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Covalent Coupling of a Short Polyether on Sodium Alginate: Synthesis and Characterization of the Resulting Amphiphilic Derivative

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ABSTRACT

A covalent sodium alginate–polyoxyethylene model derivative was prepared by reductive amination of aldehydic sodium alginate, in order to obtain a polymer with amphiphilic properties. Characterization of this derivative was carried out by NMR spectroscopy, viscosity measurements and low-angle laser light scattering. The data obtained suggest a limited capacity of the polymer structure to expand, resulting from intramolecular self-aggregation and formation of hydrophobic microdomains.

INTRODUCTION

With their rich abundance, low cost and general versatility, natural polysaccharides play prominent roles in various industrial and biomedical applications. In addition, the demand for derivatives with specific properties such as viscosity, gelling ability, metal-chelating capacity or drug-releasing potential, has prompted the research of increasingly

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sophisticated chemical modifications of these polymers (Yalpani, 1985, 1988).

The alginate salts (polyuronic acid salts) which are found in brown seaweed cell walls, differ from most other polysaccharides in that they show a sol gel transition when simply submitted to modifications of their ionic environment (substitution of Na^+ by divalent cations such as Ca^{2+}). The use of Ca^{2+} alginate gels or beads has been described for diffusional chromatography and solids separation techniques (Kierstan, 1981), but many of their uses concern the immobilization of a variety of cell types, subcellular organelles, enzymes etc. (Kierstan & Bucke, 1977).

We are starting a study on the covalent immobilization of various polyethers onto alginic acid. Although polyoxyalkylene glycols (POAG) are mainly considered and used as hydrophilic species, they may also be regarded as mildly hydrophobic compounds, and have already been exploited as such, in hydrophobic chromatography (Ling & Mattiason, 1983; Shibusawa *et al.*, 1987; Mathis *et al.*, 1989). The covalent coupling of POAG onto Na^+ alginate may therefore give amphiphilic derivatives, whose hydrophobic character can be modulated by varying the type of POAG, its chain length and the degree of substitution of the alginate.

The present article reports the covalent coupling of a short polyether onto previously activated sodium alginate and the properties of the resulting derivatives.

MATERIALS AND GENERAL METHODS

Sodium alginate (medium viscosity, derived from *Macrocystis pyrifera*) was obtained from Sigma (USA). It contained mannuronic and guluronic acid residues in the ratio 1·56:1, as determined by ^{1}H-NMR according to Grasdalen *et al.* (1979). It had an intrinsic viscosity of 10 dl/g in 0·1 M NaCl at 25°C and its molecular weight ($\bar{M}_w$) was 295 000, as determined by low-angle laser light scattering (LALLS) measurements ($dn/dc = 0{\cdot}155$).

Tetraoxyethylene glycol monobenzyl ether was prepared according to the method described by Gartiser (1982), under phase transfer conditions. All other chemicals were reagent grade and used without further purification. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck, RFA) using ethylacetate or ethylacetate–petroleum ether as eluents.

Alginate-containing reaction mixtures were purified by dialysis against distilled water, using cellulosic Visking dialysis tubes (molecular weight cut-off 6000–8000) obtained from Poly-Labo (France). Solutions

were concentrated under reduced pressure below 40°C and subsequently freeze-dried. Gel permeation chromatography (GPC) experiments were performed on Ultrogel AcA 54 and Ultrogel AcA 22 (IBF, France) with 0·25 M NaCl as eluent. Refractometric detection of the effluent was monitored with an IOTA RI detector (Jobin-Yvon, France).

Intrinsic viscosities were determined at 25°C in an Ubbelhode viscosimeter with 0·1 M NaCl polymer solutions. Weight average molecular weights ($\bar{M}_w$) were determined from LALLS measurements on a Chromatix KMX 6 (Milton Roy, USA). Polymer solutions ($\approx$ 1 mg/ml) in 0·1 M NaCl, prepared from ultrapure water (Milli-Q water purification system, Millipore) were clarified by filtration through single use 0·22 or 0·45 μm Millex filter units (Millipore) and slowly flowed into the scattering cell. Excess Rayleigh factors $\bar{R}_\theta$ ($\bar{R}_\theta = R_\theta$ solution $- R_\theta$ solvent) were measured at various polymer concentrations and plotted versus the concentration. The best linear fit to the data affords the inverse weight average molecular weight ($1/\bar{M}_w$), by extrapolation to zero concentration. The second virial coefficient value is obtained from the slope. Refraction index increments (dn/dc) were measured on a Bryce–Phoenix differential refractometer (Virtis, USA) equipped with a helium–neon laser light source (633 nm).

^{1}H-NMR spectra were run on D_2O or $CDCl_3$ solutions, on a Bruker AM 200 MHz spectrometer, respectively, with sodium 3-(trimethylsilyl)-1-propane sulfonate (DSS) and tetramethyl silane (TMS) as internal references. ^{13}C-NMR spectra of aqueous samples were recorded on the same apparatus at 50 MHz with DSS as internal reference. UV spectra were recorded from aqueous solutions on a lambda 5 Perkin Elmer UV/Vis spectrophotometer.

Preparation of α-amino, ω-benzyloxy tetraoxyethylene (BzlO–TEG–NH_2)

Methane sulfonyl chloride (5 ml, 7·4 g, 64·6 mmol) in methylene chloride (20 ml) was slowly added at 0°C to BzlO–TEG–OH (12·24 g, 43 mmol) and triethylamine (12 ml, 8·7 g, 86·1 mmol) dissolved in methylene chloride (30 ml). At the completion of the reaction (4 h at 0°C, monitored by TLC), the mixture was poured into ice-water and extracted with methylene chloride. The combined organic phases were washed successively with dilute HCl, saturated aqueous $NaHCO_3$ and then brine. After drying over $MgSO_4$ and removal of the solvent under reduced pressure, the crude mesylate was obtained (15·24 g). Sodium azide (1·3 g, 20 mmol) was added to 3·54 g of the crude mesylate thus obtained, dissolved in dimethyl formamide (20 ml). The mixture was

stirred at 40°C for 5 h. After cooling, it was poured into water and extracted with ether. Drying over $MgSO_4$, then removal of the solvent, afforded a crude product (2·92 g) which was used in the next step without further purification. $NaBH_4$ (0·76 g, 20 mmol) was added to the BzlO-TEG-N_3 thus obtained dissolved in 2-propanol (10 ml). The reaction mixture was stirred at 50°C for 5 h. After cooling, the reaction mixture was poured into water, acidified with 1 N HCl and extracted with ether in order to remove non-aminated products. The aqueous layer was made alkaline with 1 M NaOH, then extracted with methylene chloride. Drying over $MgSO_4$ and removal of the solvent under reduced pressure afforded the desired product (2·14 g, 7·61 mmol, 76% from BzlO-TEG-OH). It gave the same characteristic spectral data as previously described (Gartiser, 1982): ^{1}H-NMR ($CDCl_3$)δ: 2·25 ppm (2H, *s*, NH_2), 2·81 ppm (2H, *t*, -CH_2-NH_2), 3·40–3·83 ppm (14H, *m*, -$OCH_2(CH_2OCH_2)_3$-), 4·57 ppm (2H, *s*, Ar-CH_2-), 7·21–7·51 ppm (5H, *m*, Ar). ^{13}C-NMR (D_2O)δ: 140·5, 131·6, 131·2 ppm (Ar, C), 75·6, 74·2, 72·5, 71·4 ppm (CH_2-CH_2O), 42·5 ppm (CH_2-NH_2).

Preparation of the aldehydic alginate (2) (Fig. 1)

Sodium alginate (1) (Fig. 1) (1% w/v, 100 ml H_2O) was treated by a mixture of 1-propanol (25 ml) and 0·25 M sodium metaperiodate (4 ml) theoretically sufficient to produce a 20% oxidation ratio. Experiments were carried out in the dark, at 4°C for 24 h. After addition of ethyleneglycol (1 ml) to reduce any residual periodate, then aqueous $NaHCO_3$ until neutrality, the reaction mixtures were extensively dialysed against

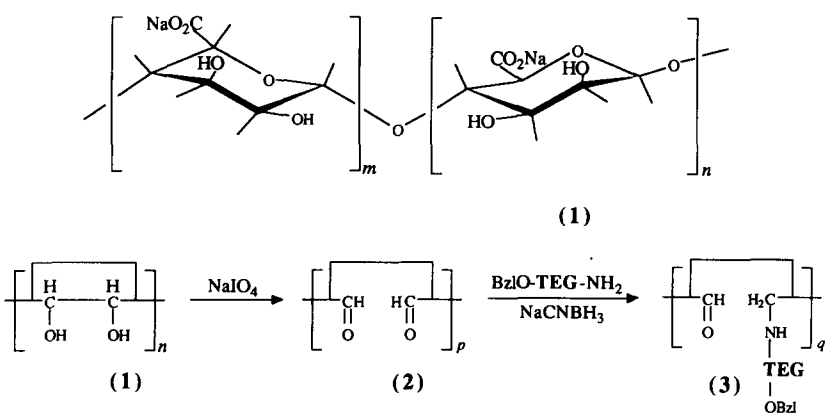


Fig. 1. Schematic representation of the periodate oxidation of sodium alginate (1) and of the subsequent reductive amination of the resulting aldehydic alginate (2) to afford the alginate TEG-OBzl derivative (3).

water and subsequently freeze-dried. Average molecular weight: $\bar{M}_w = 44\,000$ ($dn/dc = 0{\cdot}150$).

Preparation of the alginamine (3) (Fig. 1)

A solution of the BzlO–TEG–NH_2 (0·437 g, 1·54 mmol) in phosphate buffer (0·2 M, pH 7, 2 ml), was added to a solution of the aldehydic alginate (0·455 g) and $NaCNBH_3$ (0·29 g, 3·17 mM) in the same buffer (20 ml). The reaction mixture was stirred at room temperature for 24 h, dialysed and then freeze-dried to afford the crude alginamine. The unreacted BzlO–TEG–NH_2 ws removed upon precipitation of the sodium alginamine by aqueous $CaCl_2$, filtration and washing of the precipitate with ethanol. The calcium–alginamine was then dissolved into aqueous Na_2CO_3. After dialysis and freeze-drying, the pure alginamine was obtained free of residual BzlO–TEG–NH_2, as demonstrated by gel filtration chromatography. Average molecular weight was $\bar{M}_w = 37\,000$ ($dn/dc = 0{\cdot}143$).

RESULTS AND DISCUSSION

Treatment of Na^+ alginate by sodium metaperiodate, in order to introduce aldehyde groups, was the selected approach to activate this polysaccharide. As a matter of fact, the activation of hydroxyl or carboxyl groups directly available on the polysaccharide backbone presents the following drawbacks:

1. weak reactivity of hydroxyl groups — only the recently described acetylation proceeds successfully (Skjak-Braek *et al.*, 1989);
2. the synthesis of esters or amides may result in difficulties arising from an excessive consumption of alginate carboxyl groups. As a matter of fact, when sol–gel transition is concerned, these groups are essential to the gelation process.

The coupling reaction was carried out with α-amino, ω-benzyloxy tetraoxyethylene, taken as a simple and preliminary model to a more wide-spread study, according to the scheme in Fig. 1.

Synthesis of α-amino, ω-benzyloxy tetraoxyethylene (BzlO–TEG–NH_2)

Tetraethylene glycol monobenzylether (BzlO–TEG–OH) was prepared according to the method described by Gartiser (1982), under the general conditions of phase transfer between an aqueous (H_2O + NaOH) and an

organic (BzlCl + HO–TEG–OH) phase with HO–TEG–OH itself as the phase transfer catalyst. To obtain preferentially the monobenzyl ether in good yield and simultaneously limit the dibenzyl ether formation, particular attention must be paid to the slow addition (8 h) of the benzyl chloride to the well-stirred mixture.

The unprotected hydroxyl end of the TEG ether was then activated through mesylation (using mesyl chloride and triethylamine), because of the susceptibility of the mesyl group to nucleophilic displacement. In respect of the ease of preparation, lack of chain cleavage and high reactivity, the mesylate effectively appears as the most satisfactory leaving group (Harris *et al.*, 1984). Substitution with NaN_3 in dimethyl formamide and subsequent $NaBH_4$ reduction of the azide in 2-propanol, afforded the amino derivative with a 76% overall yield. The different steps of the synthesis were followed by TLC and the final product presented the same ^{1}H- and ^{13}C-NMR spectral data as those described (Gartiser, 1982).

Synthesis of the aldehydic alginate (2)

The aldehydic alginate was prepared by oxidation of sodium alginate with sodium metaperiodate.

The synthesis of aldehydic alginate was carried out according to the method previously described by Andresen *et al.* (1977) by treatment of sodium alginate with sodium metaperiodate at 4°C in the dark, for 24 h, in a water:1-propanol mixture (4:1). This approach resulted in an extensive decrease of the intrinsic viscosity of the polymer, which was even more pronounced when the reaction was performed on a larger scale.

If this decrease can be partly attributed to an increase in the flexibility of the chain (Smidsrod & Painter, 1973), it is also well known from the literature (Painter & Larsen, 1970; Scott *et al.*, 1976; Andresen *et al.*, 1977) that alginate oxidation by metaperiodate generally results in some depolymerization of the chain, even when experiments are carried out in the dark, at low temperature or in the presence of 1-propanol. Yet, in our case the oxidation was still more degradative. This difference may arise from the different nature of our starting alginate. As a matter of fact, the work of Andresen *et al.* (1977) was carried out on a sample derived from *Laminaria digitata* whereas an *M. pyrifera* sample was used in the present study. These two alga, not only have a different ratio of mannuronic/guluronic acids (1·3–1·6 for *L. digitata*; 1·4–1·8 for *M. pyrifera*) (Martin, 1986), but above all a different distribution. *M. pyrifera* sample comprises much more alternating MG blocks (15% for *L. digitata*

(Gacesa, 1988) compared to 26% in our sample, as determined by ^{1}H-NMR spectroscopy). As was pointed out in the literature (Scott *et al.*, 1976), chain-scission during periodate oxidation, preferentially takes place at atypical sugar units in the alginate molecule. The high ratio in our samples, of alternating sequences much more sensitive to hydrolysis than homogeneous blocks, may explain the extensive breakdown of our polymer.

Synthesis of the alginamine (3)

Imine formation requires an optimum pH interval (~ 6–7). In this pH range, reduction of imines with $NaCNBH_3$ occurs, whereas the carbonyl reduction into the corresponding alcohol is very limited. Thus, despite the unfavourable equilibrium for imine formation in aqueous solutions, secondary amines resulting from reductive amination can be obtained in good yield when the reactions are conducted in water at pH 7 (Borch *et al.*, 1971).

The aldehydic alginate was treated with an excess of both BzlO–TEG–NH_2 and $NaCNBH_3$ (see experimental section). This reaction was performed in a number of different solvents: acetate buffer pH 5·9; 1:1 mixture of methanol and 2% acetic acid; phosphate buffer pH 7. Rapid estimation by UV analysis gave immobilization ratios ranging from 5% to 12% and showed that the phosphate buffer was the most appropriate solvent. More detailed studies discussed in the following text were carried out on the coupling derivative obtained in this buffer.

At the end of the reactions, the products purified by extensive dialysis were still contaminated by unreacted BzlO–TEG–NH_2, as demonstrated by GPC analysis. To remove this contaminant, the more efficient procedure involves transforming the crude polymer into its Ca^{2+} salt. The resulting precipitate was filtered and extensively washed with ethanol, then dissolved again in aqueous Na_2CO_3. After dialysis and freeze-drying, the alginamine was obtained in pure form, free of any BzlO–TEG–NH_2, as demonstrated by GPC analysis.

^{1}H-NMR spectrum (Fig. 2b) affords immediate evidence of covalent coupling. The presence of aromatic proton resonances, together with the absence of the signal characteristic of the amine-bearing methylene group are convincing arguments. This distinctive methylene signal is observed (Fig. 2a) when artificial mixtures of BzlO–TEG–NH_2 + aldehydic alginate are prepared. Owing to overlapping of the resonances both in the high field and in the anomeric proton region, quantitative estimation of the immobilization ratio proved unreliable, even when the spectrum is run at 80°C (Fig. 2c).

Additional confirmation of the covalent coupling was unambiguously afforded by ^{13}C-NMR analysis. The spectrum does not exhibit the signal of the carbon bearing the amine function (42·5 ppm) (Fig. 3a) and reveals the presence of signals due to the aromatic carbon atoms (140 and 131 ppm) (Fig. 3b). Furthermore, the ratio of the integral value of the five aromatic carbons (131 ppm) to that of the carboxylic carbon (177 ppm) affords a good estimation of the amount of immobilized polyether. In the present example, this amount was close to 0·12 mol polyether/mol uronic acid unit.

This derivative was analysed, in terms of molecular weight, by different techniques. The LALLS measurement evidenced a slight decrease in the weight average molecular weight (37000 instead of 44000 for the starting aldehydic-alginate), confirmed by GPC analysis on Ultrogel AcA 22 (exclusion limit for proteins: $1{\cdot}2 \times 10^6$ D) (Fig. 4).

This result contrasts with that obtained when coupling BzlO–TEG–NH_2 or MeO–PEG–NH_2 (α-methoxy, ω-amino polyoxyethylene) with

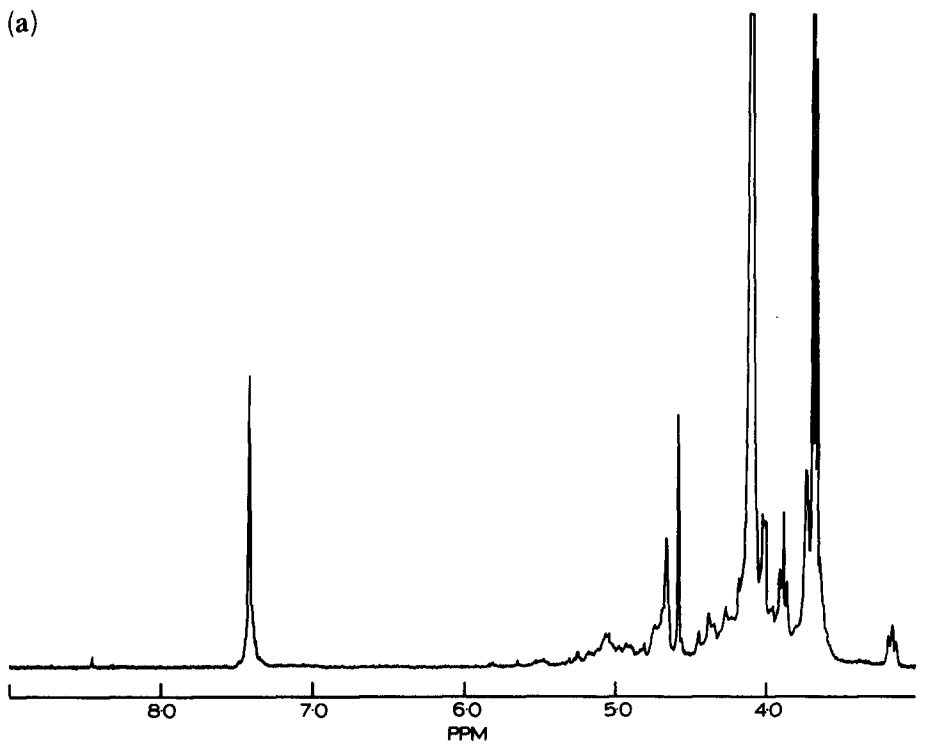
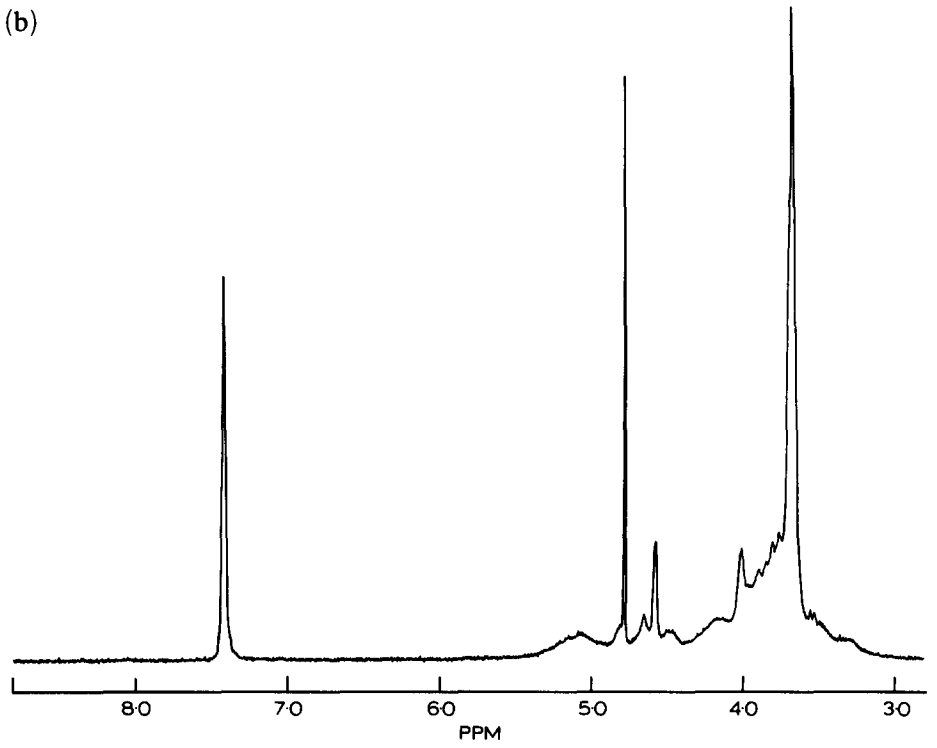


Fig. 2. ^{1}H-NMR spectrum of an artificial mixture of BzlO–TEG–NH_2 + aldehydic alginate, in D_2O at 80°C (2a); ^{1}H-NMR spectra of the coupling derivative, in D_2O at room temperature (2b) and at 80°C (2c).



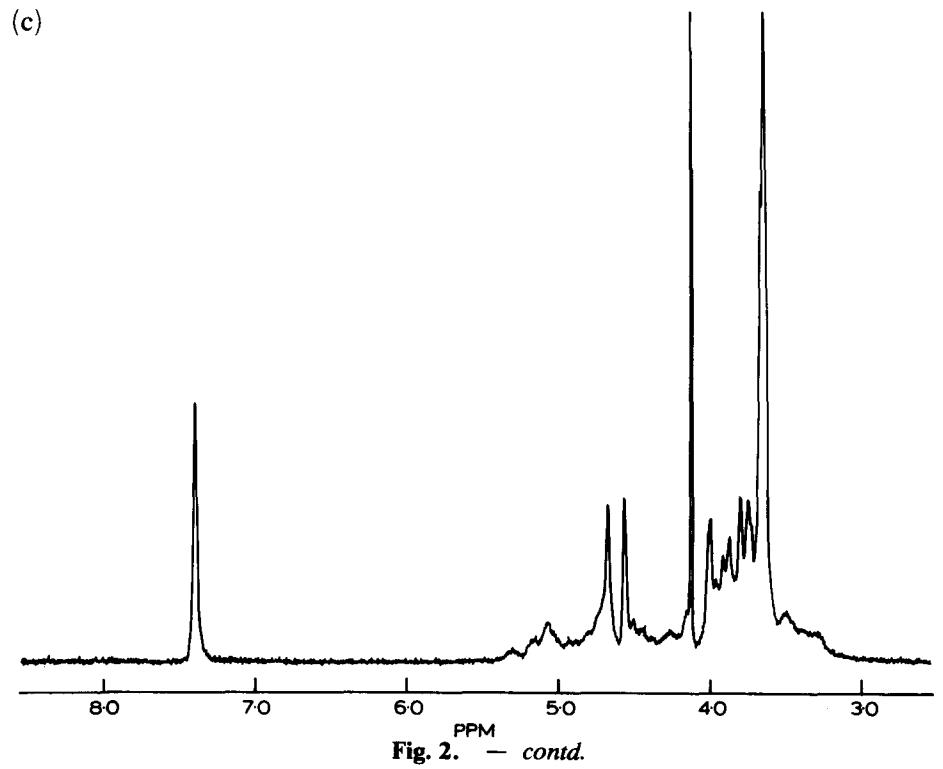


Fig. 2. — *contd.*

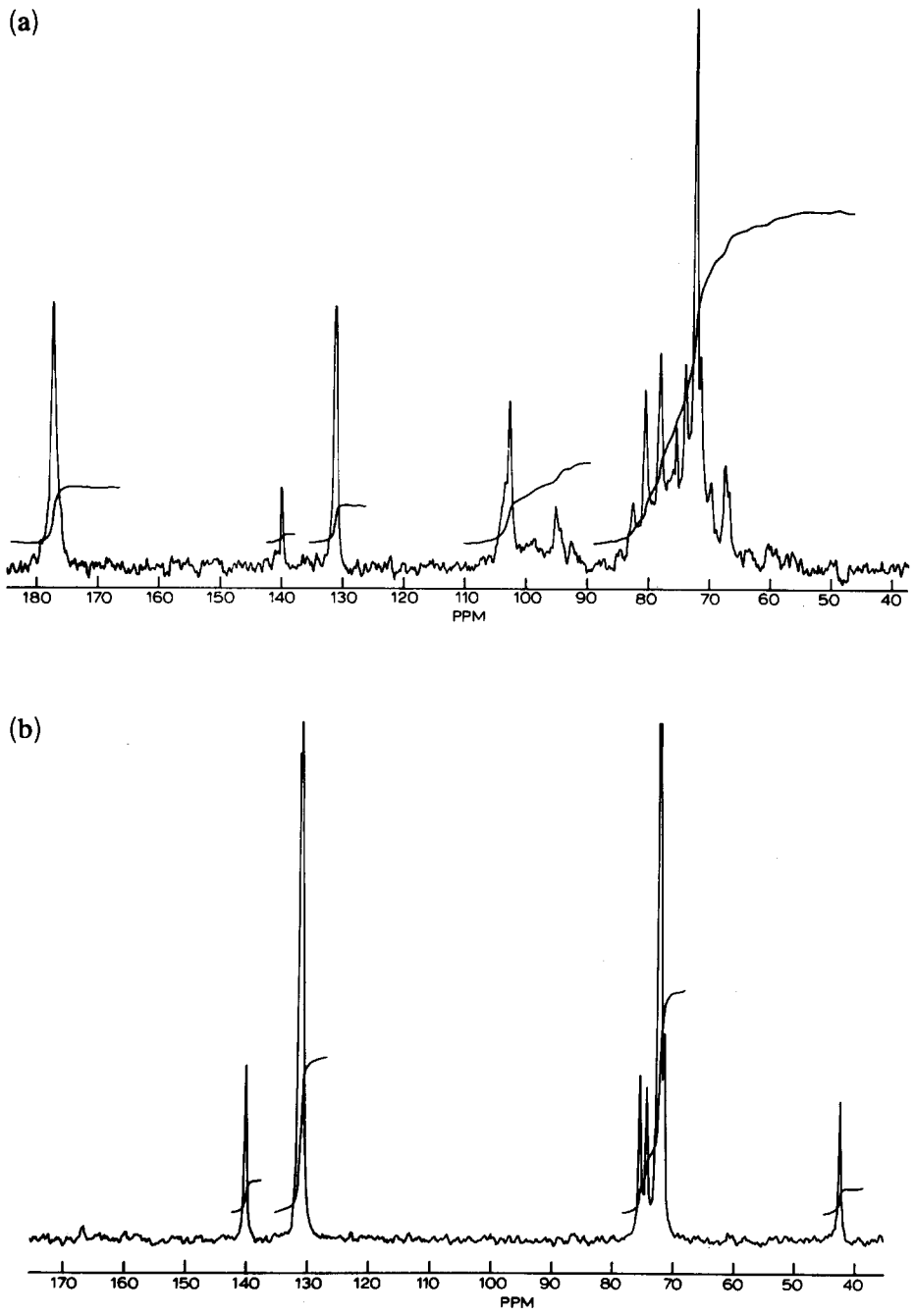


Fig. 3. ^{13}C-NMR spectrum of BzlO–TEG–NH_2 in D_2O at 30°C (3a); ^{13}C-NMR spectrum of the coupling derivative, in D_2O at 30°C. Use of inverse gate sequence, pulse angle 30°, pulse delay 15 s (3b).

dextran ($\bar{M}_w = 40\,000$) (Duval *et al.*, 1990). As a matter of fact, these polysaccharide derivatives behave, when analysed by GPC or LALLS, as high molecular weight aggregates (3×10^5–10^6 D) with second virial coefficient values close to zero or even negative, characteristic of strong preferential polymer–polymer interactions.

Additional LALLS experiments were carried out at high ionic strength (2 M NaCl) or in the presence of a lyotropic salt (0·3 M sodium citrate), in order both to decrease repulsive electrostatic forces due to the carboxylic groups of the alginate backbone and to favour attractive hydrophobic interactions between the polyether chains. None of these two modifications induced the formation of high-molecular-weight structures.

A large decrease in viscosity is observed (0·33 dl/g instead of 1·37 dl/g for the corresponding aldehydic-alginate). In contrast to light scattering which affords absolute molecular weights, viscosity is not only related to molecular weight but is also highly dependent on the shape and the expansion state of the macromolecule.

This decrease of the viscosity after the reductive amination is neither due to a further depolymerization of the chain (aldehyde–alginate (2) and alginamine (3) have very similar molecular weights: 44 000 and 37 000, respectively) nor to the influence of the reduction by borohydride on the rigidity of the chain ($[\eta] = 1{\cdot}33$ dl/g for aldehydic–alginate (2), $[\eta] = 1{\cdot}0$ dl/g for alcoholic–alginate obtained by $NaCNBH_3$ treatment of aldehydic–alginate in the absence of amino–polyether), as previously pointed out by Smidsrod & Painter (1973).

This indicates the prominent role played by the immobilized polyether side chains and probably reflects a limited capacity of the polymer structure to expand. As similarly considered for other amphiphilic polyelectrolytes (Morishima *et al.*, 1981, 1989; Auvray *et al.*,

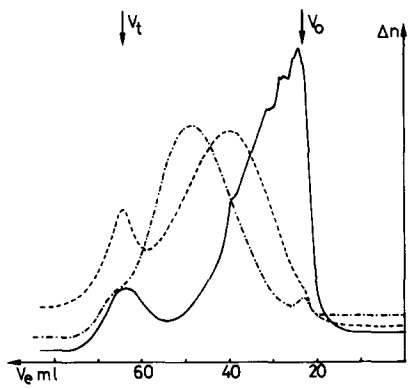


Fig. 4. Elution profiles of sodium alginate (1) (——), aldehydic alginate (2) (– – –) and alginate-TEG–OBzl (3) (– · – · –) on an Ultrogel AcA 22 column (1·6 × 30 cm). Eluent 0·1 M NaCl, 20°C, flow rate 13 ml/h. Refractometric detection. The arrows indicate the void volume (V_0) and the total permeation volume (V_t).

1986), the present situation may be interpreted in terms of competition between the repulsive electrostatic interaction of the carboxylic groups — which results in fully expanded structure — and the attractive interaction of the hydrophobic side-chains — which conversely produces shrinkage. When the hydrophobic interaction prevails over the Coulombic repulsion, *intramolecular* self-aggregation and formation of microdomains occurs.

This hypothesis is consistent with the results concerning the dextran–polyether derivatives (Duval *et al.*, 1990). In this case, the absence of charged groups able to produce repulsive interactions, allows the occurrence of *intermolecular* hydrophobic interactions between polyether side chains and the formation of high-molecular-weight aggregates.

Additional investigations of these two types of polysaccharide–polyether structures by photon correlation spectroscopy, dye solubilization of fluorescence quenching are currently in progress.

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