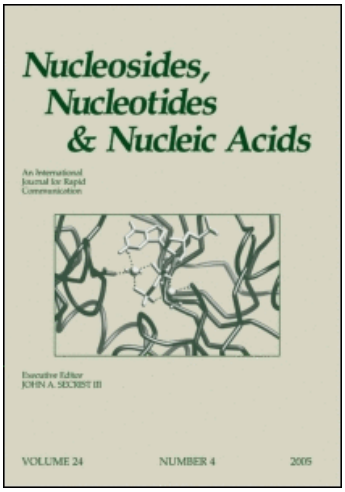

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LARGE SCALE SYNTHESIS OF OLIGORIBONUCLEOTIDES ON HIGH-LOADED POLYSTYRENE (HLP) SUPPORT

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Abstract: Large quantities of oligoribonucleotides (up to 200 μmole) were synthesized on the high-loaded polystyrene (HLP) support with phosphoramidite nucleosides and 5-ethylthio-1*H*-tetrazole as activator. The HLP support significantly reduces solvent and reagent consumption. RNA synthesized on HLP support at large scale was shown to have full biological activity by a comparative ribozyme-substrate assay.

Chemical synthesis is the method of choice to obtain large quantities of RNA fragments suitable for therapeutic and biomedical purposes, structural and physical analysis and for RNA containing modified nucleotides. Multiple grams of synthetic RNA are required for therapeutic based investigations currently in clinical trials targeted against HIV, viral, venereal and other diseases. The application of ribozymes as therapeutics, specifically as anti-HIV agents, their emergence as a cure for a myriad of plant diseases, ribozyme engineering, and other related studies, offer exciting possibilities for large scale RNA synthesis.[1-7] Also, efficient synthesis and purification of synthetic RNA are necessary for X-ray crystallography, NMR and other physical studies. Full activity of synthetic RNA, comparable to that derived from transcription methods, depends on efficient synthesis and purification methods.[8-10]

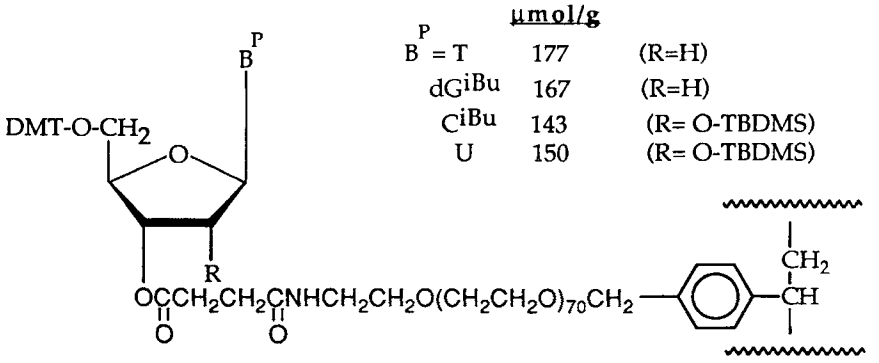
Ideally, large scale synthesis requires nucleoside loading levels greater than 100 μmole/g. Typically, RNA has been synthesized on either CPG or high-cross linked polystyrene supports at scales of 10 μmole or less.[11-13] The loading on these supports is in the 15-40 μmole/g range. When these low

loaded supports are used in large scale synthesis, reagent and solvent consumption is excessive and synthesis cost is high. At loading levels greater than 40 μmole/g, the traditional supports lose their efficiency (due to vigorous agitation, as well as mechanical instability at large scale syntheses). Also, since most commercial automated synthesizers are generally designed for smaller scale oligoribonucleotide syntheses, the scale-up causes various non-linear effects. To address these limitations we have applied our High-Loaded Polystyrene (HLP) supports which we have reported previously for large scale DNA synthesis[14] for large scale oligoribonucleotide syntheses. In this paper, we report successful synthesis of RNA up to 200 μmole scale on both the DNA and RNA HLP supports. The purity and yield of the product is comparable to the one synthesized on low loaded CPG or high-cross linked polystyrene supports, with significant reductions in reagent and solvent consumption.

RESULTS AND DISCUSSION

The HLP supports were prepared from 1% cross-linked polystyrene (co-polymer of styrene with 1% divinylbenzene) beads which were grafted with ethylene oxide to form a polystyrene/polyethylene copolymer.[15] Average chain length of the ethylene oxide is 70 units. The polyethyleneoxy moiety of the co-polymer represents 70-80% of the total mass and dominates the physical and chemical behavior such as hydrophilic nature and uniform swelling. This physical composition allows the supports to swell to a uniform volume in very polar solvents (H_2O, alcohol) as well as aprotic organic solvents (DCM, DMF, THF, CH_3CN). Termination of the polyethylene glycol functionality with a primary amine loaded in the range of 200-220 μmole/g,[14] addition of succinyl nucleosides and acetylation (capping) of the unreacted amines provides the HLP supports. The loading of these supports was obtained in the 140-180 μmole/g range (FIG. 1).

HLP supports conduct efficient and rapid oligoribonucleotide syntheses at scales ranging from 25 μmole to 200 μmole on an Applied Biosystems 390Z DNA/RNA synthesizer. The average time per cycle (detritylation, coupling, capping and oxidation) at 200 μmole scale was optimized at about 74 minutes. For most RNA sequences, 8 equivalents of phosphoramidite per coupling



P: base protecting group; TBDMS: t-butyldimethylsilyl.

FIG. 1. Structure and loading of the HLP supports used in large scale RNA synthesis.

and a 30 minute coupling time result in high quality synthesis. Complete coupling on HLP is slower than CPG or high cross-linked polystyrene support due to the diffusion limits reagents within the gel matrix. Activation of phosphoramidites was effected with a 0.75 M solution of 5-ethylthio-1*H*-tetrazole in acetonitrile.[16] A 0.1 M solution of phosphoramidites in acetonitrile was used for all the syntheses except at the 200 µmole scale where a 0.2 M solution was used to keep reagent volume below the volume of the reaction vessel. From this synthesis, we obtained in excess of 20,000 ODs (500 mg) of the oligoribonucleotide (sequence 1, TABLE 1) after cleavage. Cleavage was performed with 100 mL of 3 : 1 ammonium hydroxide (30%) : ethanol. The quality of the syntheses on the 390Z was markedly enhanced by a DMF wash before and after the detritylation with 2% dichloroacetic acid in dichloromethane. DMF was found to very effective in removing traces of iodine and acids still retained on the support after acetonitrile washes. The longest oligoribonucleotide synthesized on HLP support is a 32 nt hammerhead ribozyme (sequence 5). Kinetic and thermodynamic analyses were performed on this ribozyme and the information has elucidated the rate-determining step in hammerhead ribozyme-catalyzed reactions.[17, 18]

The overall reagent consumption per cycle for HLP syntheses is much less than that for a high-cross linked polystyrene support[19] (TABLE 2). For a 25

TABLE 1. Synthesis data of RNA sequences synthesized on ABI Models 394 and 390 Z.

Sequence	Scale (μmole)	3' Loading	ASWY (%)	Crude Yield
1	25	177 μmol/g	98.2[A]	75 mg[G]
1	50	177 μmol/g	98.3	140 mg[G]
1	100	177 μmol/g	98.8[B]	260 mg[G]
2	5	20 μmol/g	98.1[C]	12.5 mg
2	200	150 μmol/g	99.0[D]	544 mg
3	25	16.6 μmol/g	99.2[E]	52.5 mg
4	25	167 μmol/g	98.2	49 mg[G]
5	25	17.7 μmol/g	97.9	62.5 mg
5	25	143 μmol/g	97.5	51 mg[G]
6	25	143 μmol/g	98.4	70 mg[G]
6	25	17.7 μmol/g	96.6[F]	53 mg

Sequence 1: 5'> UGCCCGUCUGUUGUG **T** <3'
Sequence 2: 5'> UGCCCGUCUGUUGUG **U** <3'[20]
Sequence 3: 5'> CUCAAUAAAGCUUGCCUUGA **G** <3'[21]
Sequence 4: 5'> CUCAAUAAAGCUUGCCUUGA **dG** <3'
Sequence 5: 5'> CGGGGCUGAUGAGGCCGAAAGGCCGAAACGG **C** < 3'[17, 18]
Sequence 6: 5'> GAGCCUGGGAGCU **C** <3'[16]

ASWY : Final average stepwise yield by trityl cation absorbance assay.
A : ASWY was 97.7% when a 20 minute coupling time was used.
B : 8.9 fold excess phosphoramidite, 60 fold excess 5-ethylthio-1*H*-tetrazole.
C : Synthesized on 394, using the 10 μmol RNA cycle and a 0.075 M solution of phosphoramidites.
D : 7.2 fold excess phosphoramidite, 53 fold excess 5-ethylthio-1*H*-tetrazole.
E : 8 fold excess phosphoramidite, 15 minute coupling.
F : A 0.5 M solution of 5-ethylthio-1*H*-tetrazole in acetonitrile was used for activation.
G: Estimated total yield (crude) based on partial cleavage.

μmole RNA synthesis the savings is about 135 mL/cycle. With increasing scales and for longer oligoribonucleotides, these savings translate into significant reductions in reagent consumption and synthesis costs, making HLP an ideal support for large scale oligoribonucleotide synthesis.

In an effort to demonstrate that the RNA synthesized on the HLP support is biologically active we synthesized an RNA substrate on HLP support (sequence 2) and compared its cleavage to the one synthesized on the high-

TABLE 2: Comparison of reagent consumption per cycle for RNA synthesis on HLP and high-cross linked polystyrene supports (PS) at 25 μmole scale on the ABI 390Z Large scale DNA/RNA synthesizer.

	HLP	**PS**
Loading	*150-170 μmol/g*	*15-40 μmol/g*
Tetrazole/Acetonitrile	2.8 mL	3.2 mL
Phosphoramidites (0.1 M)	2.0 mL	2.0 mL
Dichloroacetic acid/CH_2Cl_2	35.4 mL	60.8mL
Acetic Anhydride/Lutidine/THF	2.2 mL	2.7 mL
1-Methyl Imidazole/THF	2.2 mL	2.7 mL
Iodine/Pyridine/THF/Water	5.3 mL	5.3 mL
Acetonitrile	116 mL	203 mL
DMF	52 mL	73 mL
DCM	25.2 mL	51.1 mL
Volume / Cycle	**240** mL	**375** mL

cross linked polystyrene support. Cleavage was performed with a previously synthesized ribozyme.[16,20] The 16 nt substrate (sequence 2) was synthesized on the the RNA HLP support (150 μmole/g; FIG. 5) and also on the high cross-linked polystyrene support (20 μmole/g) at 5 μmole scale (ABI Model 394; FIG. 2). Catalytic activity was determined at 37 °C by combining substrate with ribozyme and analyzing the products by gel electrophoresis. The results show cleavage levels of the substrates by the ribozyme to be roughly equivalent and show full biological activity of the chemically synthesized oligoribonucleotides (FIG. 9). Chemically synthesized substrates and ribozymes on high cross-linked polystryrene support have previously been shown to have activity comparable to T7 RNA polymerase transcribed RNA.[13,16]

In summary, we have developed a highly efficient support which enables the large scale production of oligoribonucleotides. The product quality of RNA synthesized on HLP support is comparable to RNA obtained from low-loaded, high-cross linked polystyrene or CPG supports with substantial reductions in solvent consumption and waste. These and other continuing developments and improvements should enable more exciting investigations in the RNA field.

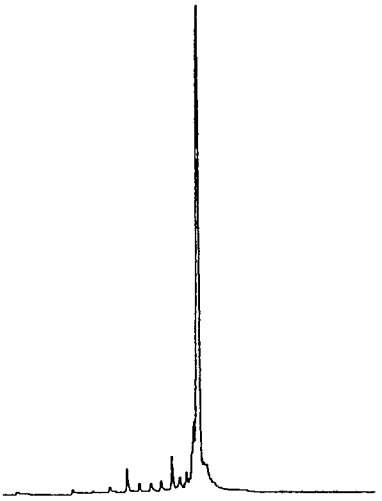


FIG.2. Anion-exchange HPLC analysis of sequence 2 synthesized at 5 μmol scale.

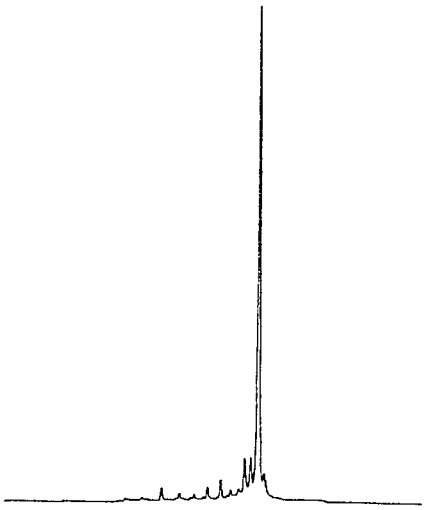


FIG.3. Anion-exchange HPLC analysis of sequence 1 synthesized at 50 μmol scale.

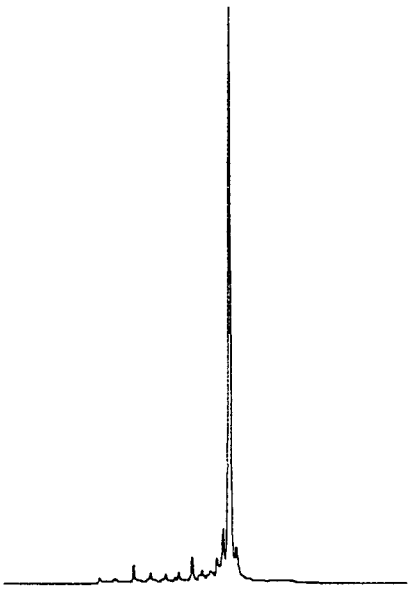


FIG.4. Anion-exchange HPLC analysis of sequence 1 synthesized at 100 μmol scale.

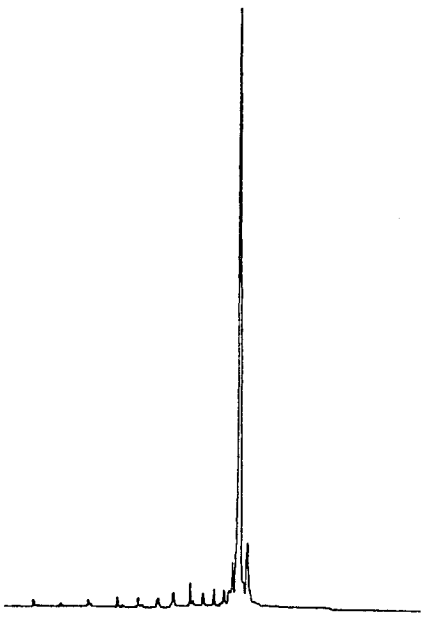


FIG.5. Anion-exchange HPLC analysis of sequence 2 synthesized at 200 μmol scale.

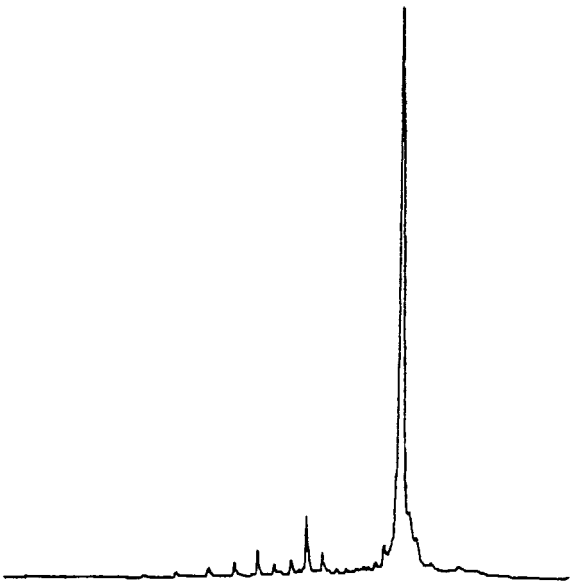


FIG.6. Anion-exchange HPLC analysis (at 50 °C) of sequence 4 synthesized at 25 μmol scale.

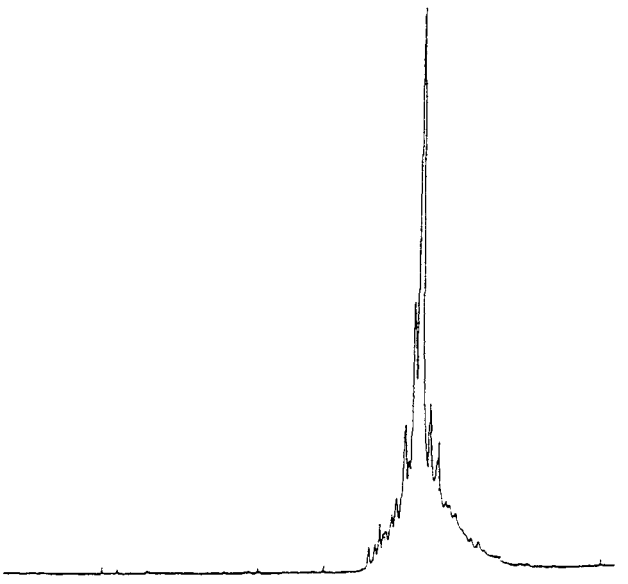


FIG.7. MicroGel Capillary electrophoresis analysis of sequence 5 synthesized at 25 μmol scale.

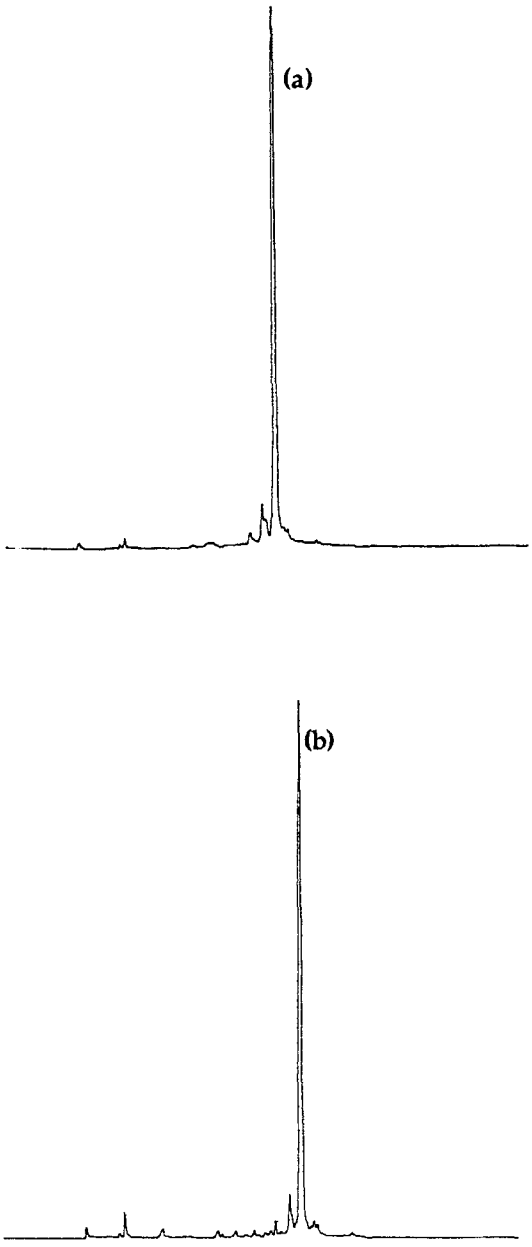


FIG.8. Anion-exchange HPLC analysis of sequence 6 synthesized at 25 μmol scale and analyzed at 25 °C (a) and at 50 °C (b).

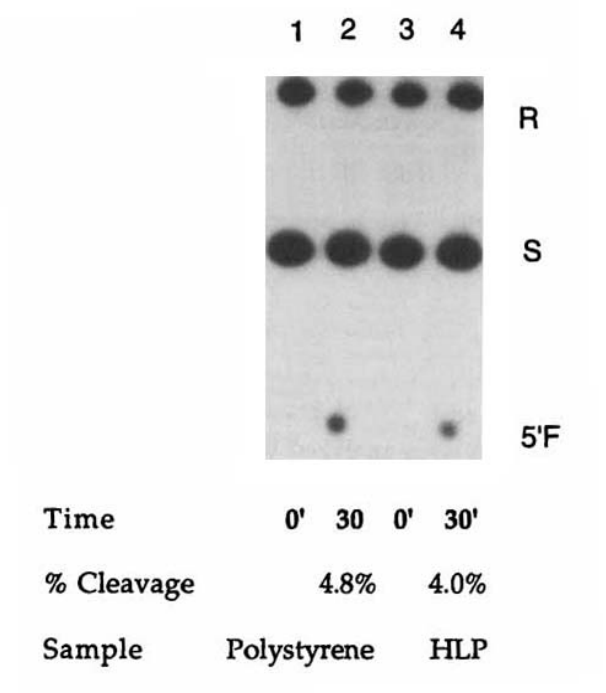


FIG. 9. Cleavage of chemically synthesized HIV substrate (sequence 2) by the HIV specific hairpin ribozyme. Lanes 1,2 (Substrate synthesized on high-cross linked polystyrene support): Cleavage was for 0 and 30 min; Lanes 3,4 (Substrate synthesized on HLP support): Cleavage for 0 and 30 min. The abbreviations R, S and 5'F represent ribozyme, substrate and the 5' cleavage fragments respectively. No 3' fragment is seen as only the 5' end was labelled.

EXPERIMENTAL

Triethylamine trihydrofluoride {$Et_3N(HF)_3$}, 1-propanol, 1-butanol (HPLC grade), sodium acetate and lithium perchlorate were purchased from Aldrich. The RNA phosphoramidites and supports (Applied Biosystems) used in synthesis employ 6-N-phenoxyacetyl protection for the exocyclic amine of adenosine, 2-N-dimethyl(dimethylamino)methylene for guanosine and 4-N-isobutyryl for cytidine.[22] The syntheses were carried out using a 0.75 M solution of 5-ethylthio-1*H*-tetrazole (Parish Chemical Company, Orem, Utah) in acetonitrile as an activator. Ancillary reagents for automated syntheses were also obtained from Applied Biosystems. Concentrated ammonium hydroxide (28-30%) was purchased from Baker.

GENERAL PROCEDURES

The oligoribonucleotides were synthesized on Applied Biosystems Models 390Z and 394 DNA/RNA synthesizers. For oligoribonucleotides synthesized on Model 390Z, cleavage from the support was conducted off the instrument (3-4 h) at ambient temperature in ammonium hydroxide (30%) : ethanol (3:1). Removal of the exocyclic amine protecting groups was complete within 4-5 hours in ammonium hydroxide (30%) : ethanol (3:1) at 55 °C. The silyl protecting groups were removed with a neat solution of triethylamine trihydrofluoride (20-24 hours, room temperature) and the crude RNA precipitated by the addition of 1-butanol.[16,22] The oligoribonucleotides (synthesized trityl-off) were analyzed (crude) by anion-exchange HPLC (A_{260}) at 50 °C (NucleoPac PA-100 column, 250 x 4 mm, Dionex Corporation) by the conditions described below. MicroGel capillary electrophoresis (Applied Biosystems Model 270 HT) was used for analysis of sequence 5.

Anion-Exchange HPLC Conditions:

Solvent A : 20 mM $LiClO_4$ + 20 mM NaOAc in H_2O: CH_3CN (9:1) (pH 6.5);
Solvent B : 600 mM $LiClO_4$ + 20 mM NaOAc in H_2O: CH_3CN (9:1) (pH 6.5);
Flow Rate : 1.0 mL/min. (Gradient: 0 to 70% B in 40 min).

Chemically synthesized substrates were passed through a spin column to desalt, kinased with 5'^{32}P γ labeled ATP and purified by polyacrylamide gel electrophoresis. HIV specific ribozyme was prepared by T7 polymerase transcription using α-P^{32} CTP as previously described.[20] Labeled substrate (20 nM) and ribozyme (8 nM) were combined for cleavage in 12 mM $MgCl_2$, 40 mM Tris (pH 7.5) and 2 mM spermidine for 30 min at 37 °C.[23,24]

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