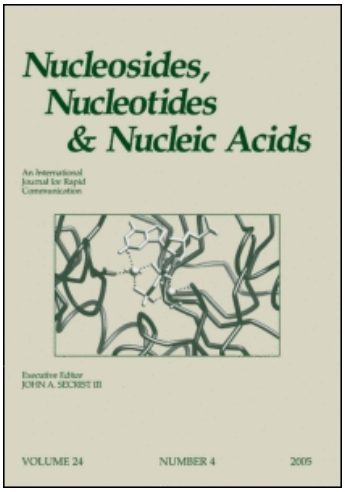


**Nucleosides, Nucleotides and Nucleic Acids**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

# Synthesis and Analytical Characterization of RNA-Polyethylene Glycol Conjugates

Andres Jäschke[ab]; Rolf Bald[b]; Eckhart Nordhoff[c]; Franz Hillenkamp[c]; Dieter Cech[a]; Volker A. Erdmann[b]; Jens P. Fürste[b]
[a] Institut für Chemie, Humboldt-Universität zu Berlin, Berlin, Germany [b] Institut für Biochemie, Freie Universität Berlin, Berlin, Germany [c] Institut für Medizinische Physik und Biophysik, Westfälische Wilhelmsuniversität, Münster, Germany

**To cite this Article** Jäschke, Andres , Bald, Rolf , Nordhoff, Eckhart , Hillenkamp, Franz , Cech, Dieter , Erdmann, Volker A. and Fürste, Jens P.(1996) 'Synthesis and Analytical Characterization of RNA-Polyethylene Glycol Conjugates', Nucleosides, Nucleotides and Nucleic Acids, 15: 9, 1519 — 1529
**To link to this Article: DOI:** 10.1080/07328319608002451
**URL:** http://dx.doi.org/10.1080/07328319608002451

NUCLEOSIDES & NUCLEOTIDES, 15(9), 1519-1529 (1996)

# SYNTHESIS AND ANALYTICAL CHARACTERIZATION OF RNA-POLYETHYLENE GLYCOL CONJUGATES

Andres Jäschke[1,2], Rolf Bald[2], Eckhart Nordhoff [3], Franz Hillenkamp[3], Dieter Cech[1], Volker A. Erdmann[2] and Jens P. Fürste[2*]. [1]Institut für Chemie, Humboldt-Universität zu Berlin, Hessische Str. 1-2, D-10099 Berlin, Germany, [2]Institut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany, [3]Institut für Medizinische Physik und Biophysik, Westfälische Wilhelmsuniversität, Robert-Koch-Str. 31, D-48149 Münster, Germany.

**Abstract:** Polyethylene glycols with degrees of polymerization from 5 to more than 100 were incorporated into synthetic oligoribonucleotides by automated solid phase synthesis at 3'-terminal, 5'-terminal and internal positions. The conjugates were characterized by chromatographic, electrophoretic and mass-spectrometric methods. The influence of coupling site, polymer size and number of coupled polymers per oligonucleotide on the molecular properties of the conjugates is investigated.



The interactions of synthetic oligoribonucleotides with organisms are considered to be of prime importance for the development of novel diagnostics and therapeutics[1]. Promising approaches include the utilization of sequence-specific ribozymes and RNA ligands (aptamers) with high affinities for selected targets[2,3]. The use of synthetic oligoribonucleotides as therapeutic agents, however, requires that several criteria can be satisfied, i.e. the ribozymes or aptamers should (i) be efficiently transported through cellular membranes to reach their destination, (ii) be stable towards cellular nucleases, (iii) specifically recognize their targets and (iv) maintain a high catalytic or binding activity[4].

Although only few data are published on *in vivo* stability and reactivity of oligoribonucleotides, information obtained for oligodeoxyribonucleotides indicates that unmodified phosphodiester oligonucleotides are less suitable due to their rather poor cellular uptake (high charge density) and their rapid degradation by cellular nucleases[5,6]. Two general chemical strategies were applied to circumvent these problems: i) the chemical modification at the sugar phosphate backbone and ii) the coupling of unnatural residues to oligonucleotides, i.e. the formation of conjugates[6,7]. Compared to the wealth of information obtained for DNA, RNA chemistry still needs further development. The

chemical synthesis of oligoribonucleotides modified at the sugar phosphate backbone and the bases have been reviewed[8,9]. Conjugates of synthetic RNA have scarcely been described[10].

We were interested in coupling polyethylene glycol (PEG) to RNA oligonucleotides because of its unique chemical and biological properties. PEG is non-toxic and non-immunogenic[11]. It interacts in a complex manner with cellular membranes. At high concentrations PEG induces cell fusions[12]; it facilitates uptake of exogenous nucleic acids by different cells and is often used in transfection experiments. PEG has been covalently coupled to proteins for epitope shielding and for increasing their cellular lifetimes[13,14]. Oligoethylene glycols with degrees of polymerization up to 10 have been incorporated into synthetic DNA and RNA oligonucleotides[15-19]. Recently, the substitution of structural domains in ribozymes by tri- and hexaethylene glycol units has been reported[20,21].

In previous publications, we described methods for the automated incorporation of polyethylene glycol (PEG) with degrees of polymerization from 5 to about 120 into synthetic oligodeoxyribonucleotides[22] and investigated the influence of the PEG size on the molecular properties of the conjugates[23]. We found an improved exonuclease stability and an undisturbed hybridization by terminal PEG coupling.

As a precondition for our studies on the design of chemically stabilized synthetic ribozymes and aptamers, we report here the automated synthesis of RNA-PEG conjugates. Besides the incorporation of PEG at 3'- and 5'-termini, we describe PEG coupling at internal positions. The conjugates are characterized by chromatographic and electrophoretic methods. Moreover, we describe the detailled analytical characterization of the RNA-PEG-conjugates by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS).



## MATERIALS AND METHODS

DMT-PEG phosphoramidites and PEG-derivatized supports were synthesized as described[23].

*Synthesis of triisopropylsilyl-protected ribonucleoside phosphoramidites*

Fully protected ribonucleosides were prepared as described with certain modifications[24,25]. Benzoyl (A and C) and isobutyryl (G) groups were used for the protection of the exocylic amino groups, while the 5'-hydroxyls were protected using the 4,4'-dimethoxytrityl group. Secondary hydroxyls were protected by triisopropylsilyl groups. The isomeric mixtures of 2'- and 3'-O-triisopropylsilyl derivatives were separated

by column chromatography on silica using a linear gradient of methanol in dichlormethane (0-5%) in the presence of 0.5% triethylamine. Fractionations were monitored by TLC on silica gel (solvents: 2.5 or 5 % methanol in $CHCl_3$). The 2'-O-triisopropylsilyl derivatives were then converted into the respective 2-cyanoethyl-N,N-diisopropyl phosphoramidites according to standard procedures[25].

*Oligoribonucleotide synthesis*

Oligoribonucleotides were prepared by phosphoramidite chemistry on an Applied Biosystems 394 automated synthesizer using the 1μmole synthesis cycle as programmed by the manufacturer. Phosphoramidite concentrations were 0.15 M, and a coupling time of 15 minutes was employed. After removal of the DMT groups, the oligonucleotides were first deprotected using concentrated $NH_3$ / ethanol 3:1 (55 °C, 24 h), followed by removal of the triisopropylsilyl groups using a 1 M tetrabutylammonium fluoride solution in THF (room temp., 72 hours). Excess reagents were removed by chromatography on QIAGEN columns. The lyophilized oligonucleotides were redissolved in water and purified by reversed phase HPLC (Nucleosil 300/5 C4 Macherey and Nagel, 1 ml/min.) using a gradient from 0.8 to 32% acetonitrile in 100 mM triethylammonium acetate, pH 7.0, in 30 min.



*Synthesis of oligonucleotide-PEG conjugates*

For the incorporation at the 3'-end, synthesis columns were packed with the PEG-derivatized glass supports[23] . Chain elongation was carried out using the standard cycles. Conjugation at the 5'-end and at internal positions was performed by coupling of 0.15 M solutions of the (4,4'-dimethoxytrityl)polyethylene glycol-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites in acetonitrile using the standard cycles. Deprotection and purification were carried out as described for the unmodified oligomers.

*Oligonucleotide labeling*

Oligoribonucleotides (0.005 $A_{260}$-units) were dissolved in buffer and 1 μl [$\gamma$-$^{32}$P]ATP (2 μCi, Amersham) and 1 μl T4 polynucleotide kinase (Gibco-BRL) were added (final concentrations: 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 50 mM Tris-HCl, pH 7.6). After incubation (1 hour, 37 °C), the labeled oligonucleotides were purified and characterized by gel electrophoresis using native polyacrylamide gels.

*MALDI mass spectrometry*

Mass spectrometry was carried out using the hardware and method described[23,26]. Samples were lyophilized overnight and redissolved in ultrapure water to a final concentration of ca. 0.002 $A_{260}$-units/µl. Aliquots of 0.5 µl of analyte solution and 1µl of matrix solution (300 mM 3-hydroxypicolinic acid) were mixed on a flat metallic sample support and dried in a stream of cold air. Ubiquitous alkali metal ions were removed by adding 0.5-0.7 µl of a suspension of $NH_4^+$-loaded cation exchanger beads (BioRAD 50W-X8) in water to the analyte/matrix solution prior to drying[26].

## RESULTS

Since our previous work has shown that PEG can be introduced into DNA oligonucleotides by using the standard cycles of automated solid phase phosphoramidite synthesis[22,23], we examined how the different synthons and the altered reaction conditions influence the synthesis of RNA-PEG conjugates. We applied solid phase RNA synthesis using 5'-O-(4,4'-dimethoxytrityl)-2'-O-triisopropylsilyl-nucleoside-3'-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites employing the benzoyl and isobutyryl groups for protection of the exocyclic amino groups. The synthesis of these phosphoramidites was performed using published procedures[24,25]. N-protected nucleosides were first reacted with 4,4'-dimethoxytrityl chloride, then with triisopropylsilyl chloride, and the resulting mixtures of 2'- and 3'-isomers were separated by column chromatography. The 2'-O-triisopropylsilyl derivatives were then converted into the respective 2-cyanoethyl-N,N-diisopropyl phosphoramidites by reaction with (2-cyanoethyl-N,N,N',N'-tetraisopropyl)phosphordiamidite and tetrazole.



Due to the low reactivity of ribonucleoside phosphoramidites, the phosphoramidite concentration during automated synthesis was increased to 0.15 M, and a coupling time of 15 minutes was chosen. Under these conditions, coupling efficiencies of 98 - 99 % per cycle were obtained with unmodified RNA, as monitored by DMT cation release. After removal of the dimethoxytrityl groups, oligoribonucleotides were first treated with a mixture of concentrated aqueous ammonia and ethanol, followed by the removal of the triisopropylsilyl groups using tetrabutylammonium fluoride/THF. Excess reagents were removed by chromatography on QIAGEN columns. Depending on the size and sequence of the oligoribonucleotide, either reversed phase HPLC or preparative denaturing PAGE were employed for purification of the crude mixtures.

The preparation of the synthons for the coupling of PEG to oligoribonucleotides, i.e. the (4,4'-dimethoxytrityl)-polyethylene glycol-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites of PEG 400 and PEG 1000 and the (4,4'-dimethoxytrityl)-polyethylene glycol

derivatized glass supports of PEG 400, PEG 1000 and PEG 4000 has been described previously[22,23]. To compare the properties of the conjugates with those of the DNA-PEG conjugates, we chose the corresponding nucleotide sequence (5'-UUCGA-3') and synthesized a series of various terminally and internally coupled pentaribonucleotides with PEG 400, PEG 1000, and PEG 4000, respectively.

Functionalization at the 3'-terminus was performed using DMT-PEG-derivatized supports. As monitored by the dimethoxytrityl cation assay, a coupling efficiency of 98 % in the first coupling step and 99 % for subsequent steps were detected for PEG 400, while values slightly above 100 % were measured for PEG 1000 and PEG 4000 during the first cycles (see below). The analysis of the reaction mixtures after deprotection by reversed phase HPLC and by MALDI mass spectrometry is shown in FIG. 1.

Since the polyethylene glycols used for the preparation of the synthons were polydisperse samples, the coupling products gave numerous peaks with characteristic patterns in the chromatograms. As expected from DNA synthesis, coupling to PEG leads to an increased hydrophobicity indicated by an increase in retention time. While the unmodified oligonucleotide is eluted after 11.4 min, the PEG 400-conjugates appear between 14 and 20 minutes, and the PEG 1000-conjugates between 20 and 29 minutes. The PEG 4000-conjugates could not be resolved in this chromatographic system and appeared as a broad peak mostly between 30 and 41 minutes (data not shown). All RNA-oligonucleotides and conjugates are eluted 3-4 minutes earlier than the respective DNA molecules in the same chromatographic system (previous publication[23]). The MALDI mass spectra of the raw products show a regular spacing between adjacent peaks of 44 dalton corresponding to a $CH_2CH_2O$-unit. Discrete degrees of polymerization were assigned to the HPLC peaks by mass spectrometric analysis of collected single peak fractions (see FIG. 2). The byproducts eluting around 10 min gave molecular weights of 1592.7, 1610.7 and 1281.3, respectively, consistent with the assumption that 2'-(3'-) phosphates or 2'-,3'-cyclophosphates of the pentanucleotide UUCGA and the tetranucleotide UUCG were formed. Considering the occurence of coupling efficiencies larger than 100 % in the first cycles (see above) and the relative increase of those peaks in the HPLC chromatograms with increasing size of coupled PEG, we assume that the coupled PEG shielded adjacent amino groups during the derivatization of the solid supports. These amino groups could have become accessible successively during automated synthesis leading to 3'-shortened products linked to the support via phosphoramidate linkages, which are finally hydrolyzed during deprotection. The byproducts in the chromatogramm of the unmodified oligonucleotide, however, cannot be explained by this mechanism. These products are most likely 5'-shortened oligonucleotides resulting from coupling yields lower than 100 %.

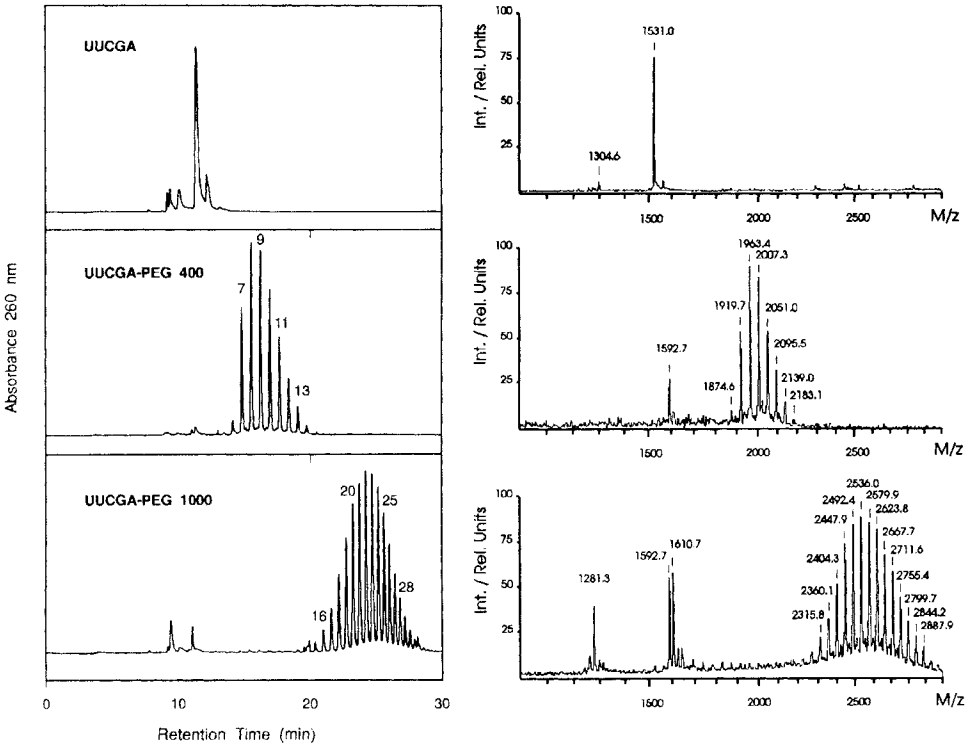


FIG. 1: HPLC chromatograms (left) and corresponding MALDI-mass spectra (right) of unpurified oligoribonucleotide conjugates.

To get a better insight into side reactions and to investigate the potential of MALDI mass spectrometry for the elucidation of reaction mechanisms, we prepared several conjugates using unpurified PEG phosphoramidites, which gave coupling efficiencies between 40 and 80% as determined by DMT cation release. In contrast to DNA synthesis, our purification scheme involved only one HPLC purification, and most of the byproducts appear in the chromatograms shown. The 5'-conjugates PEG 1000-UUCGA are separated with a good resolution, and the spacing between adjacent HPLC peaks is 44 dalton (see inserts in FIG. 2). The major contamination of the PEG 1000 conjugate is eluted at 11.4 min and has a molecular weight of 1625.2, which corresponds to the 5'-O-methyl-phosphodiester of UUCGA that results from quenching the reaction mixture with methanol during the preparation of phosphoramidites. The byproducts, however, could be removed by preparative HPLC.

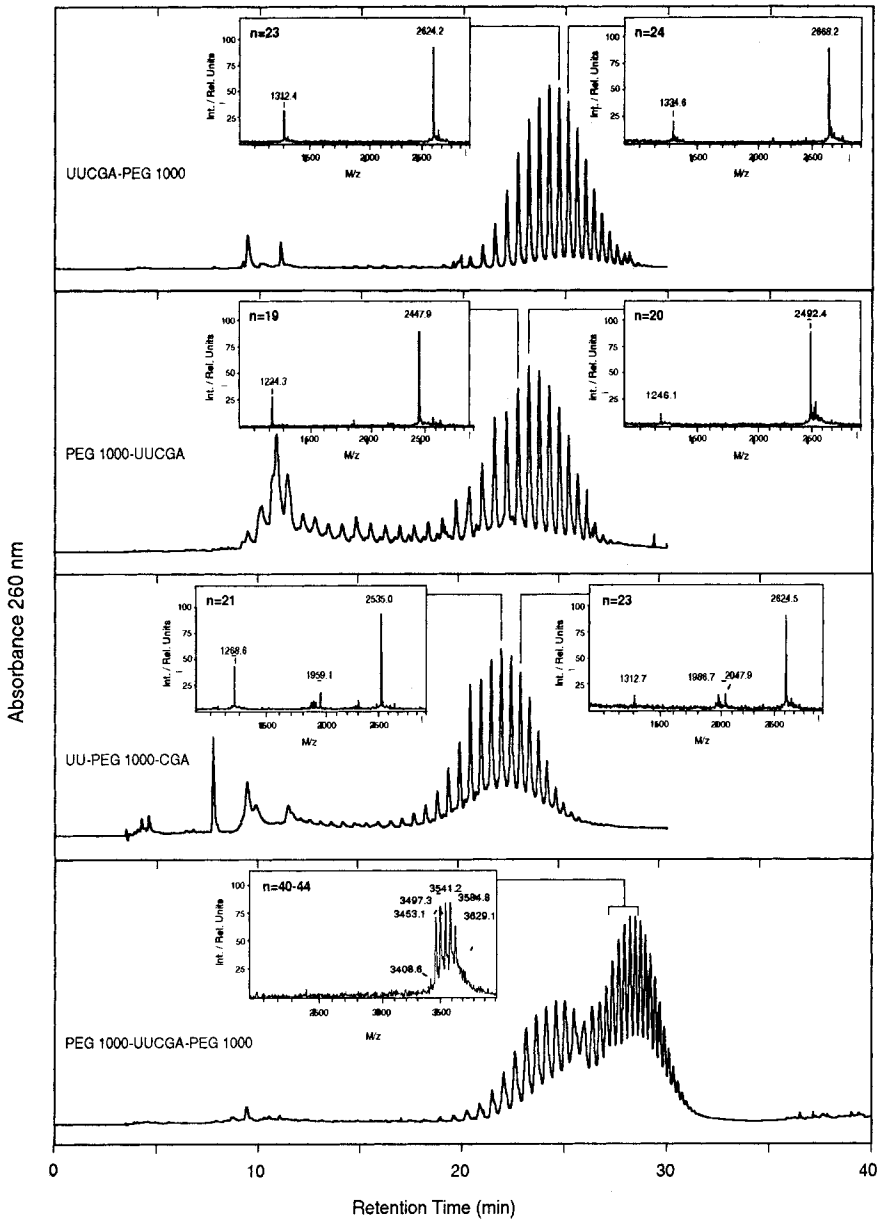


**FIG. 2:** HPLC chromatograms and MALDI-mass spectra of PEG 1000-oligoribonucleotides with 3'-terminal, 5'-terminal, internal and 3',5'-terminal conjugation

The internally PEG-coupled pentanucleotides UU-PEG 1000-CGA were synthesized by coupling of a PEG phosphoramidite, subsequent removal of the DMT group and automated chain elongation. The main products are eluted earlier than the terminally coupled PEG conjugates. Successful coupling is again proved by MALDI mass spectrometry. The calculated and measured values agree by +/- 1 Dalton (FIG. 2).

The 3',5'-PEG 1000-coupled oligonucleotides were synthesized by using PEG-derivatized glass supports and DMT-PEG-phosphoramidites. Due to the permutation of the molecular weight distributions, the number of product peaks is much higher, and the combination of side reactions leads to the occurrence of various byproduct peaks. Collected fractions from the HPLC purification, however, gave clear spectra with limited numbers of peaks. E.g., the fraction eluting from 27.5 min to 28.7 min contained the 3',5'-conjugates with degrees of polymerization of 40 to 44 (m.w.= 3453.1 to 3629.1, FIG. 2).

Similar results were obtained for 5'- and 3',5'- terminal and internal PEG 400-conjugates (data not shown). The coupling of PEG 4000 was restricted to 3'-terminal modifications, since the high viscosity of a PEG 4000-phosphoramidite solution prevented its use in automated synthesis.



As described for the DNA conjugates, RNA-PEG-conjugates with unmodified 5'-terminus could be phosphorylated by T4 polynucleotide kinase and $\gamma$-$^{32}$P-ATP. Coupling of PEG leads to an increase in the molecular size without increasing the charge similarly. Therefore, the electrophoretic mobility of the conjugates decreases with increasing degrees of polymerization. FIG. 3 shows a the 3'-PEG-coupled and internally PEG-coupled oligoribonucleotides compared to the unmodified RNA-molecule and an analogous DNA-pentanucleotide.

## DISCUSSION

The results described here demonstrate that synthetic oligoribonucleotides can be easily conjugated to synthetic polymers during automated synthesis at various positions. The strategy allows to incorporate the conjugate groups (PEG) into oligoribonucleotides during automated synthesis at defined positions and with defined stoichiometries. By using polydisperse polyethylene glycols, whole populations of conjugates with varying degree of polymerization $n_{PEG}$ are produced. The polydisperse product mixtures can be fractionated by HPLC; whereby in several cases the resolution of single degrees of polymerization is possible. The chromatographic procedures can be scaled up to a loading of 10 $A_{260}$-units and more. Chromatographic resolution generally decreases with the size of both the oligo and the coupled PEG. This effect can be compensated in part by decreasing the slope of the acetonitrile gradient.

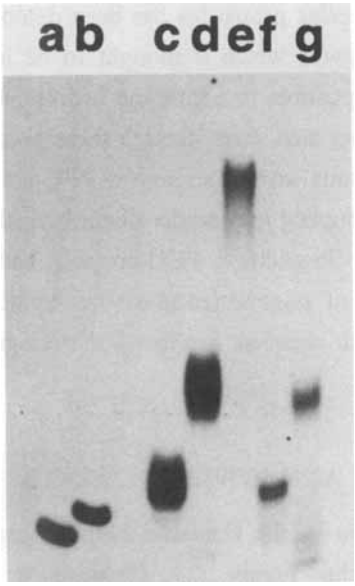


**FIG 3:** PAGE of $^{32}$P-labelled PEG-oligoribonucleotide conjugates. Product fractions were collected during HPLC purification, $^{32}$P-phosphorylated and analyzed on a 20 % native polyacrylamide gel (20 V/cm). lane a: DNA oligonucleotide d(TTCGA); lane b: RNA oligonucleotide UUCGA; lane c: UUCGA-PEG 400; lane d: UUCGA-PEG 1000; lane e: UUCGA-PEG 4000 conjugate; lane f : UU-PEG 400-CGA; lane g: UU-PEG 1000-CGA.



Matrix-assisted laser desorption/ionization mass spectrometry proved to be an elegant method of reaction analytics. In all mass spectra, the molecular weights of the product peaks agree by +/- 1 dalton with the theoretical values, thereby confirming the successful coupling of the synthons. Adjacent peaks in the HPLC chromatograms show a regular m.w. difference of 44 dalton. In addition, MALDI mass spectrometry proved useful for the elucidation of byproducts occuring during synthesis of RNA conjugates. It should be emphasized, however, that the interpretation of the chemical nature of byproducts is based solely on the mass spectra. We cannot preclude the possibility that some byproducts may have different chemical compositions but similar molecular weigths within the limits of accuracy.

The modulation of molecular properties has been demonstrated with respect to the hydrophobicity of the conjugates which is thought to be important for the uptake by eukaryotic cells. Thus, it is possible to adjust the hydrophobicity of a conjugate by the selection of a suitable polymer size. Even though these points have not been addressed within this study, our previous investigations on PEG-coupled oligodeoxynucleotides indicate that terminal PEG-coupling may render oligoribonucleotides more stable towards exonucleolytic degradation[23]. In addition, PEG-coupling had only marginal influence on the hybridization properties of oligonucleotides. Investigations on the combination of automated PEG coupling with chemical modification strategies, e.g. O-2'-alkylation, are under way.

## ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 344 and SPP "RNA-Biochemie"), the Fonds der Chemischen Industrie e.V., and the Bundesministerium für Forschung und Technologie. The authors are grateful to A. Eickmann (Freie Universität Berlin) for technical assistance.



## REFERENCES

1. Edgington, S.M. *Biotechnology* **1992**, *10*, 256-262.
2. Szostak, J.W. *Trends Biochem. Sci.* **1992**, *17*, 89-93
3. Yarus, M. *FASEB J.* **1993**, *7*, 31-39.
4. Bertrand, E., Pictet, R., and Grange, T. *Nucleic Acids Res.* **1994**, *22*, 293-300.
5. Uhlmann, E. and Peymann, A. *Chem. Rev.* **1990**, *90*, 543-584.
6. Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165-187.
7. Beaucage, S.L. and Iyer, R.P. *Tetrahedron* **1993**, *49*, 1952-1963.
8. Heidenreich, O., Pieken, W. and Eckstein, F. *FASEB J.* **1993**, *7*, 90-96.
9. Usman, N. and Cedergren, R. *Trends Biochem. Sci.* **1992**, *17*, 334-349
10. Oberhauser, B. and Wagner, E. *Nucleic Acids Res.* **1992**, *20*, 533-538.
11. Harris, J. M. In *Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*; Harris, J. M. Ed.; Plenum Press: New York, 1992; pp. 1-14.
12. Arnold, K., Krumbiegel, M., Zschörnig, O., Barthel, D. and Ohki, S. In *Cell and Model Membrane Interactions*. Ohki, S. Ed.; Plenum Press: New York, 1991; pp. 63-87.
13. Abuchowski, A., van Es, T., Palczuk, N.C. and Davis, F.F. *J. Biol. Chem.* **1977**, *252*, 3578-3581.

14. Delgado, C., Francis, G.E. and Fisher, D. *Crit. Rev. Therap. Drug Carrier Syst.* **1992**, *9*, 249-304.
15. Maskos, U. and Southern, E.M. *Nucleic Acids Res.* **1992**, *20*, 1679-1684.
16. Durand, M., Chevrie, K., Chassignol, M., Thuong, N.T. and Maurizot, J.C. *Nucleic Acids Res.* **1990**, *18*, 6353-6359.
17. Rumney, S. and Kool, E.T. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1617-1619.
18. Cload, S.T. and Schepartz, A. *J. Am. Chem. Soc.* **1991**, *113*, 6324-6326.
19. Ma, M.Y.-X., Reid, L.S., Climie, S.C., Lin, W.C., Kuperman, R., Sumner-Smith, M. and Barnett, R.W. *Biochemistry* **1993**, *32*, 1751-1758.
20. Benseler, F., Fu, D.-J., Ludwig, J. and McLaughlin, L.W. *J. Am. Chem. Soc.* **1993**, *115*, 8483-8484.
21. Thomson, J.B., Tuschl, T. and Eckstein, F. *Nucleic Acids Res.* **1993**, *21*, 5600-5603.
22. Jäschke, A., Fürste, J.P., Cech, D. and Erdmann, V.A. *Tetrahedron Lett.* **1993**, *34*, 301-304.
23. Jäschke, A., Fürste, J.P., Nordhoff, E., Hillenkamp, F., Cech, D. and Erdmann, V.A. *Nucleic Acids Res.* **1994**, *22*, 4810-4817.
24. Usman, N., Ogilvie, K.K., Jiang, M.-Y. and Cedergren, R.J. *J. Am. Chem. Soc.* **1987**, *109*, 7845-7854.
25. Milecki, J.F., Dembek, P. and Antowiak, W.Z. *Nucleosides Nucleotides* **1989**, *8*, 463-474.
26. Nordhoff, E., Cramer, R., Karas, M., Hillenkamp, F., Kirpekar, F., Kristiansen, K. and Roepstorff, P. *Nucleic Acids Res.* **1993**, *21*, 3347-3357.

Received September 9, 1995
Accepted May 23, 1996