_{150}$. This loss may perhaps be further reduced by an additional careful sizing of the silica gel microbeads after loading them with nucleoside. No build up of back pressure was observed with the apparatus and under the conditions described here.

The application of the microbead support system to the preparation of various oligonucleotides, containing different constituents, is presently under investigation in our laboratory.

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[a] PAGE shows a resolution of ±2 bases at a chain length of 150 bases (see ref. 1).

REFERENCES

1 J. W. Efcavitch, L. J. McBride and J. S. Eady, in K. S. Bruzik and W. Stec (Editors), *Biophosphates and Their Analogues, Synthesis, Structure, Metabolism and Activity*, Elsevier, Amsterdam, 1987, p. 65.
2 H. Seliger, A. Herold, U. Kotschi, J. Lyons and G. Schmidt, in K. S. Bruzik and W. Stec (Editors), *Biophosphates and Their Analogues, Synthesis, Structure, Metabolism and Activity*, Elsevier, Amsterdam, 1987, p. 43.
3 G. Gröger and H. Seliger, *Nucleosides Nucleotides*, 7 (1988) 773.
4 H. Köster, *Tetrahedron Lett.*, 16 (1972) 1527.
5 H. Köster and S. Geussenhainer, *Angew. Chem.*, 84 (1972) 712.
6 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 359 (1986) 61.
7 R. A. Henry and W. G. Finnegan, *J. Am. Chem. Soc.*, 76 (1954) 290.
8 H. Seliger, A. Herold, U. Kotschi, J. Lyons, G. Schmidt and F. Eisenbeiss, *Nucleosides Nucleotides*, 6 (1987) 137.
9 K. Unger, H. Giesche and J. Kinkel, *Ger. Pat. Appl.*, DE 3534143 (1985).
10 W. Stöber, A. Fink and E. Bohn, *J. Colloid Interface Sci.*, 26 (1968) 62.
11 W. Stöber and H. Flachsbart, *J. Colloid Interface Sci.*, 30 (1969) 568.
12 M. D. Matteucci and H. M. Caruthers, *J. Am. Chem. Soc.*, 103 (1981) 3185.
13 H. Seliger, S. Klein, C. K. Narang, B. Seemann-Preising, J. Eiband and N. Hauel, in H. G. Gassen and A. Lang (Editors), *Chemical and Enzymatic Synthesis of Gene Fragments*, Verlag Chemie, Weinheim, 1982, pp. 81ff.
14 N. D. Sinha, J. Biernat, J. McManus and H. Köster, *Nucleic Acids Res.*, 12 (1984) 4539.
15 H. Seliger and G. Schmidt, *J. Chromatogr.*, 397 (1987) 141.
16 T. Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning —A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
17 G. Schmidt, R. Schlenk and H. Seliger, *Nucleosides Nucleotides*, 7 (1988) 795.
18 R. T. Pon, N. Usman and K. K. Ogilvie, *Biotechniques*, 6 (1988) 768.