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Poly(*N*-acryloylmorpholine) as a new Soluble Support for the Liquid-Phase Synthesis of Oligonucleotides.

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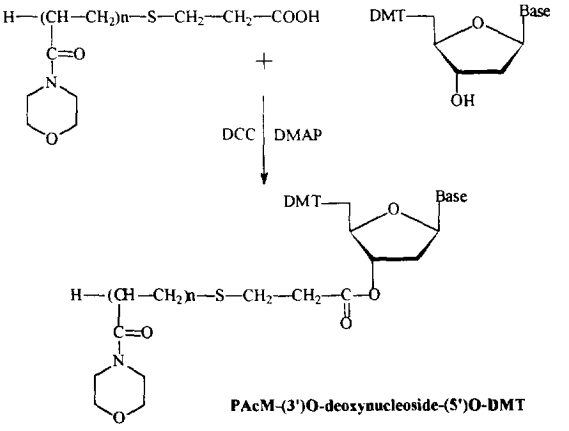
ABSTRACT: *The utilization of poly(N-acryloylmorpholine) as a new soluble polymeric support for the liquid phase synthesis of oligonucleotides is described.* Copyright © 1996 Elsevier Science Ltd

The therapeutic utilization of oligonucleotide derivatives seems close,[1] and the consequent need for commercial production has led to investigations of alternative synthetic polymers aimed at large scale preparation of oligonucleotides.[2] From this point of view, recently we proposed the use of polyethylenglycol (PEG) as a soluble polymeric support for a new, "friendly", scalable solution synthesis of these compounds.[3] We proposed this method as an alternative to the well-established solid-phase procedure, since, as also recently underlined,[4] the use of insoluble supports hampers scaling up of the process owing to phase heterogeneity and diffusion problems of reagents.

In a recent paper, a new end-functionalized, low molecular weight poly(*N*-acryloylmorpholine) or PAcM has been described.[5] The molecular dimensions, together with the structural and physico-chemical properties, appeared, at first glance, suitable for utilization as a new soluble polymeric support in our so-called HELP method.

In this paper we are reporting an investigation on the use of PAcM as a possible alternative to PEG for the liquid-phase synthesis of oligonucleotides. A PAcM end-functionalized with a carboxy group, prepared as previously reported,[5] was used, in order to test its direct derivatization with the first nucleoside of the chain through the formation of an ester bond with its 3'-OH group (Scheme 1).



Scheme 1. Functionalisation of PAcM.

This procedure avoids any pre-modification of the nucleoside unit as demanded by the normal OH terminating PEG chains, where a succinylated nucleoside is employed to form the first, reversible ester bond. The average molecular weight of PAcM moieties range from 6000 to 8500 daltons, corresponding to 190-120 μmol/g of COOH groups. (Average M.W. of 3000 and 12000 are also available). The use of dicyclohexylcarbodiimide (DCC) as activating agent, with dimethylaminopyridine (DMAP) as catalyst, in 1:1 ratio, allowed a final loading of different nucleosides from 50 to 90 μmol/g, depending upon the PAcM employed, using 3 equivalents of the reacting nucleotide. This value, determined by the dimethoxytrityl (DMT) absorption at 498 nm, corresponds to an average yield of about 50 %. Any attempt to increase this value by using a larger excess of reagents and increasing the reaction time, or by repeating the condensation procedure, failed. To ascertain the reason for this incomplete reaction, a COOH titration before and after the derivatization was performed. After the reaction, only a few free COOH units (less than 5 % of the initial value) were present, indicating that some irreversible modification had occurred. As confirmed by treating PAcM in the same conditions but with no nucleoside component, the DCC appeared able, as previously observed on PEG [6], to modify the COOH groups. Unfortunately, the ^{1}H NMR analysis of the PAcM derivatives cannot give any further information, due to the superimposition of the signals of backbone on those expected for DCC-modified groups. In fact, only the spectral window from 4.0 to higher ppm values is accessible (Figure 1).

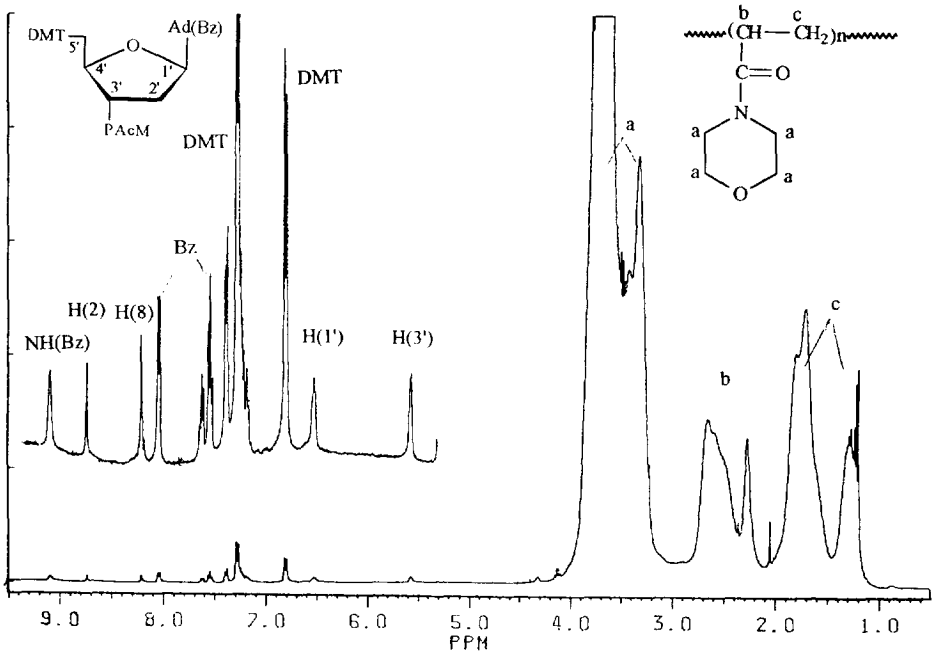


Figure 1. ^{1}H NMR spectrum of PAcM-(3')O-dA-(5')O-DMT (20 mg/.4ml $CDCl_3$ - Bruker AM 400)

We tried to avoid this drawback by utilizing a *N*-hydroxysuccinimmido-activated PAcM; however, the low reactivity of the 3'-OH nucleoside group limits the maximum yield to 20-30 %. At this point a low-loaded PAcM-nucleoside-DMT was used as starting support, because, in any case, no further reactive COOH groups appeared still present in the polymer, and it was still possible to test the behaviour of the PAcM as a soluble support in the liquid-phase synthesis of oligonucleotides. Further studies will be conducted to find alternative activating processes to obtain higher functionalization of the PAcM chains.

With PAcM as soluble support it was possible, as with PEG, to follow the condensation process by UV/Vis spectroscopy, owing to transparency of these polymer chains above 250 nm. This is an important advantage of liquid phase synthesis over solid phase synthesis.

The general procedure for the oligonucleotide assembly followed the one previously employed in the PEG-supported synthesis based on the phosphoramidite chemistry.[7] During the detritylation step an advantage of the PAcM support over the PEG one was found. In fact, a single acidic treatment was sufficient for complete deblocking of the DMT groups; on the contrary the PEG support requires repeated treatments, especially at the beginning of the synthesis, likely due to the "buffering" effect of the oxygen atoms of the polyether PEG. Then, with PAcM the risk of partial depurination of the oligonucleotide chain appears lowered.

During the synthesis particular care was taken with the intermediate purification steps which are performed through a precipitation/filtration procedure. With PAcM goods results were obtained with a tert-butyl methyl ether (TBME)/isopropanol mixture (3:1; v/v). With this mixture a better degree of purification was generally observed in comparison with the one obtained with ether alone, the usual precipitating agent of PEG. On the other hand, with PAcM, it appeared almost impossible to employ EtOH as an alternative crystallizing solvent, as often adopted for PEG-derivatives, owing to the incomplete dissolution of PAcM by warming, and to the formation of a gelatinous precipitate after cooling.

A careful study was devoted to the effect of the oxidation step, as demanded for the production of the phosphate bond from the intermediate phosphite. PAcM contains a thioether bond between the terminal COOH and the remaining part of the chain that is potentially subjected to oxidation. The use of a 10% tert-butyl hydroperoxide (TBHP) solution in acetonitrile, at 0° C, for the time demanded for the complete oxidation of phosphites (15 min), did not reveal any oxidation of sulfur to sulfoxide or sulfone, as judged by the benzidine test [8] and FTIR spectroscopy (data not shown). As a consequence, reduced stability between the support and the growing chain was not expected during the synthesis.[9]

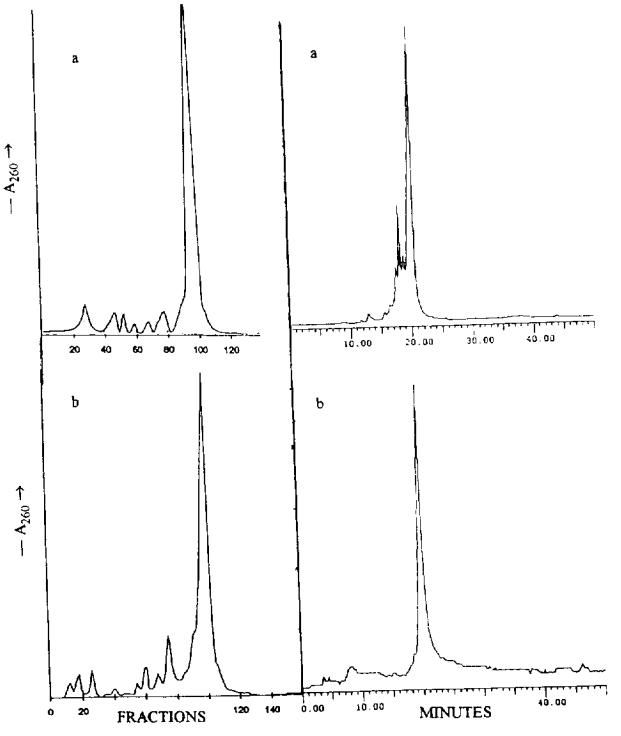


Figure 2. Left side: Ion exchange low-pressure chromatography on Sephadex CM-25 of fully deblocked, crude 8mer obtained a) from a PEG-supported synthesis and b) from a PAcM-supported synthesis
Right side: RP-HPLC on Super Pac PepS column of fully deblocked 8mer a) crude and b) purified.

To test the efficacy of the process and to optimize reaction parameters, a series of experiments was performed at the homodinucleotide level. A standard synthetic protocol, employed with the PEG-supported procedure, was set up and, for purpose of comparison, applied to the production of the same oligonucleotide sample, namely the d(TAGCGCTA) sequence already used for the optimization of the PEG-based approach [7]. During synthesis with PAcM (M.W. = 6000; 90 μmol/g of starting nucleoside) solubility problems were not found, similar to the use of PEG of similar chain size. Starting from 1.0 g of polymer, 0.9 g of PAcM-8mer-DMT was isolated. The overall yield was 83 %, corresponding to an average yield of 97.5 %, as determined by the A_{498} of the DMT group and confirmed by the ^1H NMR analysis. After the usual deblocking and purification procedure,[7] more than 100 mg of pure liophylized TEA salt of the 8mer sample was obtained, whose identity was ascertained by chromatographic comparison with an authentic sample. These values are close to those previously obtained with PEG, confirming that PAcM could represent a valid alternative in the liquid-phase synthesis of oligonucleotides. The amount obtained of pure product reveals that, as with PEG, little, if any, absorption of the deblocked oligonucleotide to the polymer carrier can be envisaged.

The fully deprotected oligomer was purified by low pressure ion-exchange chromatography on DEAE-Sephacell. The elution profile is shown in the left part of Figure 2, compared with the same profile obtained with the same 8mer synthesized on a PEG support; the similarity of the two samples is clearly recognizable. In the right part of Figure 2 the elution patterns of the RP-HPLC analyses of crude and purified octamer are reported.

In conclusion the results obtained demonstrate that PAcM represents a valid alternative to PEG as a soluble supporting polymer for liquid-phase synthesis of oligonucleotides. This encourages further studies aimed at the improvement of nucleoside binding, either using different coupling procedures or different end-functionalized PAcM derivatives, such as the OH terminating polymer, a compound already synthesized. The effect of the PAcM chain on the *in vivo* stability and cellular permeability of covalently bound oligonucleotides will also be studied in future.

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REFERENCES

1. *Antisense Research and Applications*; Crooke, S.T.; Lebleu, B. Eds.; CRC Press, Inc., 1993.
2. Padmapriya, A.A.; Tang, J.; Agrawal, S. *Antisense Res. & Dev.* **1994,** 4, 185-199.
3. Bonora, G.M. *Applied Biochem. and Biotech.* **1995**, 54, 3-17.
4. Han, H.; Wolfe, M.M.; Brenner, S.; Janda, K.D. *Proc. Natl. Acad. Sci. USA* **1995**, 92, 6419-6423.
5. Ranucci, E.; Spagnoli, G.; Sartore, L.; Ferruti, P.; Caliceti, P.; Schiavon, O.; Veronese, F. *Makromol. Chem. Phys.* **1994**, 195, 3469-3479.
6. Zalipski, S.; Gilon, C.; Zilkha, A. *Eur. Polym. J.* **1983**, 19, 1177-1183.
7. Bonora, G.M.; Biancotto, G.; Maffini M.; Scremin, C.L. *Nucleic Acids Res.* **1993**, 21, 1213-1217.
8. Feigl, F.; *Spot Tests*; vol.II, Elsevier Publ. Co., 1954; p. 180.
9. Folder, E.; Schwyzer, R.; Charubala, R.; Pfleiderer W.; Schulz, B. *Tetrahedron Lett.* **1984**, 25, 3967-3970.

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