Pergamon

Tetrahedron Vol. 50, No. 8, pp. 2617-2622, 1994

0040-4020/94 $6.00+0.00

0040-4020(93)E0217-4

Criteria for the Economic Large Scale Solid-Phase Synthesis of Oligonucleotides

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Abstract: The experimental conditions necessary for reducing the cost of large-scale solid-phase oligonucleotide synthesis by the phosphite triester approach have been studied using highly loaded polystyrene resins.

For many years synthetic oligonucleotides have been obtained at the milligram scale mainly when needed for structural studies[1]. Nowadays, with the discovery of the potential use of antisense oligonucleotides as therapeutic agents against cancer and viral deseases even larger quantities (gram amounts) will be required[2]. The problem appears when the economic variable is considered since large excesses of monomer building blocks are usually employed to ensure efficiency in microgram scale standard solid-phase syntheses. In this respect, it is worth studying the criteria for developing economic procedures for the large scale synthesis of oligonucleotides and analogues.

Although controlled-pore glass (CPG) is still the most widely employed support for small, or even medium, scale synthesis of oligonucleotides[3], the limited nucleoside loadings (typically 30-40 μmol/g)[4] that can be obtained are a serious drawback to performing large scale solid-phase synthesis. Liquid-phase synthesis has emerged as an attractive alternative to solid-phase methods: it makes use of a soluble polyethylene glycol (PEG) polymeric support which allows higher loadings (up to 180 μmol/g) to be obtained and has been used in combination with the phosphotriester approach[5] and very recently with the phosphite triester method[6] for the milligram scale synthesis of oligonucleotides.

In our opinion, a method for the large scale synthesis of oligonucleotides should rely on the phosphite triester approach[7], which is the most rapid and effective method, and on the solid-phase methodology for its simplicity, speed and convenience of operation. We have already shown that polystyrene supports (PS) are compatible with phosphoramidite chemistry[8]. Polystyrene offers some advantages over other supports for the synthesis of large amounts of oligonucleotides: i) it has a large surface area and can be loaded to the highest extent (340 μmol/g)[9] compared to other supports, ii) its hydrophobicity helps to avoid the problem of water contamination that reduces the amount of activated phosphoramidite available and therefore the coupling efficiency, iii) it is chemically and mechanically stable and allows gram amounts of support to be stirred.

Copolymers of polyethyleneglycol and polystyrene (PEG-PS) can be also highly functionalised (190 μmol/g) and have been used for the synthesis of oligonucleotides by the H-phosphonate[10] method or by the phosphite triester approach at a very large scale[11]. It has been argued that the main advantage of these PEG-PS supports over the crosslinked polystyrene-divinylbenzene that we have previously used[8] is that the resin swells

almost equally well in nonpolar organic solvents [dichloromethane(DCM), tetrahydrofuran(THF), toluene] as in more polar solvents [acetonitrile(ACN), alcohol, water][11]. However, the behaviour of PEG-PS copolymers is dominated by the hydrophilic PEG component, and this decrease in the overall hydrophobicity of the support might be a disadvantage from the point of view of increasing the risk of moisture contamination. A rigid macroporous PS support has also been reported to be useful for the preparation of large amounts of oligonucleotides[12].

Probably, the best support for large scale oligonucleotide synthesis remains to be discovered and as stated by Caruthers[13] "perhaps the key feature is a relatively impenetrable support with major surface channels to enhance synthesis capacity" and that "may be accesible to reagents but lack the porosity that leads to irreversible entrapment of reagents in the matrix".

We report here on our studies on the criteria for defining the conditions, in particular those related to the phosphoramidite coupling, that are necessary in order to reduce the high cost of large scale oligonucleotide synthesis. We show that very highly loaded polystyrene resins allow large amounts of oligonucleotides to be obtained without a significant decrease in synthesis efficiency.

We have recently shown that high substitution degrees can be obtained by DCC- and HOBt-mediated coupling of DMT-nucleoside-succinates on aminomethyl-PS supports[9]. Furthermore, we have shown that using the described anchoring conditions the starting resins, which usually have a too high an amine loading, can be functionalised to the desired extent within a small error[9]. Throughout this study we have used several nucleoside-resins with loadings ranging from 80 to 340 μmol/g that have been prepared according to ref. 9.

Before undertaking the synthesis of large amounts of oligonucleotides we have first studied the conditions under which the excesses of phosphoramidites can be reduced.

Studies on phosphoramidite coupling conditions

Kinetics of coupling onto differently loaded resins. We have studied the coupling rate of DMT-thymidine β-cyanoethyl-N,N-diisopropyl-phosphoramidite onto the reactive OH sites of differently loaded thymidinyl-resins (80, 130, 220 and 330 μmol/g), using 4 equivalents of phosphoramidite and 16 equivalents of the tetrazole catalyst[14]. In these studies, the total volume of the coupling solution is kept constant[15] so that a higher support loading also corresponds to a higher concentration of the phosphoramidite and tetrazole catalyst used. The main conclusion is that all the resins perform equally well and coupling rates and yields[16] are essentially the same (results not shown). A detailed analysis of the coupling rates shows that: i) 50% yields are normally reached in 3-4 minutes but 15-20 minutes are required to obtain a 90% yield and half an hour or more to get to completion (>99% yield)[17], ii) when using the most highly loaded support (330 μmol/g), and therefore the highest concentration of amidite (0.33 M), the coupling rates are faster in the first minutes but it takes longer to go to completion, probably because the more concentrated amidite/tetrazole solution speeds up the coupling onto the more accesible OH sites of the polymer but, as the coupling solution is also more viscous, there is a slower diffusion of the activated nucleoside into the less accessible reactive sites.

Influence of the nature and excess of the catalyst. Tetrazole (T), 5-(*o*-nitrophenyl)tetrazole (oNPT) and 5-(*p*-nitrophenyl)tetrazole (pNPT) have been tested as acid catalysts. As shown in Table 1, the efficiency of the catalysts has been found to be, as described[18], pNPT > oNPT > T in direct relation to their acidity (pKa values of pNPT, oNPT and T are 3.69, 4.03 and 4.79 respectively[19])[20]. By keeping constant the excess of amidite to

3 equivalents with respect to OH sites and increasing the excess of catalyst we have seen that the higher the excess of catalyst the faster the coupling rate. Therefore, a reduction in the excess of phosphoramidite can be counterbalanced by increasing the excess of catalyst, the economic balance being favourable as the catalyst is cheaper than the amidite.

Table 1. Influence of the Nature and the Excess of the Catalyst on Coupling Yields*

equiv amidite	equiv catalyst	T	oNPT	pNPT
3	6	-	33 %	-
3	12	70 %	96 %	> 99.5 %
3	18	-	> 99.5 %	-

* Resin loading : 110 μmol/g , coupling time : 15 min

Although from the results shown in Table 1 it can be concluded that pNPT is the best catalyst, we prefer to use oNPT rather than pNPT in syntheses with the more highly loaded resins, when concentrated solutions are required, because of the greater solubility of oNPT in THF, the solvent we use for the catalysts[21]. We should emphasize that the choice of the solvent for the phosphoramidite/tetrazole coupling need not be restricted to ACN, although it has been the universal solvent when using silica gel or CPG as solid supports. Other solvents may work equally well provided that they can also be obtained anhydrous and that the reagents are sufficiently soluble. This is the case with THF, in which the solubility of the three tetrazole derivatives that we have tested is greater than in ACN. Furthermore, when using a PS support THF has the advantage that it swells the resin almost as well as DCM and much better than ACN.

Single versus double coupling procedures. As we have already stated, using solid supports with a high substitution degree it becomes possible to employ higher concentrations but lower excesses of the reagents and the cost of the syntheses is reduced. The results shown in Table 2 are a striking example of how important the concentration of the reagents (phosphoramidite and catalyst) is in order to allow a reduction in their excesses.

Table 2. Comparison between Single and Double Coupling Procedures

	Single Coupling	Double Coupling
Excess phosphoramidite	3 eq	2 eq + 1 eq
Phosphoramidite concentration	0.09 M	0.06 M + 0.03 M
Excess pNPT	12 eq	8 eq + 4 eq
pNPT concentration	0.36 M	0.24 M + 0.12 M
Coupling time	15 min	15 min + 15 min
Coupling yield	> 99.5 %	54% + 18% = 72%

As can be seen in Table 2, better coupling yields are obtained by a single coupling with highly concentrated solutions than by dividing the same number of equivalents of reagents into a double coupling

procedure with more diluted solutions. Since the reaction time of the double coupling procedure is twice that of the single coupling, the only explanation is that the reaction kinetics is greatly affected by the concentration of the reagents.

Large scale syntheses

The ease of amidite coupling may vary as the oligonucleotide chain grows and although the above-described coupling experiments give a good insight into the conditions that are necessary to perform large scale syntheses, the definitive proof of their validity can only come from the synthesis of oligonucleotides.

In Table 3 we summarise the conditions and yields of several oligonucleotide syntheses that have been carried out on PS resins with loadings ranging from 130 to 340 μmol/g[22].

Table 3. Large Scale Syntheses

	Conditions			
Sequence	CGTTTTCG (**1**)[8]	CCAGGCCTGG (**2**)	GAGATCTC (**3**)	GCAATTGC (**4**)
Scale(μmol)	35	32	85	20
Resin loading (μmol/g)	140	130	340	200
Excess phosphoramidite	10 eq	5 eq	3 eq	5 eq
Catalyst	Tetrazole	pNPT	oNPT	oNPT
Excess catalyst	40 eq	20 eq	16.6 eq	25 eq
Coupling time	30 min	30 - 60 min	15 min	20 min
Mode	Manual	Manual	Manual	Automatic
		Yields		
Overall yield	82 %	74 %	68 %	86 %
Average coupling yield	96.8 %	96.4 %	94.1 %	97.8 %
Average yield : T , C	97.4 %	97.9 %	96.8 %	100 %
Average yield : A , G	94.0 %	94.4 %	92.0 %	96.3 %

Although the excesses of phosphoramidites and catalysts have been reduced to 3-5 equivalents of amidite with respect to reactive OH sites on the support, the overall and average coupling yields are similar to those obtained in standard small syntheses with CPG. The most extreme conditions have been employed in the synthesis of the DNA fragment GAGATCTC (**3**): highest loaded support (340 μmol/g), lowest excess of phosphoramidites (3 equivalents) and shortest coupling times (15 min). In this synthesis, the average coupling yield (94.1%) is somewhat lower because of the decrease in the coupling yield of the most hindered purine nucleosides (92.0%), whilst those of the pyrimidine nucleosides are maintained at a reasonable level (96.8%). One may assume that yields could have been bettered simply by increasing the coupling time.

The first three synthesis were carried out manually in a syringe system[3b] which represents a serious drawback in terms of preventing water contamination from ambient humidity which reduces the coupling efficiency. On the contrary, the synthesis of the octamer **4** was carried out automatically using an adapted protocol on an ABI 380B synthesiser and shows the best coupling yields.

The oligonucleotides were deprotected and cleaved from the support by a treatment with concentrated aqueous ammonia/dioxane (1:1) at 60°C for 24 h. The crude oligonucleotides (Fig.1) were purified by a single chromatography on DEAE-Sephadex except for the octamer **3** that was submitted to an extra purification step by C18 medium pressure liquid chromatography. The purified oligonucleotides were shown to be homogeneous (>99% pure by analytical HPLC, Fig.1) and gave the expected ratios of deoxynucleosides after enzymatic digestion with snake-venom phosphodiesterase and alkaline phosphatase[23].

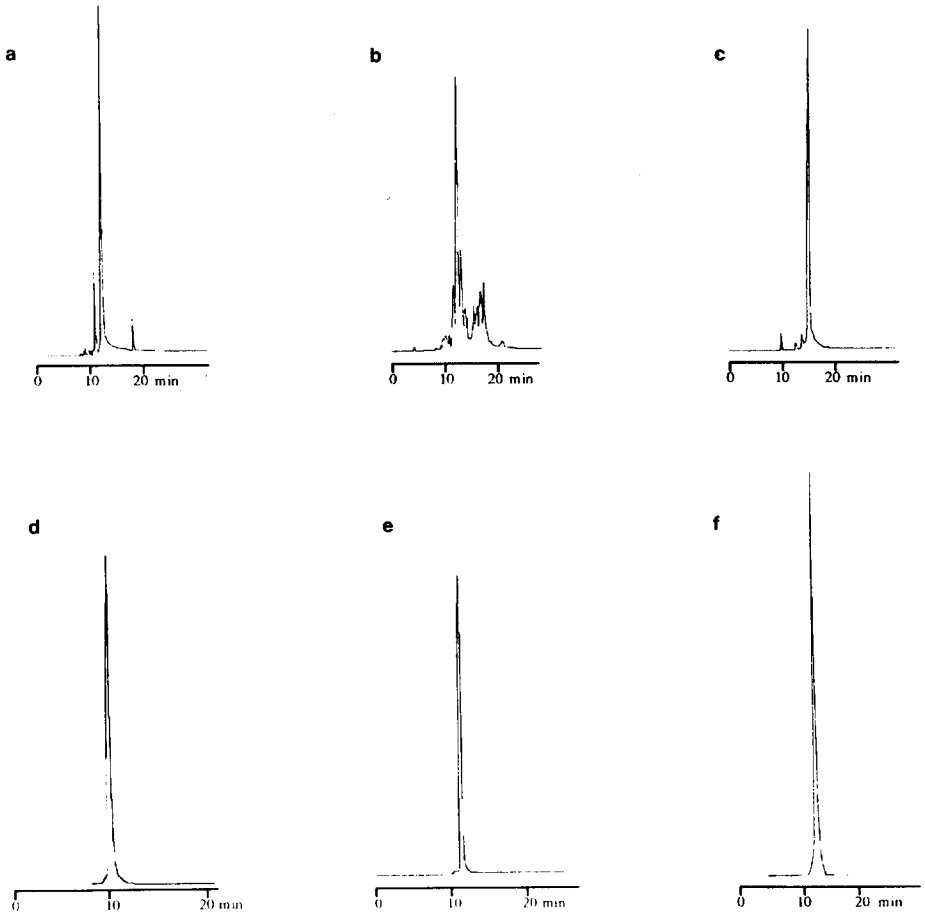

Fig.1. Analytical HPLC profiles[24] of the oligonucleotides: crude a) **2**, b) **3**, c) **4** and purified d) **2**, e) **3**, f) **4.**

In summary, we have studied the conditions under which the excesses of reagents can be reduced in order to minimise the economic cost of solid-phase oligonucleotide synthesis by the phosphite triester approach and we have applied the resulting conclusions to the synthesis of some oligomers at a reasonably large scale. The PS support used has proved to be convenient for our purposes and may be a feasible alternative for economically synthesising even larger amounts of oligonucleotides using the appropriate automatic instrument.

Acknowledgement. This work was supported by funds from the DGICYT (grant PB91-0270)

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14. The synthesis protocol is that described in ref. 8 except that tetrazole was dissolved only in THF.
15. The total volume of the coupling solution was maintained constant at 8 mL/g of resin in order to properly swell the resin without unnecessarily diluting the reagents.
16. Yields were determined by spectrophotometric quantitation of the dimethoxytrityl cation released upon acid deprotection.
17. Obviously, these results indicate that amidites are still activated after this reaction time. We have monitored a coupling reaction mixture by gel-phase ^{31}P-NMR (Bardella, F.; Eritja, R.; Pedroso, E.; Giralt, E.; *BioMed. Chem. Lett.* in press) and have observed the formation of the desired phosphite triester together with the activated phosphoramidite and its hydrolysis product. After 60 min, between 30% and 60 % of the phosphoramidite remains activated, the precise amount being dependant on the extent of hydrolysis in each particular experiment.
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20. TLC analysis shows that DMT-deoxynucleosides are completely stable to the more acidic catalyst (pNPT) for 4 h, and no release of the DMT group is observed. Care has to be taken, however, to use HCl-free dichloromethane to dissolve the phosphoramidite derivatives.
21. The solubility of the catalysts in THF is: 0.8M for pNPT, 1.4M for oNPT and 0.9M for T.
22. Standard DMT-deoxynucleoside-β-cyanoethyl-N,N-diisopropylphosphoramidites (T, C^{bz}, A^{bz}, G^{ibu}) were used in all the syntheses.
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24. Spherisorb C-18 column. Elution with A: 0.01M aq. triethylammonium acetate, B: ACN/water (1:1); Linear gradient from 0% to 40%B in 30 min.

(*Received in UK* 3 *August* 1993; *revised* 7 *December* 1993; *accepted* 10 *December* 1993)