Journal of Chromatography, 326 (1985) 311–320
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 599

NEONUCLEOPROTEINS

PREPARATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF SUCCINYL-LYSOZYME-DIAMINOOCTYL-POLYCYTIDYLIC ACID AND RELATED POLYCYTIDYLIC ACID CONJUGATES

MARKUS EHRAT, DOUGLAS J. CECCHINI and ROGER W. GIESE*
Department of Medicinal Chemistry in the College of Pharmacy and Allied Health Professions, and Barnett Institute of Chemical Analysis and Materials Science, Northeastern University, 360 Huntington Avenue, Boston, MA 02115 (U.S.A.)

SUMMARY

Transamination conjugates of cytidine-3′-phosphate and polycytidylic acid (poly C) with diaminopropane, diaminohexane and diaminooctane (DAO) are formed both at 25°C and 60°C. The extent of reaction and formation of side products, with intermittent hydrolysis to mononucleotides in the case of aminoalkyl-poly C, is monitored by reversed-phase high-performance liquid chromatography. Both Dns-DAO-poly C and succinyl-lysozyme-DAO-poly C covalent conjugates are then prepared and similarly characterized, including separation on a size-exclusion diol high-performance liquid chromatography column. The retention of the latter on a wide-pore reversed-phase column seems to be controlled by the protein moiety.

INTRODUCTION

Nucleic acids are increasingly being employed as probes to detect and characterize the biochemical molecules with which they interact. These interacting molecules include both complementary nucleic acids involving hybridization reactions[1], and proteins such as the complementary enzyme for a transfer RNA[2]. In many cases, it is important in these reactions to enhance the detection properties of the polynucleotide probe by incorporating a signal or signal-related group such as a radioactive phosphate[3], an enzyme[4], biotin[5], or potentially an electrophoric release tag[6]. Polynucleotides are attractive for this purpose because of their highly polar nature, variety of functional groups, and increasing ease of preparation, both chemically and biochemically.

For instance, polycytidylic acid (poly C) or transfer RNA have been used for a variety of conjugation reactions by Schulman and co-workers[7–9]. These workers transaminated the polynucleotides with carbohydrazide or diaminoalkane reagents in aqueous sodium bisulfite at the 4-position of the cytosine residues. The new amino

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groups were then demonstrated to be reactive with fluorescein isocyanate and also cross-linking agents, the latter allowing subsequent extension of this technique to the conjugation of a transfer RNA to its complementary enzyme[2]. Throughout these studies, characterization of the reactions and products was based on classical liquid chromatography (LC), along with other techniques.

Here, the prior work of Schulman and co-workers is extended primarily along chromatographic lines by the development of some high-performance liquid chromatographic (HPLC) conditions useful for the analysis of such conjugation reactions and products of poly C. In particular, columns packed with diol and reversed-phase silica-based materials are found to be useful. Also, the use of a higher temperature to accelerate the transamination reaction has been investigated.

EXPERIMENTAL

Chemicals and reagents

Poly C and cytidine phosphate (2′ and 3′) as potassium salts, Dns chloride, lysozyme, and the alkyldiamines 1,3-diaminopropane (DAP), 1,6-diaminohexane (DAH), and 1,8-diaminooctane (DAO) were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium metabisulfite, sodium sulfite and magnesium chloride (all as certified grade, ACS), buffer salts and solvents (HPLC grade), Spectrapor dialysis bags (3500 MW cutoff) were obtained from Fisher (Bedford, MA, U.S.A.). Dimethylsulfoxide and N-methylmorpholine were from Pierce (Rockford, IL, U.S.A.). HPLC buffer solutions were filtered through Millipore (Bedford, MA, U.S.A.) 0.45-μm HA membrane filters.

High-performance liquid chromatography

This equipment consisted of a Model 4 pump, control unit and LC 85b variable-wavelength UV detector from Perkin-Elmer (Norwalk, CT, U.S.A.); an FS 970 fluorescence detector from Schoeffel (Westwood, NJ, U.S.A.); an SP 4270 integrator for single-channel monitoring from Spectraphysics (San Jose, CA, U.S.A.) and an SF-4000 Chromcard II system for dual channel monitoring from Anadata (Glen Ellyn, IL, U.S.A.).

Diaminoalkyl poly C derivatives

A solution of 2 *M* sodium bisulfite was prepared by adding 18.9 g (0.15 mol) of sodium sulfite and 42.5 g (0.22 mol) of sodium metabisulfite to 300 ml of aqueous 10 m*M* magnesium chloride. The diaminoalkane was added to give a final concentration of 1 *M*, and the pH was adjusted to 7.0 with hydrochloric acid. The reaction was initiated by adding 100 mg of poly C to 100 ml of this solution. After the desired degree of modification was achieved, the reaction mixture was dialyzed twice against 0.02 *M* Tris–HCl buffer (pH 7), containing 0.2 *M* sodium chloride, and twice against water. (If the reaction mixture was dialyzed directly against water, most of the modified poly C precipitated irreversibly, probably due to the tendency of polyamines to form precipitates with phosphates[10].) After lyophilization the average yield of derivatized poly C was 80 mg.

The degree of hydrolysis of the poly C during the modification reaction was determined on two Brownlee OH-100 size-exclusion columns (purchased from Rain-

in, Woburn, MA, U.S.A.) in series. The mobile phase was 0.1 *M* potassium phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min, and the peaks were detected at 270 nm.

Diaminoalkyl-cytidine phosphate derivatives

The cytidine 3'- and 2'-monophosphates were derivatized as described for poly C, except that the HPLC characterization was performed directly on the reaction mixture.

Kinetics of diaminoalkyl modification of cytidine monophosphates

The cytidine 3'- and 2'-monophosphates and their diaminoalkyl derivatives were separated on a Rainin Microsorb C_8, 5-μm, 150 × 4.6 mm I.D. column (Rainin, Woburn, MA, U.S.A.) and a C_{18}, HS 3, 100 × 4.6 mm I.D. column (Perkin Elmer, Norwalk, CT, U.S.A.) in series. Solvent A was 0.1 *M* potassium phosphate buffer, pH 4.6 and solvent B was acetonitrile. A 12-min gradient from 0–15% B was run. The flow-rate was 1.2 ml/min and the peaks were monitored at 270 nm.

Kinetics of diaminoalkyl modifications of poly C

Prior to analysis by HPLC, 100-μl aliquots of the reaction mixture containing poly C and a diaminoalkane, were diluted with 0.9 ml of 0.3 *M* aqueous potassium hydroxide and incubated for 18 h at 37°C. After the pH was adjusted to 7 with concentrated hydrochloric acid, the modified and unmodified mononucleotides were determined by the HPLC system as described above for the modification of the cytidine monophosphates.

Succinyl-lysozyme

Lysozyme (300 mg, 20 μmol) was dissolved in 140 ml of 0.2 *M* potassium phosphate buffer (pH 7.8). Succinic anhydride (1.3 g, 13 mmol) was added to the stirred solution in 100-mg portions over the course of 1 h. The pH was maintained at 7.5–8.0 by the dropwise addition of 50% aqueous potassium hydroxide solution. Half an hour after the final addition of succinic anhydride, the mixture was dialyzed three times against water. The dialyzate was lyophilized and further purified by gel chromatography on a 93 × 1.6 cm I.D. Sephadex G-10 column, equilibrated with water. The product was lyophilized, giving 285 mg of a white powder.

Succinyl-lysozyme-DAO-poly C

Prior to coupling with DAO-poly C, the succinylated lysozyme was dried *in vacuo* for 24 h over phosphorus pentoxide. Succinylated lysozyme (10 mg) was dissolved in a solution comprising 1.5 ml of dimethylsulfoxide, 0.2 ml of formamide, and 1.2 μl of N-methylmorpholine. The resulting solution was cooled to 0°C, and 2 μl of isobutyl chloroformate were added. The mixture was stirred for 2 min followed by the addition of 10 mg of DAO-poly C, dissolved in 1.5 ml of formamide, containing 2.4 μl of N-methylmorpholine (the DAO content of this poly C was 53% of the number of cytidine residues, see Fig. 3). The reaction was allowed to proceed for 4 h at room temperature prior to analysis by HPLC. The DAO-poly C could be separated from succinyl lysozyme by size-exclusion chromatography (SEC) on two Brownlee Aquapore OH-100 columns (purchased from Rainin, Woburn, MA, U.S.A.) in series, having a molecular-weight-range, according to the manufacturer

(Brownlee Labs., Santa Clara, CA, U.S.A.), from 10 000 to 100 000 daltons for proteins. The eluent was acetonitrile–0.1 *M* potassium phosphatebuffer (pH 7.0) (20:80) at a flow-rate of 0.5 ml/min. The separation of DAO-poly C-lysozyme from unbound DAO-poly C was achieved by HPLC on a Vydac C_{18} column, purchased from Rainin, 33 nm pore size, 250 × 4.6 mm I.D. The mobile phase consisted of solvent A: 0.1 M potassium phosphate buffer pH 7.0 and solvent B: acetonitrile. A 10-min gradient from 10 to 50% B was run at a flow-rate of 1.5 ml/min.

Dns-DAO-poly C

Using a procedure adopted from Gray[11], poly C or DAO-poly C (53% DAO) was dissolved in 0.2 *M* sodium bicarbonate buffer (pH 8.3) to give a final concentration of 2 mg/ml. To 1 ml of this solution was added 500 μl of a Dns chloride solution (20 mg/ml) in dimethyl sulfoxide. After a reaction time of 2 h, 5 μl of the reaction mixture was analyzed by HPLC on two Brownlee Aquapore OH-100 SEC columns in series as described above. The UV absorbance of poly C and lysozyme was monitored at 270 nm and the fluorescence of lysozyme was monitored at 320 nm with an excitation wavelength of 280 nm.

RESULTS AND DISCUSSION

When poly C is modified with DAP, DAH, or DAO in the presence of bisulfite, and then hydrolyzed to nucleotide monomers by mild aqueous base[7], reversed-phase chromatograms such as that shown in Fig. 1 are obtained. For this particular chromatogram, DAH was allowed to react with poly C for 24 h at 60°C prior to the hydrolysis reaction. Peaks 1 and 2 represent cytidine phosphates arising from unreacted residues in poly C; peaks 3 and 4 are the corresponding uridine phosphates

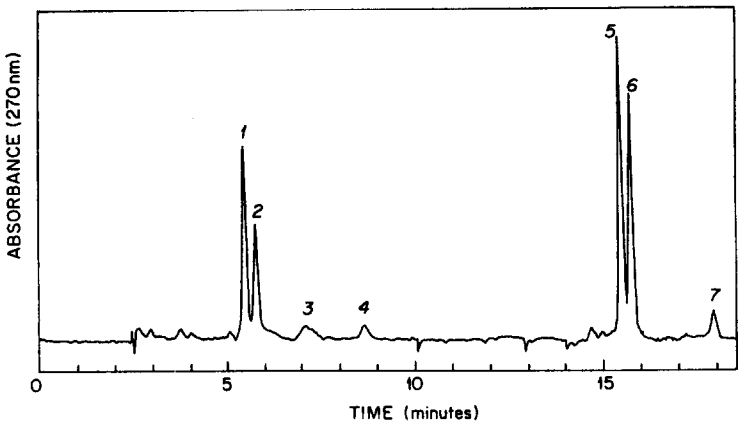


Fig. 1. Representative HPLC chromatogram of diaminohexyl-poly C after alkaline hydrolysis. The poly C was allowed to react with 1,6-diaminohexane for 24 h at 60°C prior to hydrolysis. The alkaline digest was analyzed on a Rainin Microsorb C_8 5 μm 150 × 4.6 mm I.D. column in series with a Perkin-Elmer C_{18} HS3 100 × 4.6 mm I.D. column. Peak identification was based on simultaneous injection of authentic samples: 1 = cytidine-3′-phosphate; 2 = cytidine-2′-phosphate; 3 = uridine-3′-phosphate; 4 = uridine-2′-phosphate; 5 = 4-diaminohexyl cytidine-3′-phosphate; 6 = 4-diaminohexylcytidine-2′-phosphate, 7 = unknown.

derived from hydrolysis of cytosine groups[12]; peaks 5 and 6 are the corresponding DAH-cytidine phosphates, and peak 7 is unidentified. Attempts to prove that the latter peak is a dimer, comprising two cytidine mono-phosphate groups attached to a single molecule of diaminohexane were unsuccessful. This effort involved attempts to increase this peak by prolonging the reaction or raising the ratio of poly C or cytidine-3′-phosphate to DAO. However, these efforts did not exclude the possibility that peak 7 is the postulated dimer.

For the reaction of poly C with DAO, the conjugate peaks corresponding to peaks 5 and 6 were unresolved, whereas these peaks were well resolved in the case of DAP. Because the molar extinctions of the diaminoalkane cytidine phosphate adducts were not different experimentally from those of the corresponding cytidine phosphates at the detection wavelength used, it is clear from the chromatogram that the reaction went about 2/3 to completion at this time.

In Fig. 2 is shown the time course of the reaction of DAP, DAH and DAO at both 24°C and 60°C with cytidine-3′-phosphate. The faster reaction under both temperature conditions with DAP is consistent with prior observations[7], but is unexplained. This difference in the rate of transamination between DAP and the other two, longer diaminoalkanes largely disappears when poly C is allowed to react similarly, as shown in Fig. 3. This behavior may be caused by a change in the rate-determining step, related to the conformation of poly C[13], or may be simply due to DAH and DAO being more reactive than DAP with poly C.

Largely because poly C reacts more slowly than cytidine-3′-phosphate with these diaminoalkanes, a greater percentage of side products (Fig. 1, peaks 3, 4 and 7) arise in the reaction with poly C, limiting the percentage yield of the N^4 transaminated product to 80%, as observed and defined in Fig. 3. Cytidine bisulfite adducts

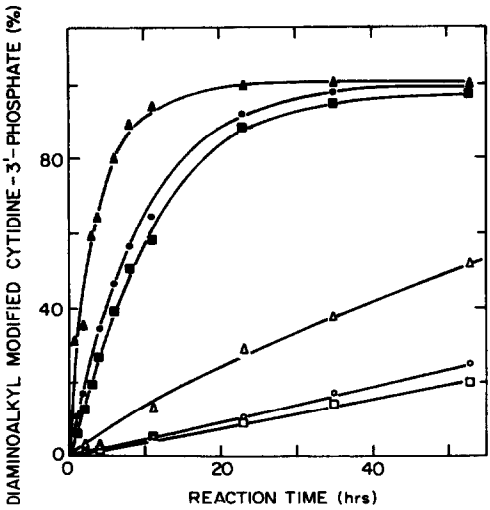


Fig. 2. Reaction of cytidine-3′-phosphate at 60°C (closed symbols) and 25°C (open symbols) with diaminoalkanes (propyl, △ and ▲; hexyl, ○ and ●; and octyl, □ and ■) in the presence of bisulfite to form the corresponding 4-diaminoalkylcytidine-3′-phosphates. The reaction was monitored by HPLC (see Fig. 1). The percent modification was calculated from the peak area of the 4-diaminoalkylcytidine-3′-phosphate divided by the total peak area of all peaks observed.

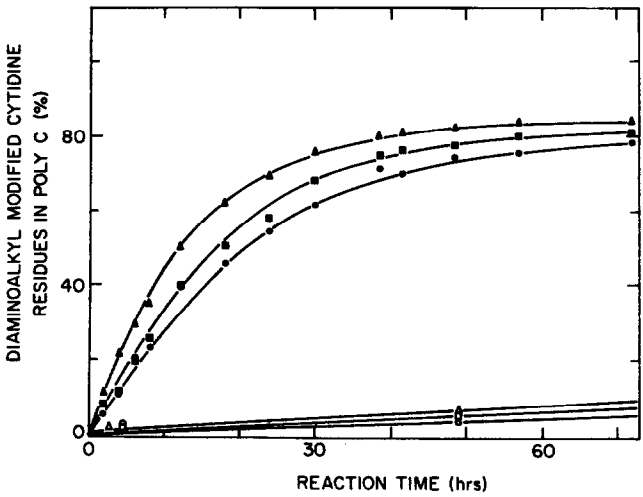


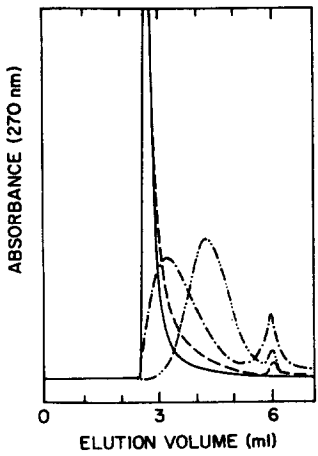
Fig. 3. Reaction of poly C at 25°C and 60°C with diaminoalkanes in the presence of bisulfite. Aliquots of the reaction mixture were hydrolyzed in aqueous sodium hydroxide to form the monomeric cytidine-2'- and -3'-phosphates and the 4-diaminoalkylcytidine-2'- and -3'-phosphates prior to analysis by HPLC (see Fig. 1). The results are presented as described in Fig. 2.

undergo deamination to form uridine (peak 3 and 4). However, in the presence of excess diamine and at neutral pH, transamination is the preferred reaction[9]. As was determined by the hydrolysis of unreacted poly C, the amount of side products formed during alkaline hydrolysis is not significant. In the corresponding reaction with cytidine-3'-phosphate, shown in Fig. 2, nearly 100% of peaks 5 and 6 is obtained at 60°C.

Raising the temperature from 25°C to 60°C accelerates the transamination reaction of cytidine-3'-phosphate about five-fold, and that of poly C about ten-fold. The disproportionate increase in reaction rate obtained with poly C at the higher temperature further supports the contention that conformational changes influence the rate of transamination.

However, whether the transamination reaction is conducted at 25°C or 60°C, a complicating feature is the simultaneous fragmentation of poly C, as seen in Fig. 4. While unreacted poly C is eluted largely in the void volume of the sec diol column (MW cut-off of 100 000 for proteins), transamination of the poly C, in this case with DAO, reveals the generation of shorter segments of poly C and/or aminoalkyl-poly C, especially at the higher reaction temperatures reported in this figure. The shift in peak retention to higher elution volumes was due to a decrease in molecular weight, rather than to the more basic or hydrophobic properties of the added DAO groups. This was confirmed by polyacrylamide gradient gel electrophoresis (data not shown). Furthermore, the HPLC pattern was essentially unchanged when the acetonitrile content of the mobile phase was varied from 0 to 20%.

It should be mentioned, that after extensive use of the column, a significant fraction of the DAO-poly C was retained beyond the total volume when eluted with phosphate buffer alone. The retained peak was not observed when 20% acetonitrile was added to the mobile phase, suggesting hydrophobic interactions. This phenom-



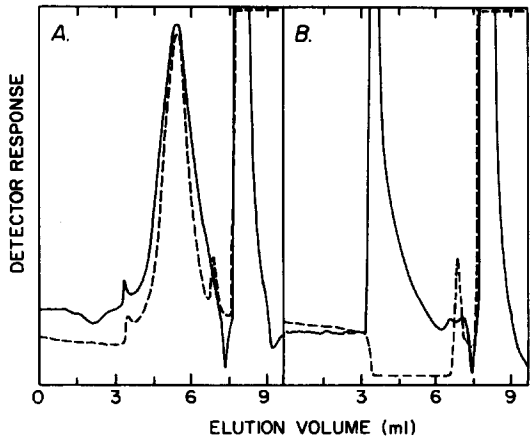


Fig. 4. Change in molecular weight during the modification of poly C with diaminooctane for 264 h at 25°C (– – –), 120 h at 40°C (– · –) and 19 h at 60°C (– ·· –). The reaction mixtures were analyzed on a pair of Brownlee OH-100 SEC columns, as described in Experimental.

Fig. 5. Analysis of the reaction products of DAO-poly C (A) or poly C (B) with Dns chloride on a pair of Brownlee OH-100 sec columns, as described in Experimental. The UV detection wavelength was 270 nm (———). The fluorescence was monitored at 470 nm (– – –) with an excitation wavelength of 250 nm.

enon could be due to the irreversible adsorption of previously injected material to exposed silanol groups, which occur with column aging.

Fig. 5 illustrates the usefulness of the same diol column in monitoring the reaction of aminoalkyl-poly C, in this case DAO-poly C, with a reactive signal compound, such as Dns chloride. Fig. 5B presents the analysis of a control sample, showing the lack of reaction of underivatized poly C with Dns chloride. In this chromatogram the Dns groups are detected by both their absorbance and fluorescence characteristics, whereas poly C gives only an absorbance peak. The drop in the fluorescence baseline under poly C peak is apparently due to quenching of a background of Dns fluorescence by poly C, the dansyl background arising from prior injection of Dns-containing samples.

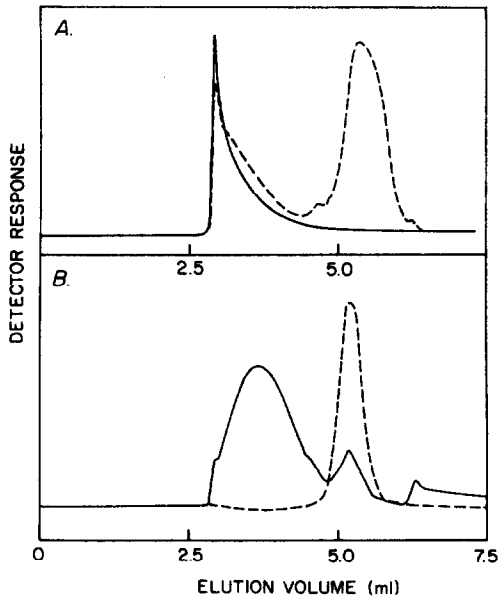
When Dns chloride is allowed to react with DAO-poly C, the chromatogram shown in Fig. 5A is obtained, having a new fluorescence peak which is Dns-DAO-poly C. The shift to later elution for this compound relative to the earlier elution for poly C is primarily a consequence of the shorter chain length of aminoalkyl-poly C.

One application of nucleic acid probes is to form covalent adducts with proteins[2,4]. In view of this interest, a model protein-aminoalkyl-poly C conjugate was prepared and investigated chromatographically. Succinyl-lysozyme was an attractive protein for this purpose, having both a small size and also extended carboxyl groups to facilitate its reaction with aminoalkyl-poly C. The carboxyl groups of the modified protein were activated with isobutyl chloroformate to form the mixed anhydride and reacted with DAO-poly C in a solution of dimethyl sulfoxide and formamide. The resultant product was analyzed on the diol column (Fig. 6A). The control (Fig. 6B) consisted of a mixture of DAO-poly C and succinyl-lysozyme without isobutyl chloroformate. An acetonitrile concentration of 20% in the mobile phase was necessary

to prevent adsorption losses of the succinyl-lysozyme-DAO-poly C adduct. The retention of the product, succinyl-lysozyme -DAO-poly C, is seen to be controlled by the DAO-poly C, due to the larger size of the latter. In this case, the tryptophan residues in lysozyme provided the basis for fluorescence detection. Covalent coupling between DAO-poly C and succinyl-lysozyme was confirmed by collecting the initial peak in the chromatogram of Fig. 6A, concentrating it to raise the ionic strength and thereby overcoming any ionic complexes, reinjecting this sample, and observing only the initial peak. Moreover, the HPLC pattern was unchanged after exposure of the conjugate to 3 *M* guanidine–hydrochloride or 9 m*M* sodium dodecyl-sulfate.

Analysis of the succinyl-lysozyme-DAO-poly C was also successful on a wide-pore C_{18} reversed-phase column, as shown in Fig. 7. On this column, succinyl-lysozyme in the control mixture was retained more than the DAO-poly C, apparently because succinyl-lysozyme is less polar. Interestingly, the retention of the conjugate, succinyl-lysozyme-DAO-poly C, seems to be controlled by the succinyl-lysozyme moiety (Fig. 7, peak 3). Whether this will be a frequent property of covalent nucleoproteins by this separation mode remains to be seen.

To investigate the stoichiometry of succinyl-lysozyme-DAO-poly C, the conjugate was first isolated on the wide-pore C_{18} column (Fig. 7, peak 3). Unreacted succinyl-lysozyme was removed by reinjecting the conjugate into the sec diol column and collecting only the high-molecular-weight-fraction. This fraction was analyzed



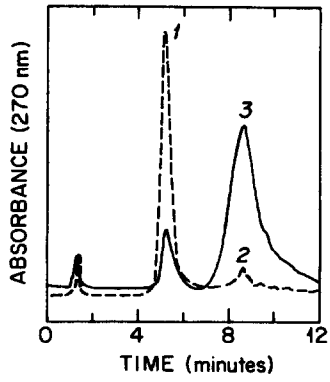


Fig. 6. Analysis of the reaction products of DAO-poly C and succinyl-lysozyme with (A) and without (B) prior activation of succinyl-lysozyme with isobutyl chloroformate. The mixtures were analyzed on a pair of Brownlee OH-100 sec columns. The UV absorbance was monitored at 270 nm (———). The fluorescence of lysozyme was followed at 320 nm (– – –) with an excitation wavelength of 280 nm.

Fig. 7. Separation of DAO-poly C (1) and DAO-poly C coupled to succinyl-lysozyme (3) on a Vydac C_{18} wide-pore column (———). The coupling was carried out by the mixed-anhydride method. The control (– – –) consisted of a mixture of DAO-poly C (1) and succinyl-lysozyme (2).

for succinyl-lysozyme by the method of Lowry *et al.*[14]. Based on the absorbance spectrum of the isolated product, it was determined that approximately one molecule of succinyl-lysozyme was bound per 45 cytidine residues. The influence of the protein/nucleic acid ratio on this chromatographic behavior requires further study. Also relevant is the tendency of proteins to be denatured on a reversed-phase chromatographic surface[15].

Future work

Now that procedures have been established for preparing and characterizing a polynucleotide-protein conjugate in which the polynucleotide contains reactive amino groups, it is of interest to extend these procedures to other proteins, and to attach signal groups to the polynucleotide component. In particular, the polymeric attachment of an electrophoric release tag-labeled polynucleotide to an antibody should allow sensitive detection of this antibody in certain types of immunoassay procedures. This includes the potential to employ several different antibodies simultaneously, each labeled with a different release tag, as has been discussed previously[6].

CONCLUSIONS

(1) The rate of the transamination reaction of poly C with diaminoalkanes in the presence of bisulfite can be increased significantly at 60°C relative to the rate at 25°C without significant formation of side products. However, simultaneous fragmentation of the poly C occurs at these elevated temperatures.

(2) An HPLC diol packing having a MW range of up to 100 000 D for proteins is useful for the analysis of conjugation products of aminoalkyl-poly C both with a small signal group such as Dns or a small protein such as succinyl-lysozyme.

(3) The apparent control of retention on a wide-pore (33 nm) C_{18} reversed-phase HPLC column by the protein moiety of succinyl-lysozyme-diaminooctyl-poly C encourages the further investigation of polynucleotides as polar backbones for poly-signal attachment to proteins.

ACKNOWLEDGEMENTS

This work was supported by DARPA Contract N00019-82-K-0811 administered by the Office of Naval Research. Contribution No. 245 from the Barnett Institute.

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