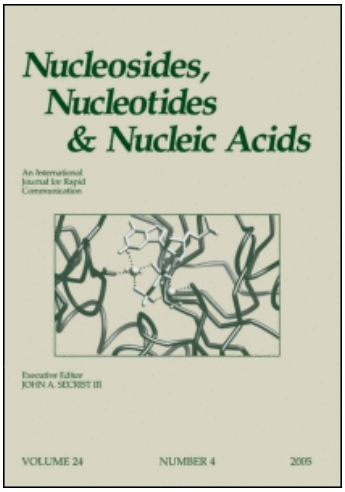


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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(8), 1281–1288 (2000)

# PAcM-AN: POLY (N-ACRYLOYLMORPHOLINE)-CONJUGATED ANTISENSE OLIGONUCLEOTIDES.

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ABSTRACT. A new amphiphilic, high-molecular weight poly (N-acryloylmorpholine) (PAcM) polymer has been used to be linked to oligonucleotide chains through a liquid-phase stepwise synthesis. This new conjugate has been investigated for its melting property, nuclease stability and capacity to elicit RNase H activity. Its antisense activity against an HIV-1 target has been also evaluated.

## INTRODUCTION

Single-stranded synthetic oligonucleotides have been demonstrated to inhibit with high specificity the expression of pathological genes [1], and, very recently, the first pharmaceutical production of these bioactive antisense molecules, proved to be safe and effective in clinical trials, has been authorized by the FDA [2].

To overcome the main drawback of its utilization, namely the rapid in vivo degradation, and to increase consequently their bioavailability, many chemical modifications of their natural structure have been introduced [3]. Alternatively, enhanced stability of oligonucleotide as drugs can be obtained by the introduction of exogenous conjugating molecules along the chain, able to mask the identity of the original sequence, avoiding the in vivo recognition by the ubiquitous degrading enzymes [4]. Moreover, the attachment of new moieties with higher lipophilic character was also suggested in order to increase the low cellular permeability of these nucleic acid derivatives [5].

As an improvement of the therapeutic properties of the oligonucleotides, their conjugation with high-molecular weight polymers has been recently investigated [6]. Among various long-chain molecules, polyethylene glycol (PEG) has demonstrated interesting features both from the synthetic point of view and for its capability to modify the biological properties of the chemically joined molecules [7]. With regard to the first point, a new liquid-phase synthetic procedure, based on PEG, has been set up to overcome any limitations due to the heterogeneous solid-phase processes commonly employed for the synthesis of these antisense molecules [8]. A further modification of this procedure allowed the introduction of a stable phosphate bond between the oligonucleotide chain and PEG, polymer that was so far employed only as a removable, inert, macromolecular synthetic helper [9].

In a recent paper, the utilization of the poly (N-acryloylmorpholine) (PAcM) as a new soluble polymeric support for the liquid-phase synthesis of the oligonucleotides has been described [10]. Consequently, by introducing the same modification adopted for the preparation of stable PEG-conjugated oligonucleotides, the use of PAcM both as synthetic support as well as conjugating moiety has been investigated. Furthermore, the biological properties of a new PAcM-conjugated antisense oligonucleotide have been analyzed here and compared with those of the same sequence bound to a MPEG (monomethoxy polyethylene glycol) chain of similar molecular weight.



## RESULTS

The synthesis of the PacM-conjugated oligonucleotides was performed by the liquid-phase method following the procedure previously reported for fully deprotected oligomers [11].

To allow the introduction of a stable phosphodiester bond between the polymer and the oligonucleotide, an OH terminating polymer have been utilized. Its molecular weight was comparable with that of the MPEG sequence (9,500 Da) previously utilized for the synthesis of the same nucleic acid. The oligonucleotide sequence tested in this study was formerly demonstrated to exhibit an antisense activity against an HIV-1 target [12]. Moreover, the same molecule conjugated to MPEG have been yet analyzed for its biological properties as melting behavior, nuclease stability, and RNase H activation [9, 13]. The scheme of the synthetic process is reported in Figure 1.

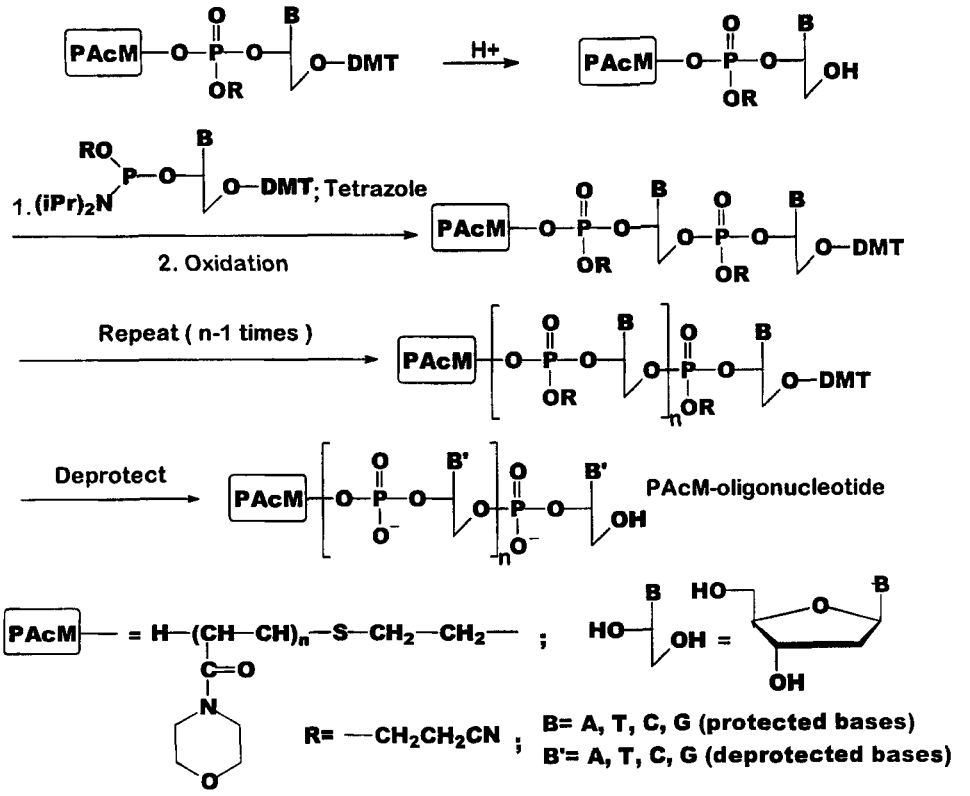


FIG. 1 Scheme of the synthesis of the conjugated PAcM-oligonucleotide.



Owing to the peculiar solubility properties of the PAcM polymer, a slight modification of the intermediate purification procedure have been introduced. As usually, the polymer-bound growing chain is freed from the byproducts of the reaction mixture by precipitation with a non-solvent, and filtration. In the case of the PAcM-derivatives, the best recovering yield have been obtained with a mixture of diethyl ether: i-ProOH = 3:1, since the alternative crystallization from EtOH, successfully employed in the PEG series, was not usable in this case. The $^{1}H$ and the $^{31}P$ spectra of the PAcM polymer bearing the first nucleotide of the planned chain are reported in Figure 2. Within the limits of the instrumental error, an almost quantitative reaction can be estimated from the ratio of the integration values of the nucleotide signals with those of the polymeric unit. The $^{31}P$ NMR spectrum demonstrated the efficacy of the oxidation procedure, since only the

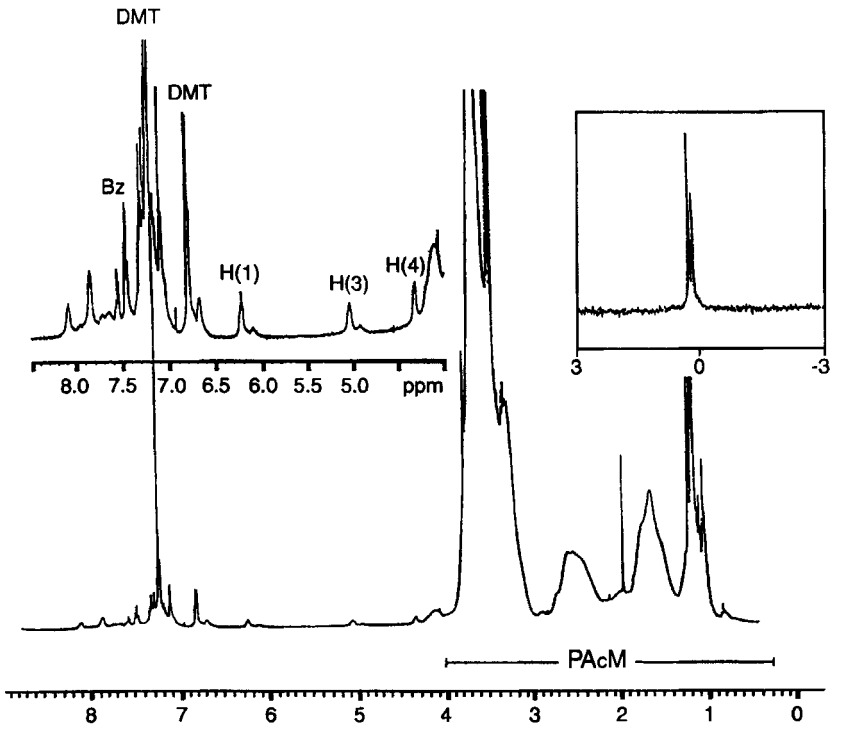


FIG. 2 $^{1}H$ NMR spectrum in $CDCl_3$ of the PAcM bearing the first nucleotide [dC(Bz)] of the conjugated chain. In the insert $^{31}P$ NMR spectrum.



signal due to the terminal phosphate group has been observed. These data were supported by the UV/Visible analyses based on the absorption at 498 nm of the nucleoside-protecting dimethoxytrityl-group (DMT).

The complete PacM-12mer was obtained following the same synthetic protocol based on the phosphoramidite chemistry previously reported [10]. An improvement of the overall procedure has been introduced, since only a single acidic treatment was needed for the complete removal of the intermediate trityl group. It must be recalled that on the MPEG-based procedure a repeated treatment was required, at least during the first steps. The reaction data of the synthesis are listed in Table and compared with those of the previously synthesized MPEG-conjugated oligonucleotide. These data are fully comparable, testifying the applicability of the new support, even if the total amount of the final product was a little lower, very likely due to the less advantageous solubility

TABLE 1. Overall and average yields [a], and total weighed recovered amount of the PacM-12mer, compared with the same oligo conjugated to MPEG.

| SAMPLE | Overall yield | Average yield | Amount recovered |
|---|---|---|---|
| PAcM-12mer | 78.5 % | 98.0 % | 48.5 % |
| MPEG-12mer | 82.0 % | 98.5 % | 60.0 % |

a. from absorption at 498 nm of the DMT group.

properties of the new polymer. IE HPLC has achieved the purification of the final product, even if in this particular case some minor drawback has reduced the efficiency of the chromatographic process; in fact, using the same Mono Q column employed for the purification of the PEG-conjugates [9], a minor separation capability has been observed. In additon an unidentified UV absorption hampered the recognition of the final product. The effectiveness of the purification process have been verified by RP and IE HPLC analyses.

A first characterization of the PAcM-conjugated oligonucleotide was performed through its thermal denaturation study. As usually, the increase of the UV absorption at 260 nm with the temperature has been followed upon the formation of the duplex with its complementary target. The thermal behavior, as demonstrated by the first derivative curve of the $A_{260}$ against temperature reported in Figure 3, is strictly superimposable to that of the same MPEG-conjugated oligonucleotide. As previously reported [9], the melting temperatures of the duplexes formed by the MPEG-supported and the free 12mer were practically identical. This indicates that even the PAcM, as the MPEG, does not affect the hybridization properties of the conjugating molecule

. A second investigation was devoted to ascertain the stabilizing effect of the conjugating polymer toward the enzymatic degradation of the nucleic acid components. The experimental conditions were those previously employed [9]. In Figure 4 the disappearance of the starting PAcM-12mer is compared with the free oligonucleotide and the MPEG-conjugated form. A comparable behavior is given by the two polymer-bound derivatives, with a little increase of stability supplied by the new PAcM moiety (after 2 hours 13% of intact oligo is still present, instead of 10 % with the MPEG).

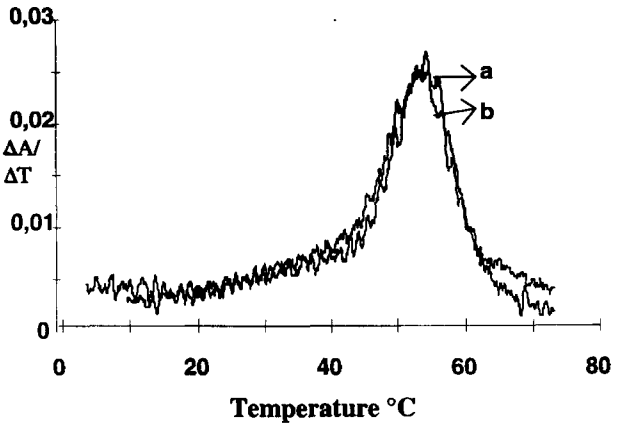


FIG. 3 First derivative of the thermal denaturation profiles of duplexes with a. MPEG-12mer and b. PAcM-12mer.



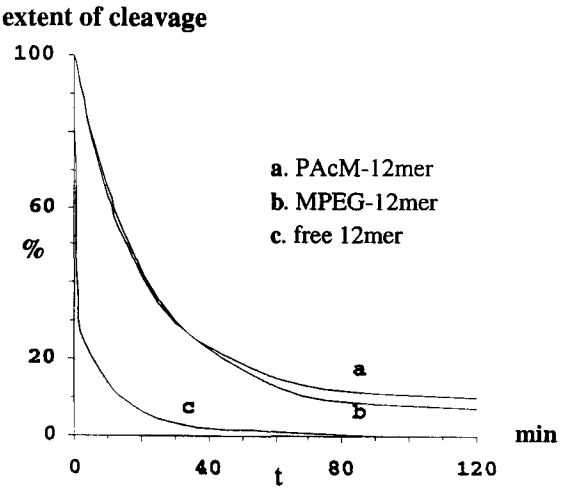


FIG. 4 Digestion of free and polymer-conjugated 12mers in presence of a mixture of diesterase and nucleotidase. The concentration of the starting oligonucleotide has been quantified by HPLC.

It is well known that the degradation of the hybrid duplex given by the antisense molecule and its RNA target is performed by the ubiquitary RNase H, and that this action contributes effectively to the inhibition of the gene expression. For these reasons, the effect of the PAcM-conjugating moiety toward this enzyme activity has been examined. The kinetic experiments, already performed on the same MPEG-conjugated sequence [13],

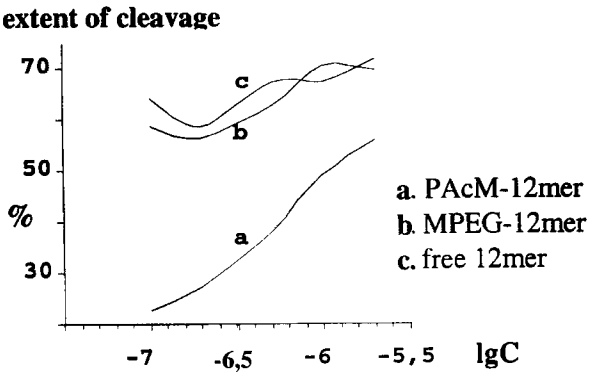


FIG. 5 Extent of the RNA cleaved by RNase H as a function of the concentration of the polymer-conjugated and free oligonucleotide.



have demonstrated that even in presence of a high-molecular weight PAcM the activity of RNase H is preserved. Moreover, it has been confirmed that the cleavage takes place mainly at the level of the two nucleosides $A^{17}$and $U^{14}$. However, as reported in Figure 5, a clear decreasing of the cleavage extent as function of the oligonucleotide concentration is observed, when compared to the same MPEG-bound and free 12mer. In particular, with a $10^{-7}$ molar oligonucleotide, a 25 % of hydrolysis of the target RNA has been observed (about 60% with the two other samples). Equally, as function of time, only 55% of the starting sequence are degraded after two hours, instead of almost 70% (data not shown). All these results indicate that the PAcM is less effective than MPEG toward the RNase H activity.

The biological activity of the synthesized antisense oligonucleotide, once conjugated to this new polymer, have been investigated for its protection capability against HIV-1 infection, using the experimental conditions adopted for the MPEG-conjugated oligonucleotide [14]. Unfortunately, the investigated sequence resulted inactive up to 50 micromolar concentration, where the complete loss of cell viability has been observed.

## CONCLUSIONS

This paper reports that it is possible to obtain stable conjugates between oligonucleotides and PAcM trough the same procedure so far successfully adopted with high-molecular weight MPEGs. Only minor drawbacks that hamper an easy

chromatographic purification of the product are observed. From the point of view of the biological properties it was found that the high-molecular PAcM chain does not interfere with the formation of a regular duplex of the linked oligonucleotide with its nucleic acid target, while it infers a clear stability against the nuclease degradation of the molecule. Moreover, its presence allows the expression of the enzymatic activity by the RNase H, even if at a lower level in comparison with a MPEG chain of similar size. However, the PAcM polymer looks quite unfavorable as conjugating moiety of oligonucleotides, owing to the absence of a measurable antisense activity, at least in case of the sequence here investigated.

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