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Synthesis and Characterization of Some Covalent Dextran-Polyoxyethyleneglycol Derivatives

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ABSTRACT

Covalent dextran-polyoxyethyleneglycol compounds were prepared in order to obtain amphiphilic polymers which may, possibly due to incompatible structures, undergo phase separation at the molecular level. Characterization of these derivatives was carried out by n.m.r. spectroscopy, gel permeation chromatography and low angle laser light scattering (LALLS) measurements.

INTRODUCTION

Since polysaccharides are involved in an impressive and ever growing number of studies and applications concerning biomedical and other topics, it is not surprising a wide range of derivatives of these polymers have been synthesized. This field is well documented (Yalpani, 1985; Yalpani, 1988). Among those chemical modifications, the synthesis of hydrophobic polysaccharide derivatives leads to a new class of products for a variety of applications, including hydrophobic chromatography (Yon, 1972; Er-el *et al.*, 1972; Hofstee, 1973; Rosén, 1978; Morgenthaler, 1982) and applications where surfactant properties are required (Pitha *et al.*, 1979; Landoll, 1982; Nemat-Gorgani & Karimian, 1984).

We are starting a study on the covalent immobilization of polyoxyalkyleneglycols (POAG) onto dextran. The driving forces for this project

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are the following. Firstly, although polyoxyalkyleneglycols are generally considered and used as hydrophilic species, they may also be regarded as mild hydrophobic compounds and exploited as such, for instance in chromatography, for the separation of proteins or cells, without the drawbacks often presented by traditional hydrophobic interaction chromatography (Ling & Mattiason, 1983; Shibusawa *et al.*, 1987; Mathis *et al.*, 1989). The covalent coupling of POAG onto dextran may therefore afford amphiphilic derivatives, whose hydrophobic character may be modulated by various parameters, such as the type of POAG, its chain length and the degree of substitution.

In addition, dextran and POAG are well-known incompatible polymers, and have been widely used, for this property, in techniques such as 'Partition between aqueous polymer two-phase systems' (Albertsson, 1971). When covalently bound together, the resulting derivative may possibly, owing to the presence of two incompatible structures, undergo phase separation at the molecular level and form aggregates of colloidal dimension.

This first paper reports on the synthesis and the characterization by ^{1}H-n.m.r., 13C-n.m.r. spectroscopies, low angle laser light scattering technique (LALLS) and gel permeation chromatography, of derivatives obtained by immobilization onto dextran T40 of α-methoxy, ω-amino-polyoxyethylene and α-benzyloxy, ω-aminotetraoxyethylene.

MATERIALS AND METHODS

Dextran T40 was obtained from Pharmacia (Sweden). Polyoxyethylene-glycol monomethyl ether (MeO-PEG-OH) (molecular weight 750) and tetraethyleneglycol (HO-TEG-OH) were supplied by Aldrich (FRG). All other chemicals were reagent grade and used without further purification.

^{1}H-n.m.r. and ^{13}C-n.m.r. were run at room temperature on a 200 MHz Bruker AM spectrometer, respectively at 200 and 50 MHz, in D_2O solutions with sodium 3-(trimethylsilyl)-1-propane sulfonate (DSS) as an internal reference.

Gel permeation chromatography was carried out on Ultrogel AcA 34 and AcA 54 (IBF, France), at room temperature, using 0·1 M NaCl as the mobile phase. Refractometric detection of the effluent was monitored with a IOTA R.I detector (Jobin-Yvon, France).

Low angle laser light scattering (LALLS) experiments were performed at room temperature, with 0·1 M NaCl polymer solutions on a Chromatix KMX 6 (Milton Roy, USA) apparatus. Refraction index increments

(dn/dc) were measured on a Brice-Phoenix differential refractometer (Virtis, USA) equipped with a helium-neon laser light source (633 nm).

PREPARATION OF THE DEXTRAN-POLYOXYETHYLENE CONJUGATES

Activated dextran

Activated dextran was prepared according to Sacco *et al.* (1989) by reaction with epichlorohydrin, in the presence of $Zn(BF_4)_2$, under pH conditions carefully controlled to avoid extensive depolymerization. The resulting polysaccharide contained 0·68 mmol Cl/g polymer. ^{1}H-n.m.r. (D_2O) δ 3·30–4·20 p.p.m. (m, chlorohydrin, dextran C_2–C_6) δ 5·0 p.p.m. (1H, m, dextran C_1). ^{13}C-n.m.r. (D_2O) δ 48 p.p.m. (CH_2Cl) 68–78 p.p.m. (chlorohydrin, dextran C_2–C_6) 100 p.p.m. (dextran C_1).

α-Methoxy, ω-aminopolyoxyethylene

α-Methoxy, ω-aminopolyoxyethylene (MeO-PEG-NH_2) was prepared by successive treatment of monomethoxy-PEG-OH with thionyl chloride, then ammonia at 100°C under pressure, according to a procedure already described (Buckmann *et al.*, 1981). The overall yield was 60%, and the amine content of the polymer 1·02 mmol NH_2/g. (Theoretical 1·03 mmol NH_2/g). ^{1}H-n.m.r. (D_2O) δ 2·85 p.p.m. (2H, t, $-\underline{CH_2}NH_2$) 3·38 p.p.m. (3H, s, $-OCH_3$) 3·50–3·95 p.p.m. (m, $-CH_2-CH_2-O$).

α-Benzyloxy, ω-aminotetraoxyethylene

α-Benzyloxy, ω-aminotetraoxyethylene (BzlO-TEG-NH_2) was prepared in four steps, starting from tetraoxyethyleneglycol (HO-TEG-OH). First, tetraoxyethyleneglycol monobenzyl ether (BzlO-TEG-OH) was synthesized under phase transfer conditions affording almost exclusively the monobenzyl derivative, according to a procedure slightly adapted from that published by Gartiser (1982). The three following steps, resulting in the amino derivative were conducted, adapting the procedure described by Gartiser (1982). Briefly, it consists of activation of the terminal OH group by methane sulfonyl chloride, then substitution of the mesyl group by NaN_3, and finally reduction of the resulting azide by $NaBH_4$. All experimental conditions were carefully designed and intermediary products controlled by thin layer chromatography in

order to avoid the presence of any α, ω, diamino TEG, in the final derivative. The desired product (76% yield from BzlO-TEG-OH) presented the same characteristic spectral data as those described (Gartiser, 1982).

^{1}H-n.m.r. (D_2O) 2·75 p.p.m. (2H, t, $-\underline{CH_2}-NH_2$) 3·40–3·83 p.p.m. (14H, m, $-OCH_2(CH_2OCH_2)_3-$) 4·55 p.p.m. (2H, s, $Ar-\underline{CH_2}-$) 7·40 p.p.m. (5H, m, Ar). ^{13}C-n.m.r. (D_2O) δ 140·5, 131·6, 131·2 p.p.m. (Ar-C) 75·6, 74·2, 72·5, 71·4 p.p.m. (CH_2-CH_2O) 42·5 p.p.m. (CH_2-NH_2).

Coupling of aminopolyethers onto dextran-chlorohydrin

In typical experiments, dextran-chlorohydrin (0·79 g; 0·54 mmol Cl) and the desired aminopolyether (MeO-PEG-NH_2 0·53 g, 0·54 mmol NH_2 or BzlO-TEG-NH_2 0·158 g, 0·54 mmol NH_2) were dissolved in water (10 ml). After the pH had been adjusted to pK + 1 (pH ~ 10–11), the mixture was stirred under nitrogen, at room temperature, for various periods of time ranging from 6 to 96 h (for instance 63 h for MeO-PEG-NH_2, 96 h for BzlO-TEG-NH_2 for the derivatives discussed in Figs 3–5). Cl/NH_2 ratios different from 1, as well as another temperature (50°C) were also investigated.

At the end of the reaction, the mixture was precipitated into acetone. The dextran-polyether thus cleared of most of the unreacted amino derivative was dissolved in water for further purification by gel permeation chromatography (GPC) on a column (2·5 × 60 cm) packed with Ultrogel AcA 54 and eluted with 0·1 M NaCl. The desired fractions were pooled, dialysed for 48 h against water and then freeze-dried for further physico-chemical characterization.

RESULTS AND DISCUSSION

Coupling experiments with the two polyether amino derivatives were conducted in water under inert atmosphere. The initial pHs were adjusted to values (pH ~ 10–11) affording amino groups in their unprotonated forms (pH = pK + 1). The influence of reaction time, temperature and stoichiometry was investigated. The expected resulting derivatives are schematically represented in Fig. 1.

After desired reaction times, the crude mixtures were fractionated on Ultrogel AcA 54 (exclusion limit for proteins: 70 000 daltons). The fractions containing the dextran-polyether derivative were collected separately from those containing the unreacted amino compound, extensively dialysed against water, then freeze-dried for subsequent

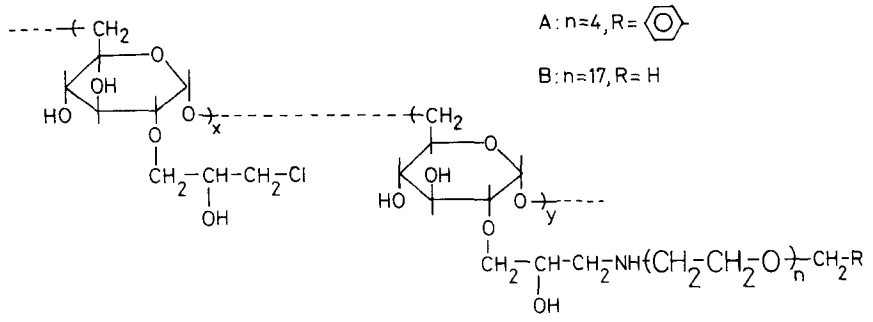

Fig. 1. Schematic representation of derivatives obtained by coupling dextran T40-chlorohydrin with α-benzyloxy, ω-aminotetraoxyethylene (A) or α-methoxy, ω-aminopolyoxyethylene (B).

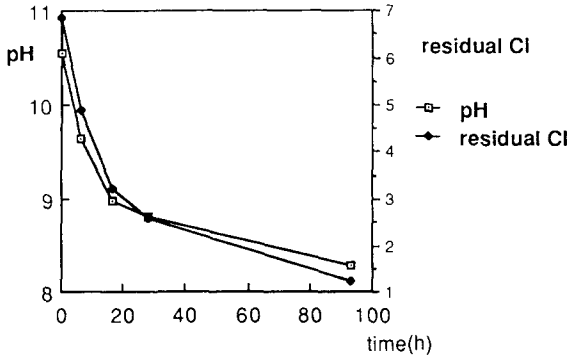

Fig. 2. Final pH of the reaction mixture and residual chlorine (10^{-4} mol/g) on the derivative obtained, after various reaction times, by coupling dextran-chlorohydrin with BzlO-TEG-NH_2.

residual chlorine determination, n.m.r. spectroscopy and LALLS measurements.

The results of polymers' residual chlorine analysis and the final pH values versus time for the reaction of dextran-chlorohydrin with BzlO-TEG-NH_2 are plotted in Fig. 2. The two curves follow the same trend. They are simple and there are immediate indications that the coupling reaction advances, since both the pH decrease and the chlorine consumption are directly related to the amount of HCl evolved as the reaction proceeds. However, control experiments carried out under the same conditions, but in the absence of polyether amino derivatives, indicate that limited, though not negligible hydrolysis of the dextran-linked chlorohydrin occurs at the basic pHs used, and therefore makes inaccurate the direct correlation of polymers' residual chlorine analysis with the

actual amount of polyether bound. Very similar results are obtained, when the coupling reaction is carried out with MeO-PEG-NH_2.

Other experiments, conducted at higher temperature or at different stoichiometries (results not shown) demonstrated, as could be anticipated, that the amount of PEG (or TEG) immobilized onto dextran, increased with time, temperature and the initial NH_2/Cl ratio.

The ^1H and ^{13}C-n.m.r. spectra of representative coupling derivatives obtained with MeO-PEG-NH_2 and BzlO-TEG-NH_2 are presented in Figs 3–5.

The ^1H-n.m.r. spectrum of the compound obtained by reaction of MeO-PEG-NH_2 with dextran-chlorohydrin (Fig. 3) shows the

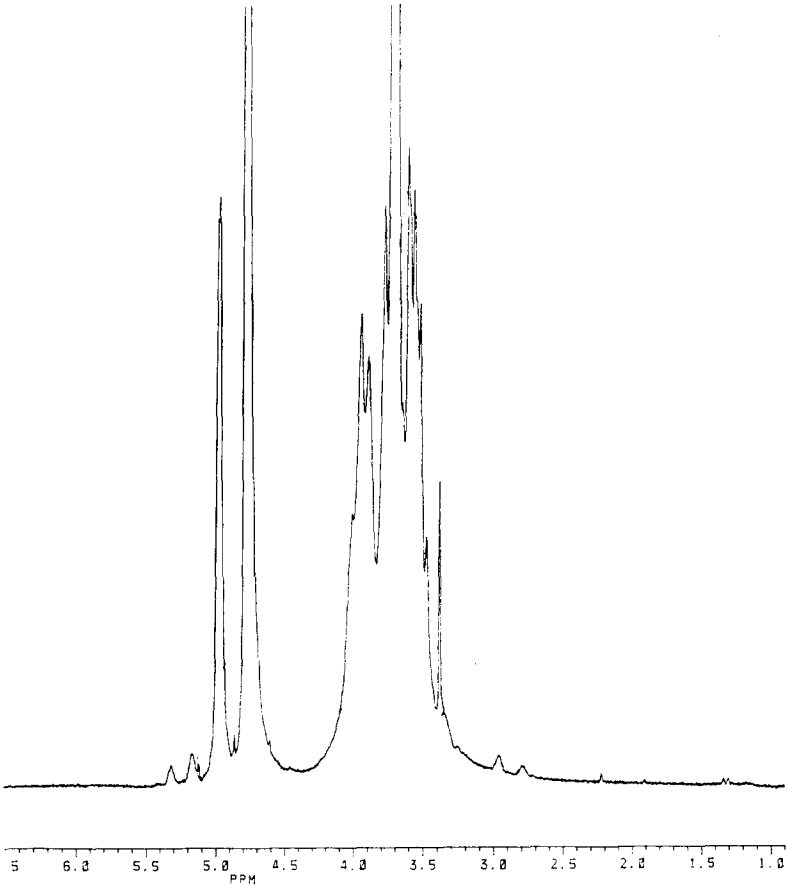

Fig. 3. ^1H-n.m.r. spectrum of the coupling derivative obtained by reaction of dextran-chlorohydrin with α-methoxy, ω-aminopolyoxyethylene (see Fig. 1(B)) (reaction time 63 h, room temperature).

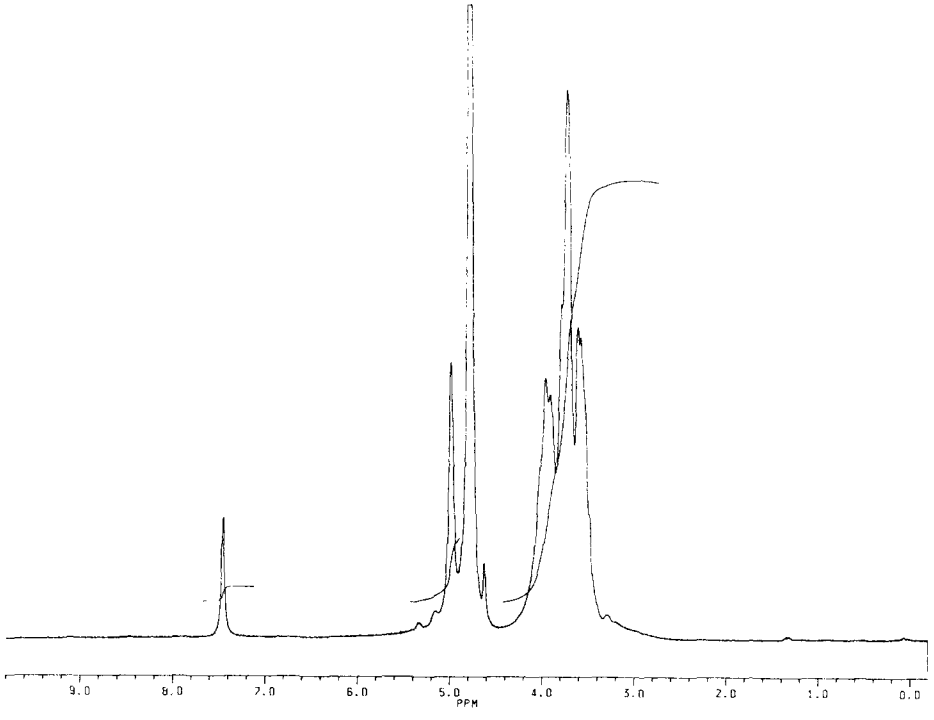

Fig. 4. ^{1}H-n.m.r. spectrum of the coupling derivative obtained by reaction of dextran-chlorohydrin with α-benzyloxy, ω-aminotetraoxyethylene (see Fig. 1(A)) (reaction time 96 h, room temperature).

appearance of a singlet at 3·38 p.p.m., characteristic of the terminal methoxy group of the polyether. In addition, no signal corresponding to $\underline{CH_2}$—NH_2, otherwise centred at 2·85 p.p.m., is detected in the coupling derivative, indicating the absence of residual unreacted polyether and thus confirming the efficiency of the GPC purification. The amount of polyether bound onto dextran can be calculated by the ratio of the integral of the —OCH_3 group (3·38 p.p.m.) to that of the anomeric proton of the polysaccharide (5·0 p.p.m.). Despite the use of spectrometer built-in signal treatments to obtain resolution-enhanced spectra and a better quantification of peak areas, the amount of polyether immobilized can be only estimated. The value of the molar ratio obtained in the example described is 0·11, i.e. about 1 polyether group every 10 glucose units.

Figure 4 shows the ^{1}H-n.m.r. spectrum of the derivative obtained by coupling BzlO-TEG-NH_2 onto dextran. One observes the absence of the $\underline{CH_2}$—NH_2 signal at 2·75 p.p.m., together with the appearance of two singlets, characteristic of the aromatic protons (7·40 p.p.m.) and of the

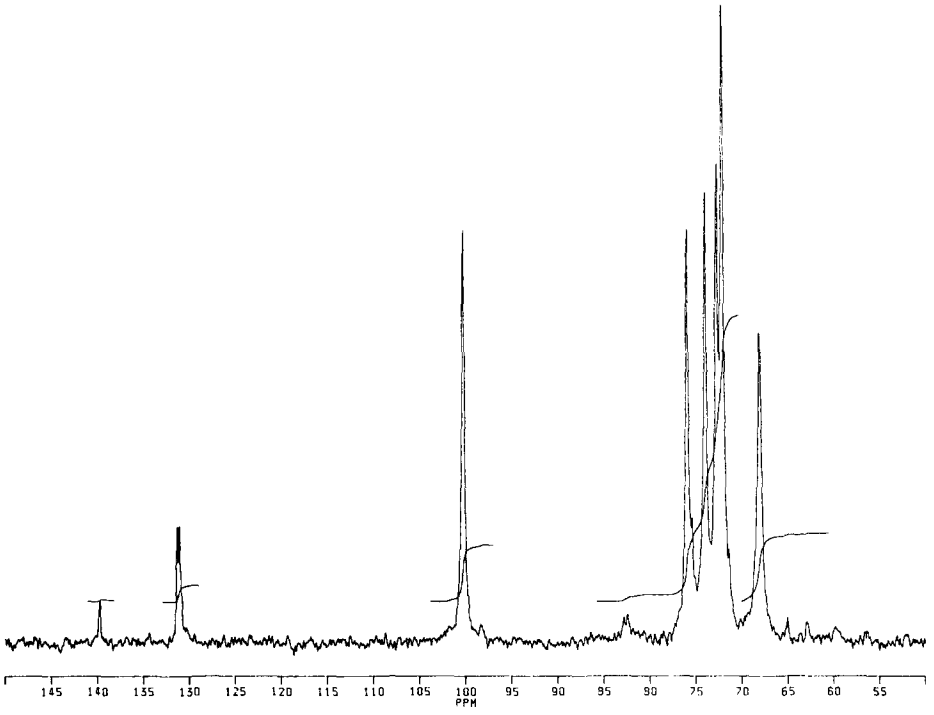

Fig. 5. ^{13}C-n.m.r. spectrum of the coupling derivative obtained by reaction of dextran-chlorohydrin with α-benzyloxy, ω-aminotetraoxyethylene (reaction time 96 h, room temperature). Use of inverse gate sequence, pulse angle 30°, pulse delay 12s, concentration 100 mg/ml for quantitative analysis).

benzylic methylene protons (4·55 p.p.m.). The amount of polyether bound can be estimated by the ratio of the integral of the aromatic protons to that of the anomeric proton of dextran. An alternative approach, intended to rule out problems due to water interferences consists of considering the integral of the multiplet (3·2–4·3 p.p.m.) rather than that of the anomeric proton. Obviously in this case, the 16 ethylene protons of the tetraoxyethylene chain must not be taken into account and are to be previously deduced from the integral value. Both approaches give similar results, i.e. 62 mmol polyether/mol glucose unit in the particular example above discussed. ^{13}C-n.m.r. spectrum (Fig. 5) affords unambiguous confirmation of the ^{1}H-n.m.r. analysis. The ratio of integral values of the 5 aromatic carbon atoms (130 p.p.m.) and of the anomeric C_1 atom of dextran (120 p.p.m.) leads to a value of 66 mmol polyether/mol glucose unit, in close agreement with that afforded by ^{1}H-n.m.r. analysis.

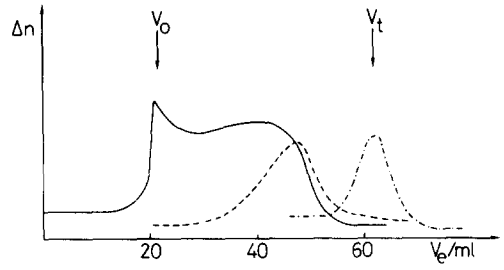

Fig. 6. Elution profiles of dextran-polyether (——) (dextran-TEG-OBzl or dextran-PEG-OMe); dextran-chlorohydrin (– – –); polyether (—·—·—) (BzlO-TEG-NH_2 or MeO-PEG-NH_2) on an Ultrogel AcA 34 column (1·6 × 30 cm). Eluent 0·1 M NaCl, 20°C, flow rate 13·5 ml/h. Refractometric detection. The arrows indicate the void volume (V_0) and the total permeation volume (V_t).

The corresponding value obtained from determination of polymer residual chlorine is 82 mmol/mol glucose unit, thus confirming, as pointed out previously in this paper, the influence of competitive hydrolysis during the aminolysis process, and the inaccuracy which would result from a direct correlation of polyether fixation with chlorine consumption.

GPC analysis on Ultrogel AcA 34 with a high exclusion limit (300 000 daltons for proteins) gave the results displayed in Fig. 6. Dextran-chlorohydrin is eluted within the permeation limits of this gel, whereas dextran-PEG-OMe as well as dextran-TEG-OBzl are eluted exclusively in the void volume of the column, indicating the presence of a high molecular weight polydisperse species. Weight average molecular weight measurements (M_W) by LALLS confirm this phenomenon. Dextran T40 and its chlorohydrin derivative have molecular weights of the same order of magnitude, respectively 35 000 and 26 000, indicating that only limited depolymerization occurs during the activation reaction. In contrast, the light scattering of derivatives obtained by coupling dextran with MeO-PEG-NH_2 as well as with BzlO-TEG NH_2, corresponds to high molecular weight aggregates ($3 \cdot 10^5$–10^6) with 2nd virial coefficient values close to zero or even negative, characteristic of strong preferential polymer–polymer interactions.

Studies by photon correlation spectroscopy, as well as other techniques such as dye solubilization or surface tension are currently in progress. They should bring additional valuable information on those dextran-polyether structures.

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