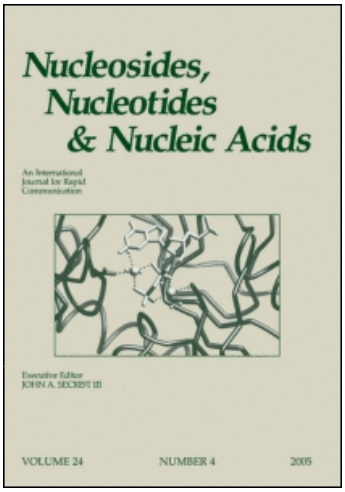

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IMPROVED PROCEDURE FOR THE REDUCTION OF N - 1 CONTENT IN SYNTHETIC OLIGONUCLEOTIDES

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Abstract: By incorporating a "capping step" at the start of an oligonucleotide synthesis ("pre-cap") and following a "SUP" work-up protocol with ammonium hydroxide, an overall improvement is observed in the quality of oligonucleotides synthesized on a large scale on controlled pore glass support (CPG). Rationalization of these results is provided.

For therapeutic development of antisense[1] and triplex strategies[2] for inhibition of protein synthesis, the availability of high purity oligonucleotides having batch-to-batch consistency in quality is crucial. Presently, the large-scale synthesis of oligonucleotides (10 μmol - 1 mmol scale) is done on a solid-phase matrix e.g., CPG, using phosphoramidite chemistry.[3] After synthesis, the crude oligonucleotide is isolated by hydrolytic cleavage from the CPG and complete deprotection of its phosphate (i.e., cyanoethyl) and base protecting groups (e.g., benzoyl and isobutyryl) using 28-30% aqueous NH_4OH and purified by reverse phase HPLC on C-18 column.[4a] Two different isolation protocols are commonly employed: (a) the CPG-linked oligonucleotide is treated with NH_4OH at ambient temperature for 1-2 hours, followed by heating the supernatant ammonia solution in a closed container for 8-16 hours at 55 °C ("SUP" protocol) *or* (b) the CPG is directly heated with NH_4OH in a closed container for 8-16 hours at 55 °C ("CPG" protocol). Our earlier work, on the analysis of various oligonucleotides synthesized and purified by standard procedures, had revealed the presence of significant (1-10%) truncated sequences corresponding to "N - 1" content.[5] Thus routine preparative HPLC purification does not eliminate the N - 1 and N - 2 sequences. Sequence analysis of the N - 1 oligonucleotides isolated by

preparative gel electrophoresis, revealed a complex heterogeneous population of oligomers (unpublished observations). Several factors such as coupling efficiency during synthesis, quality of CPG-bound nucleoside, quality of reagents and nucleoside phosphoramidites, cycles and programs used in synthesis, work-up and purification protocols etc., contribute towards the content and the heterogenous nature of the N - 1 oligonucleotide in a purified oligonucleotide.

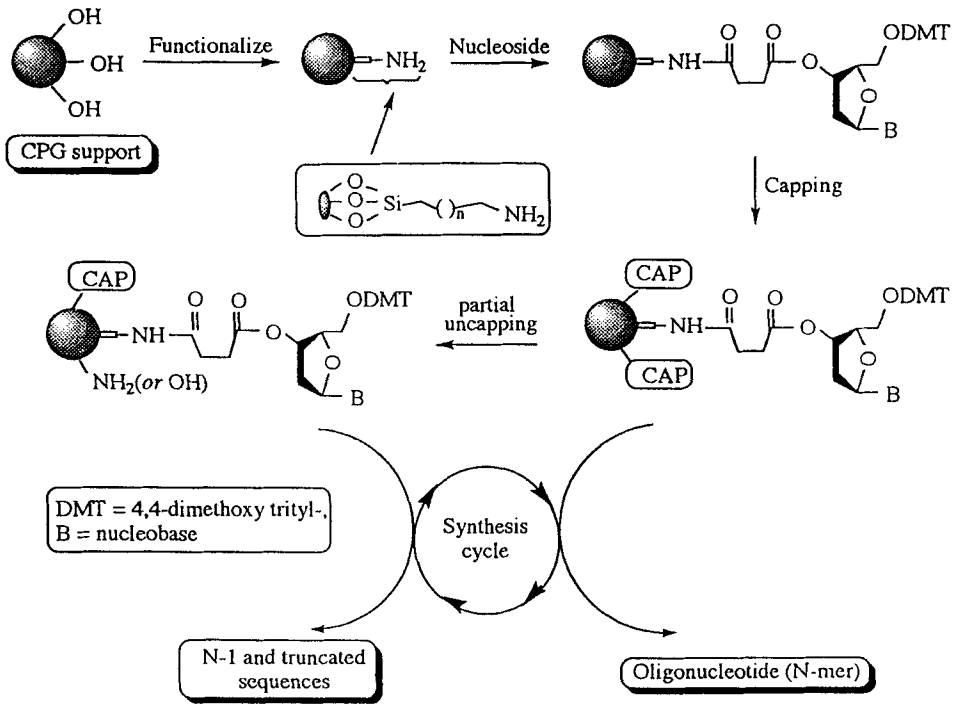
A capping step, after coupling or oxidation of a newly formed internucleotidic phosphite linkage in a synthesis cycle, is known to prevent side reactions such as branching and extensions and allow for truncation of failure sequences to aid in product purification.[4a] Capping of unreacted sites on the CPG matrix is also an important step in the preparation of CPG-linked nucleoside (Scheme 1).[4b]

Incomplete capping of CPG during preparation of support-bound nucleoside or uncapping of its nucleophilic sites upon extended storage or exposure to various reagents during synthesis cycle, could conceivably affect both the quality and yield of the desired oligonucleotide. Thus e.g., incompletely capped CPG when used in synthesis can yield an oligonucleotide with a greater proportion of N - 1 and other "truncated sequences" (Scheme 1). Quite clearly the quality of the final oligonucleotide is dependent on the quality of the initial CPG.[4b] It occurred to us that incorporation of a pre-cap step in the synthesis protocol may be beneficial in improving the quality of a synthetic oligonucleotide.

Herein we report our studies on the N - 1 and N - 2 contents of various oligonucleotides, synthesized under standard conditions, as a function of initial loading of nucleoside on CPG, effect of "pre-cap" step in the synthesis (using standard capping program) and SUP vs CPG work-up procedures.

RESULTS AND DISCUSSION:

We have routinely used ion-exchange chromatography to separate and quantitate N, N - 1 and N - 2 peaks in a 5-mer oligonucleotide. Therefore as a model study - to evaluate the effect of pre-cap, CPG loadings, and SUP vs CPG work protocols on the quality of a synthesized oligonucleotide - we prepared pentadeoxythymidine phosphoric diester (PO) oligonucleotide (dT_5). The syntheses were done using standard 1 μmol program. To study the effect of pre-cap on the synthesis, the CPG-bound nucleoside was first subjected to two cap cycles using a standard capping program, and synthesis was then started.



SCHEME 1: Outline of the origin of CPG-derived contributions to N-1 and truncated sequences in an oligonucleotide.

Control experiments were performed the usual way i.e, without pre-cap in the synthesis. After the synthesis, each of the CPG-bound oligonucleotides was dried and divided into two lots and subjected to either SUP or CPG protocol. The isolated crude homopolymer was then analyzed by ion-exchange chromatography. As seen in FIG. 1a, b, a significant reduction is observed in the N-1 content of the 5-mer with incorporation of an initial pre-cap step, in the synthesis, using either a SUP or CPG protocol. Best results however were obtained by having a pre-cap step in the synthesis followed by SUP work-up protocol. Similar results were obtained when the N - 2 content was evaluated (data not shown). Interestingly, the differences in the N - 1 and N - 2 content between oligonucleotides prepared by the above protocols, was less discernible when using CPG with a higher loading i.e., 100 μmol/g. This observation is consistent with the hypothesis that there would be lesser uncapped amino groups (see SCHEME 1) in a CPG with higher loading and those that remain

uncapped would be sterically inaccessible to electrophilic reagents during synthesis. Thus one would expect lesser "CPG-derived" contributions to the N - 1 and N - 2 content of the oligonucleotide prepared from CPG of higher loading.

We also ascertained if the N - 1 and N - 2 content of oligonucleotides using a SUP protocol is dependent on the duration of NH_4OH treatment. Thus the homopolymer $d\Gamma_5$ was synthesized with and without pre-cap. Aliquots of the CPG-linked $d\Gamma_5$ was exposed to NH_4OH for 30, 60 and 120 minutes. The supernatant was then removed and heated at 50-55 °C for 8 hours to complete the deprotection of the base and phosphate protecting groups. Upon analysis by ion-exchange chromatography, it was observed that the *difference* in N-1 content, with and without pre-cap, was 26% at 30 min and 20% at 2h, where as the difference in N - 2 content was 23% and 14% respectively (data not shown). It therefore appears that longer duration of NH_4OH treatment at ambient temperature releases more of the support-bound N - 1 and other associated truncated sequences. Complete release of support-bound oligonucleotide synthesized on a 1-10 μmol scale could be achieved by treatment with 28% NH_4OH for one hour (monitored by HPLC). Apparently, longer periods of ammonia treatment of the CPG releases more of the truncated sequences still attached to the support. A rationalization of these observations is possible. (*vide infra*).

Based upon these initial studies, we carried out the synthesis of a 25-mer phosphoric diester (PO) oligonucleotide (10 μmol scale) with and without pre-cap and using SUP and CPG protocols. Following synthesis and deprotection, the oligonucleotide was purified by reverse-phase HPLC, detritylated and dialyzed. Upon analysis by capillary electrophoresis (CE) a decrease in N - 1 and N - 2 content was noted in the oligonucleotide which received the pre-cap and SUP protocol (data not shown).

We next synthesized three batches of 25-mer phosphorothioate oligonucleotides on a 1 *mmol* scale. Aliquots of the CPG was removed and subjected to SUP and CPG protocols. The crude oligonucleotides thus obtained were analyzed by CE. Although base line separation of the N - 1 peak from the N peak could not be achieved, estimation of the integrated area under the peak revealed a reduction in N - 1 content of up to 30% in the aliquots which received the SUP protocol (data not shown).

We also carried out the synthesis of three phosphorothioate oligonucleoside constructs on a 1 mmol scale using pre-cap and SUP work up protocols.

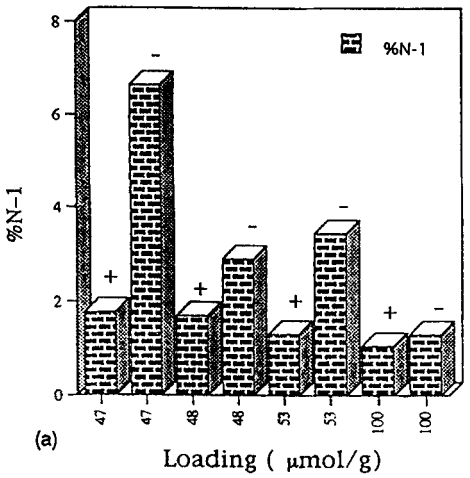


FIG.1a . %N-1 content in a 5-mer isolated by SUP protocol following pre-cap (+) or no pre-cap (-) during synthesis using different CPG loadings.

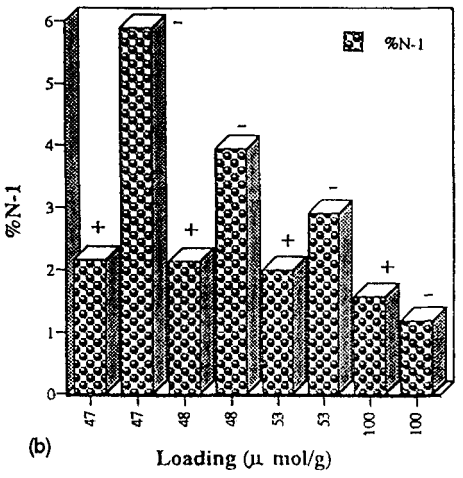


FIG.1b . %N-1 content in a 5-mer isolated by CPG protocol following pre-cap (+) or no pre-cap (-) during synthesis using different CPG loadings.

Following HPLC purification and desalting, the oligonucleotides were examined by CE which revealed a significant reduction in N - 1 content and truncated sequences in the oligonucleotide which received pre-cap treatment of the CPG (FIGs. 2).

It is possible to rationalize these observations although at present there is no direct mechanistic studies available to support them. The CPG is a silica matrix, functionalized with a long-chain alkylamine (lcaa) group. The initial nucleoside is linked *via* its 3'-OH group to the CPG through a succinyl linkage. The free nucleophilic groups on the CPG (-NH_2 and the OH groups) are then capped using a capping reagent (acetic anhydride/N-methyl imidazole/pyridine/tetrahydrofuran) (Scheme 1). In the event that the capping reaction was incomplete, the CPG will have "naked" lcaa and hydroxy groups. Alternatively, upon extended storage of the CPG-linked nucleoside, it is conceivable that the acetylated groups in the CPG (capped CPG) matrix could participate in a trityl exchange reaction with the nucleoside through neighbouring group participation, resulting in a tritylated amino group and a capped/uncapped nucleoside (FIG. 3).

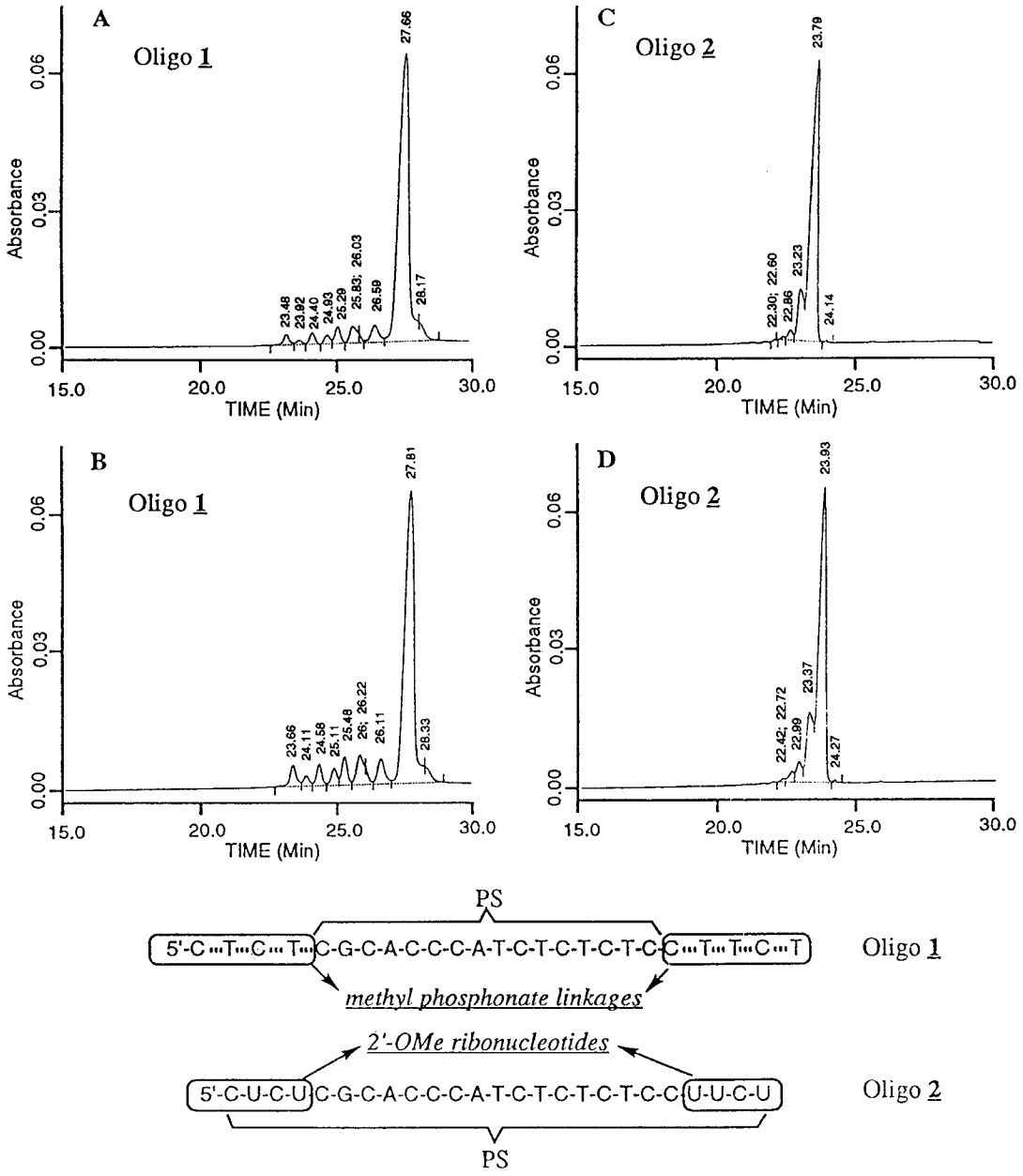
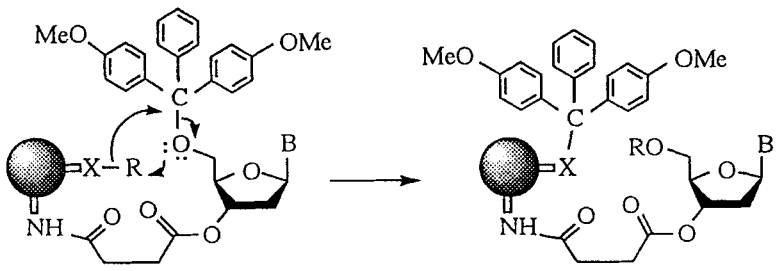


FIG. 2. CE profiles of Oligo 1 and Oligo 2. Panel A CE of Oligo 1 synthesized using pre-cap. Panel B CE of Oligo 1 synthesized without pre-cap. Panel C CE of Oligo 2 synthesized using pre-cap and Panel D. CE of Oligo 2 synthesized without pre-cap.



{X=NH, O; R=H, $COCH_3$}

Figure 3: Proposed trityl exchange reaction between nucleoside and functional group on CPG.

At the start of the synthesis, any uncapped lcaa group on the CPG will be linked to the incoming nucleoside phosphoramidite and get converted to a *phosphoramidate* after oxidation. Since phosphoramidates are less readily hydrolyzed than phosphates, one could expect that the former will be cleaved from the CPG, only on heating with NH_4OH, as in a CPG protocol. This could contribute partly to the N - 1 content and other truncated sequences in an oligonucleotide synthesized using the CPG protocol. Although direct mechanistic evidence for any of these processes do not exist at this time, our results with the pre-cap and SUP protocols point to this distinct possibility.

We also analyzed different lots of oligonucleotides synthesized by SUP and CPG protocol for their Si content. The Si content in a synthesized oligonucleotide potentially arises in part from dissolution of CPG-matrix components in ammonium hydroxide upon heating and in part by dissolution of the reverse-phase matrix (C_{18}) in buffers during HPLC purification. We have found a consistently lower Si content (< 0.03%, w/w) in the oligonucleotides prepared using the SUP protocol as compared to those produced by the CPG protocol (Si content variable, 0.05 - 0.4%, w/w), keeping other factors constant.

We have recently reported N,N-diethoxy N,N-diisopropyl phosphoramidite (DDP) as a new and efficient "phosphorylating type" capping reagent in oligonucleotide synthesis.[6] In accordance with our observation on acetylation type capping reaction, we incorporated a pre-phosphorylation capping step in oligonucleotide synthesis (10 μmol scale). We have observed and reported a

significant reduction in the N-1 content and truncated sequences by using pre-phosphorylation capping as compared to control (i.e., no pre-cap). Additional studies using SUP and CPG protocols using DDP as capping reagent is in progress.

In conclusion, in large-scale synthesis, incorporation of a pre-cap step in synthesis and adoption of a SUP work-up protocol is recommended for obtaining oligonucleotides of consistent quality and lower N-1 content.

MATERIALS AND METHODS:

Standard phosphoramidites, DNA synthesis reagents and anhydrous solvents were purchased from commercial manufacturers and used as such.

Preparation of CPG-bound nucleoside: These were prepared according to the general protocol as described.[4b]

Synthesis of Oligonucleotides: Oligonucleotides were synthesized on a Milligen/Biosearch 8700 DNA synthesizer using standard 1 μmol and 10 μmol synthesis cycles as recommended by the manufacturer. For synthesis of phosphorothioate oligonucleotides, 3*H*-1,2-benzodithiole-3-one-1,1-dioxide was used as the sulfurizing reagent.[7a,b] The 1 mmol-scale synthesis of oligonucleotides was carried out on a Milligen/Biosearch 8800 DNA synthesizer according to previously reported protocols[4a,5] with appropriate modifications as this study warranted. All synthesis reagents and reagent solutions were freshly prepared before use with standard precautions for handling moisture and air-sensitive materials. After synthesis, the CPG was removed from the column and aliquots subjected to either a SUP or CPG protocol as follows:

SUP protocol: After the synthesis, the CPG was removed from the column and was treated with 28-30% ammonia (1.5 mL for 1 μmol and 8 mL for 10 μmol scale) at ambient temperature for 30 to 120 minutes. The supernatant was removed and heated at at 50-55 ^{o}C for 8-10 hours.

CPG protocol: After the synthesis, the CPG was removed from the column was directly heated with 28-30% ammonia (1.5 mL for 1 μmol and 8 mL for 10 μmol scale) at 50-55 ^{o}C for 8-10 hours.

Additional processing of the oligonucleotides was done as described.[4a,5]

Analytical ion exchange chromatography was carried out on a Waters 660 E high pressure liquid chromatograph (HPLC) equipped with a Waters 996 photodiode Array Detector. The sample was dissolved in 0.1 M ammonium acetate and analyzed on a GEN-PAK FAX column(4.6 X 100 mm), at 45 ^{o}C, using the following gradient at a flow rate of 0.5 ml/minute.

Buffer A: 25 mM Tris HCl; pH 8.5, 10% CH_3CN

Buffer B: 25 mM Tris. HCl, 1M LiCl; pH 8.5, 10% CH_3CN

Linear gradient 100% - 50% A over 90 minutes.

Capillary electrophoretic analysis (CE) was done on a Beckman P/ace 2200 instrument operating at 14.1 Kv. Before CE, the samples were desalted on a SEP-PAK cartridge.

Analysis of Si content in oligonucleotides was carried out by Galbraith Laboratories, Knoxville, TN 37950.

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