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Large Scale Synthesis of Oligonucleotides via Phosphoramidite Nucleosides and a High-loaded Polystyrene Support

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Abstract: Large scale quantities of phosphodiester and phosphorothioate oligonucleotides are synthesized on an aminopolyethyleneglycol derivatized polystyrene (TentaGel) support. Efficient, automated synthesis up to 1 mmole scale is achieved with phosphoramidite nucleoside monomers and 5-ethylthiotetrazole activator.

Relatively large quantities of synthetic oligonucleotides are required for antisense gene expression control, diagnostic assays, and physical studies of nucleic acids. Antisense oligonucleotides, potential therapeutic agents against cancer and viral diseases, are progressing into clinical studies which require multiple gram quantities and synthesis at the near millimole scale.[1] This need is in contrast to the sub-micromole scale required for DNA sequencing and PCR oligonucleotide primers.[2] Synthesis scale-up elicits many non-linear effects due to the heterogeneous chemistry on the solid phase, immobilized support and automated synthesizers designed for smaller scales.[3]

Low-crosslink aminomethyl polystyrene beads, used successfully in solid phase peptide synthesis[4] have performed poorly in DNA synthesis.[5] While these beads can be loaded at appropriately high levels (>100 μmoles nucleoside / gm), the acetonitrile solvent used in phosphoramidite chemistry does not allow proper swelling and accessibility to reagents, resulting in slow reaction kinetics.A high-loaded solid support that operates efficiently with phosphoramidite nucleoside chemistry is very desirable.[6] Controlled pore glass (CPG) supports lose efficiency at nucleoside loading above about 40 μmole/gm and show mechanical instability under the vigorous mixing and stirring conditions of large scale synthesis.[7]

To improve the reaction kinetics and solubility properties of low cross link polystyrene, 1% DVB polystyrene beads (80 μm diameter) were grafted with ethylene oxide under carefully controlled conditions to give a polystyrene polyethylene glycol graft copolymer. The average chain length of the PEG linker is about 70 ethylene oxide units, with an average molecular mass of 3000 Dalton, constituting about 75% of the mass of the support.[8] The support-bound oligonucleotide is well solvated in acetonitrile due to the acetonitrile-soluble polyethyleneoxy linker, which dominates the physical and chemical behavior of the support 1 (Figure 1). Due to the hydrophilic and hydrophobic properties of PEG, the resin swells well in very polar solvents (e.g. water, alcohol), as well as aprotic organic solvents (e.g. CH_2Cl_2, DMF, MeCN, toluene, THF). The swelling per volume is constant (2.5 - 3.5 ml/gm resin), independent of the applied solvent.[9] Termination of the polyethylene glycol with a variety of functionalities has been reported.[10] Primary amino functionality can be attained at 200 μmoles / gm as TentaGel NH_2 1. Loading of 3' succinyl nucleosides 2 is followed by

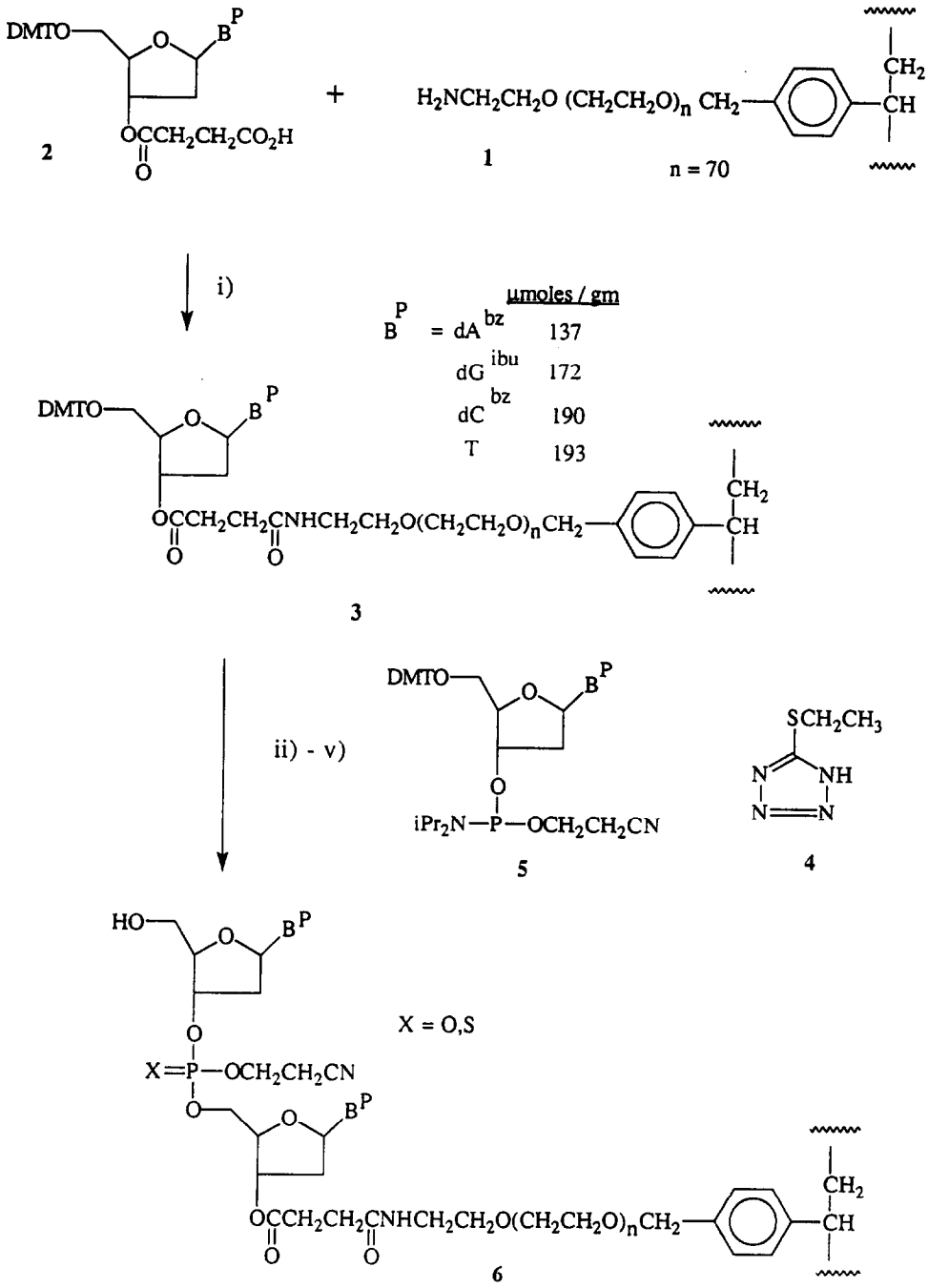

Figure 1. i) DCC, DMF, ii) 4, 5, CH_3CN, iii) Ac_2O, 1-methylimidazole, lutidine, THF, iv) I_2, pyr, THF, H_2O or $(Et_2NCS_2)_2$, CH_3CN, v) Cl_2CHCO_2H, CH_2Cl_2

acetylation capping of unreacted amines to prepare the supports **3** ready for automated, phosphoramidite nucleoside synthesis.

Supports **3**, loaded between 137-193 μmoles per gram, conduct rapid and efficient large scale oligonucleotide synthesis.[11] The synthesis cycle (45 minutes, 200 μmole scale) of four reactions (coupling, capping, oxidation, and detritylation) and washing of the support was optimized on the Model 390Z synthesizer.[12] It was found that a previously reported activator, 5-ethylthio-1-H-tetrazole **4**[13], gave about 1-2% higher average yield per cycle than 5-H-tetrazole, resulting in higher yields and purity of the oligonucleotide product. This effect was especially pronounced at the larger scales of DNA synthesis, and at lower phosphoramidite nucleoside excess. The greater acidity[14] and solubility (>2.0M in acetonitrile) may account for the enhanced performance of **4**. The phosphoramidite nucleosides **5** (0.1M) and **4** (0.6M) or 5-H tetrazole (0.5M) in acetonitrile are delivered sequentially to a pre-activation vessel and mixed by argon bubbling for thirty seconds. The tetrazolyl phosphoramidite intermediate is delivered to the growing oligonucleotide **6** in the reaction vessel (volume = 35-190 ml). Thorough mixing and agitation of the support in the reaction vessel is achieved by a vortexing mechanism.

Sequence	Scale (μmole)	ASWY (%)	Yield
A	25	96.2	0.125 gm
B	50	97.2	0.155 gm
B	100	96.5	0.337 gm
B	200	97.7	0.627 gm
B	400	98.1	1.353 gm
C	600	97.2	2.188 gm
D	600	97.7	2.204 gm
E	800	97.0	2.765 gm
E	1 mmole	96.6	3.666 gm

A: 5' AGG GCC GAG CGC AGA AGT GGT CCT GCA ACT TTA T 3' 34mer phosphodiester
B: 5' TCA CAG TCT GAT CTC GAT 3' 18mer phosphodiester
C: 5' GCG TCA CAG TCT GAT TTC GAC 3' 21mer phosphorothioate
D: 5' CGG TCA CAG TCT GAT CTC GAC 3' 21mer phosphodiester
E: 5' TCA CAG TCT GAT CTC GAC 3' 18mer phosphodiester

ASWY: final average stepwise yield by trityl cation absorbance assay
Yield: crude yield from absorbance, 1 odu (optical density unit) = 33 μgm

Figure 2.

Multigram quantities of synthetic oligonucleotides may now be prepared in a single, automated operation. A series of oligonucleotides were prepared with **3** at scales ranging from 25 μmoles to 1 mmole of starting 3' nucleoside (Figure 2).[15] The synthesis cycle and reagents were essentially the same as smaller scales (e.g. 0.2 μmole), with the exception of detritylation to remove the 5' DMT. It was found that syntheses with **3** benefitted from a dimethylformamide wash before and after detritylation with 5% dichloroacetic acid in dichloromethane. Phosphorothioate oligonucleotide analogs, e.g. (C), are efficiently sulfurized each cycle with tetraethylthiuram disulfide (0.5M TETD in CH_3CN).[16] The crude products were analyzed by MicroGel capillary electrophoresis[17] after cleavage from the support and deprotection in concentrated ammonia, 8 hr, 55°C (Figure 3). In each example in Figure 3, the product was at least 70% of the total integrated product area. TentaGel nucleoside **3** fulfills the criterion for an efficient, high-loaded support for large scale oligonucleotide synthesis.

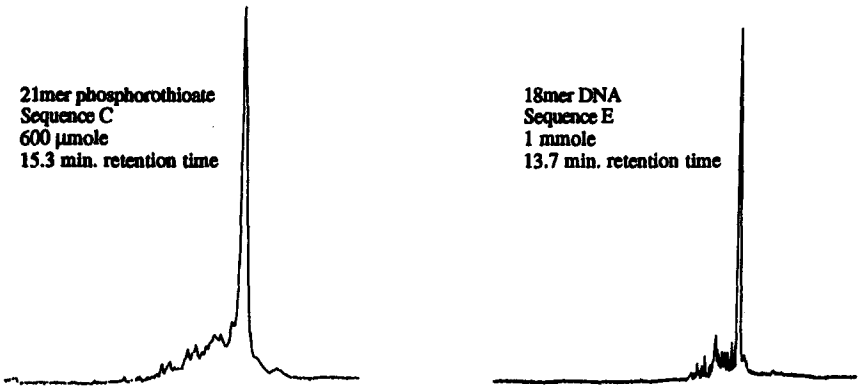

Figure 3. MicroGel capillary electrophoresis analysis of crude oligonucleotides

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