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Carbohydrate Polymers 53 (2003) 137–143

Carbohydrate Polymers

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Aggregation behavior of hydrophobically modified dextran in aqueous solution: a fluorescence probe study

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Received 5 April 2002; revised 30 May 2002; accepted 19 June 2002

Abstract

Dextrans ($M_W = 11.000$ and $M_w = 40.000$) have been modified with 4-hexyl benzoyl chloride and their aggregation behavior was studied in aqueous solution employing the fluorescent probes pyrene and 1,8 anilinonaphtalene sulfonic acid sodium salt (1,8 ANS). The photophysical studies showed that above a critical concentration the derivatives tend to form aggregates having different properties, which depend on both the degree of substitution (α) and the molecular weight of the sample. The parameter α has a marked effect on the critical aggregation concentrations (CAC) and aggregate properties. Hydrophobic microenvironments can be detected for substituted dextrans having α values varying from 0.01 to 0.19. CAC values decreased by two orders and magnitude when the molecular weight increased from 11 to 40 kDa, leading to formation of more apolar aggregates and diminishing by about 30% the polarity of the microenvironments. Pre-aggregation was evidenced by pyrene excimer emission and intermolecular interactions were responsible by the formation of aggregates leading to solution behaviour similar to that of common surfactants.

Keywords: Dextran; Hydrophobically modified; Fluorescence probe technique; Critical aggregation concentration; Micropolarity

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1. Introduction

The study of modified polysaccharides has been the subject of many recent investigations due to their surface-active properties and potential pharmaceutical, biochemical and medical applications (Chen, Jo, & Park, 1995; Crescenzi, 1994). Naturally occurring polysaccharides such as pullulan (Akioshi, Deguchi, Moriguchi, Yamaguchi, & Suanomoto, 1993; Sunamoto, Sato, Taguchi, & Hamazaki, 1992), amylopectin (Fisher et al., 1998), chitosan (Kjøniksen et al., 1997) and dextran have been modified to obtain amphiphilic polymers capable of forming micellar structures and binding organics solutes in the hydrophobic domain. In the specific case of dextran the hydrophobic modification has been used as an alternative way to improve the aqueous polymer two-phase systems, which are widely used for separation and purification of macromolecules and cell organelles (Lu, Albertsson, Johansson, & Tjerneld, 1994; Lu & Tjerneld, 1997). Recently anphiphilic dextrans have been used to modify the surface of nanospheres and microparticules used as drug delivery systems (Rouzes, Gref, Leonard, Delgado, & Dellacherie, 2000; Fournier, Leonard, & Dellacherie, 1998). The presence of hydrophobic groups bound to the polysaccharide chain can lead to the formation of large aggregates, which can change solution properties such as viscosity, surface tension and solubility. The aggregates might be similar to surfactant micelles and can be formed above a certain critical aggregation concentration (CAC), which will depend on the degree of substitution (α).

In spite of many studies on hydrophobized polysaccharides only a few reports are found on associative behavior in aqueous solution and its dependence on the degree of substitution (α). The aggregation behavior is dependent on the structure and flexibility of the polysaccharide chain, size and/or structure of the hydrophobic residue, and

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0144-8617/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0144-8617(03)00048-1

associative aggregation can take place by inter and/or intramolecular interactions (Evertsson & Nilsson, 1999). CAC for pullulan hydrophobized with cholesterol groups and long alkyl chains was reported by Akioshi, Deguchi, Tajima, Nishikawa, and Suanamoto (1997) on the basis of fluorescence spectroscopy, dynamic and static light scattering, and H^1NMR techniques (Akioshi, et al 1997). Fisher et al. (1998) have also reported CAC values for hydrophobically modified pectin derivatives bearing different degrees of substitution. On the other hand dextran modified with carboxylic acids have showed no clear CAC although hydrophobic microdomains have been detected by the ratio of the intensities I_1/I_3 of pyrene fluorescence (Zhang, Pelton, & Wagberg, 1998). Associative aggregation has been also reported for chitosan but no indication was given on the CAC (Amiji, 1995). More recently CACs have been reported (Nichifor, Lopes, Carpov, & Melo, 1999) for dextran modified with bile acids, which have showed that the aggregation process depends on both the nature of the hydrophobic moiety and the degree of substitution.

The purpose of the present work was to study the aggregation process of hydrophobically modified dextran by correlating the solution behavior with degrees of substitution (α). A series of dextrans modified with *p*-hexylbenzoyl chloride were studied using the fluorescent probes pyrene and 1,8 anilinonaphtalene sulfonic acid sodium salt (1,8-ANS), in order to determine the CACs, and the properties of the aggregates formed by interaction of the polysaccharide chains.

2. Experimental

2.1. Materials and chemicals

Dextran T40 ($M_w \approx 40{,}000$) and T10 ($M_w \approx 11{,}000$) were purchased by Pharmacia (Sweden). *p*-Hexylbenzoyl chloride was obtained from Aldrich. Pyrene (Aldrich) and 1,8 anilinonaphtalene sulfonic acid sodium salt (1,8-ANS, Aldrich) were purified by two recrystallizations from ethanol. The solvents employed were fluorescence spectroscopy grade (Merck).

2.2. Synthesis of modified polysaccharides

Dextran derivatives having different degrees of substitution were prepared by reaction of dextran in aqueous solution at 20 °C according to the method of Tjerneld (Lu, Tjerneld, Johansson, & Albertsson, 1991). Typically, 1.0 g of dextran was dissolved under stirring in 5 ml of water containing 1.8 g of triethylamine. The resulting solution was heated at 20 °C and 1.4 g of *p*-hexylbenzoyl chloride was added under vigorous stirring for 1 h. The polysaccharide solution was submitted to a double precipitation in 200 ml of ethyl alcohol, filtered and washed exhaustively with ethyl alcohol and hexane. The white powder was transferred to a soxhlet system and extracted with hexane to assure the elimination of non-bound 4-hexylbenzoyl chloride. The degree of substitution was controlled by varying the amount of 4-hexylbenzoyl chloride. The molar ratios of dextran, 4-hexylbenzoyl chloride and triethylamine are shown in Table 1.

Table 1
Properties of modified dextrans

Dextran	Molar ratio DXT:HXB:TEA	Dextran-T40 (M_w = 40.000), α_{UV}/α_{NMR}	Dextran-T10 (M_w = 11.000), α_{UV}
DXT-1	1:1:3	0.110(±0.01)/0.106	0.179(±0.016)
DXT-2	1:0.7:3	0.095(±0.01)/0.084	0.132(±0.01)
DXT-3	1:0.4:3	0.088(±0.01)/0.068	0.110(±0.002)
DXT-4	1:0.2:3	0.038(±0.004)/0.034	0.091(±0.002)
DXT-5	1:0.1:3	0.011(±0.004)/0.010	0.0192(±0.004)

2.3. Characterization

NMR was used to determine the degree of substitution (α) and all spectra were recorded on a Brucker AC 200 MHz spectrometer in deuterated dimethyl sulfoxide (DMSO) at 25 °C. α was also determined using the ester ethyl *p*-hexylbenzoate as a model compound ($\varepsilon = 2.56 \times 10^3 \times \text{mol}^{-1}\,\text{cm}^{-1}$) in UV spectrophotometry determinations using DMSO as a solvent.

2.4. Fluorescence spectroscopy

All measurements were performed at 25.0 ± 0.1 °C on a Hitachi 4500 fluorescence spectrometer. The temperature of the water-jacketed cell holder was controlled with a circulating bath. Dextrans from concentrated solutions were added to the aqueous solutions of probes under magnetic stirring and the fluorescence spectra recorded after each addition. The pyrene fluorescence was employed to monitor the solution properties of the modified dextrans. The vibrational structure of the fluorescence bands is known to be sensitive to the local polarity of the microenvironment at the binding sites. The band I (0–0) from fluorescence spectra located at 372.4 nm shows significant intensity enhancement in polar solvents. Thus the ratio between the fluorescence intensities of peaks I (372.4 nm) and III (384 nm) of the emission spectrum of pyrene (1×10^{-6} M), (I_1/I_3), was used to evaluate the polarity of the local environment (Kalyanasundaram & Thomas, 1977) and to determine the CAC. The probe 1,8-ANS was also used for the same purpose. Pyrene and ANS were, respectively, excited at 310 and 377 nm and the emission recorded from 350 to 650 nm.

3. Results and discussion

The spectral analysis of the NMR data confirms the substitution of the dextran. Fig. 1a shows comparative ^{1}H NMR spectrum of a derivatised and of a non-modified dextran, with two peaks corresponding to anomeric protons shown at δ 4.9 and 4.83 ppm. Peaks centered at 4.6 and 4.4 corresponds to methylene protons of the (α) 1 → 6 glycosidic linkage and the peaks at 3.7, 3.4 and 3.2 ppm corresponds to hydroxyl protons. The incorporation of hexylbenzoyl groups is illustrated in Fig. 1c for a dextran (T40) derivative having a degree of substitution of 0.084. The spectrum for this derivative shows the methyl and methylene protons of alkyl chain of the hydrophobic moiety, respectively, at δ 0.8 and 1.2–1.4 ppm. The aromatic protons are shown at δ 7.3 and 7.8 ppm (Fig. 1b) as observed for the model compound, the ester ethyl *p*-hexylbenzoate. The substitution was also confirmed by FTIR measurements by the appearance of a carbonyl band at 1719 cm^{-1} corresponding to the ester group of the substituent, which was absent in the native dextran (results not shown). The degree of substitution (α) was estimated by NMR and spectrophotometric measurements and both are collected on Table 1. The NMR α values were obtained by the ratio of the integrated area of aromatic protons of the substituent observed in δ 7.3 ppm to the integrated area of protons observed at 4.9 and 4.83 ppm. The α values determined by spectrophotometric measurements were obtained as described in the experimental section using the ester model measured in DMSO. As can be seen from this table good agreement was obtained by both methods (UV and NMR). The modification of dextran and the α values obtained in the present study are consistent with those reported for dextrans substituted with benzoyl and valeryl groups (Lu et al., 1994; Lu & Tjerneld, 1997).

3.1. Photophysical studies

Pyrene fluorescence. The ratio of intensities (I_1/I_3) of the pyrene fluorescence spectrum reports the micropolarity and hydrophobicity of the region in which it is solubilized, and this makes it a very useful probe for studying the formation and properties of aggregated systems (Evertsson & Nilsson, 1999; Kalyanasundaram & Thomas, 1977). Native dextran is a hydrophilic system, due to hydroxyl groups on the macromolecular chain, and the ratio I_1/I_3 of the emission

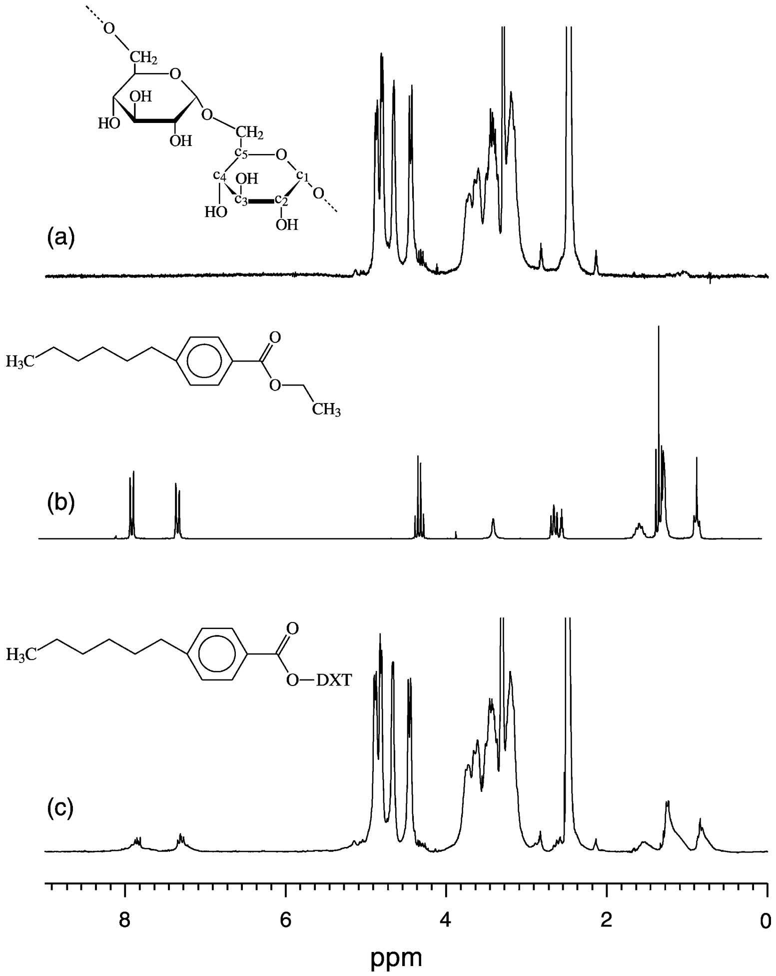

Fig. 1. ^{1}H NMR spectra of dextran (a), ester ethyl *p*-hexylbenzoate (b) and the substituted dextran (c) in deuterated DMSO.

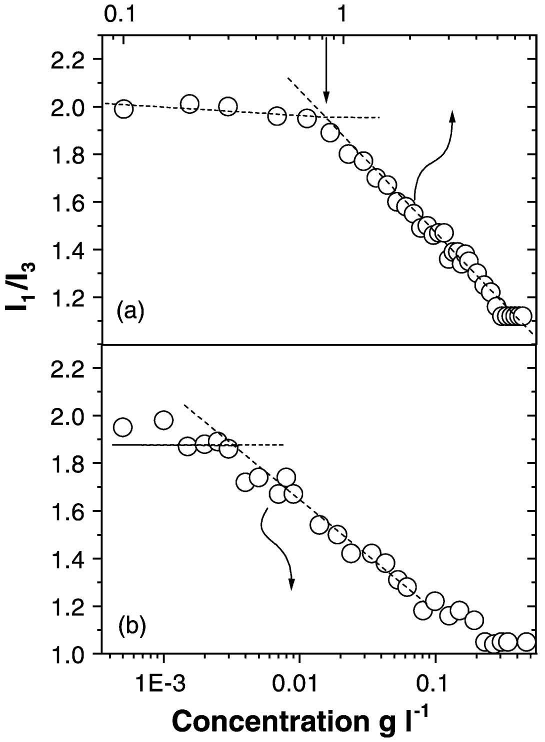

Fig. 2. I_1/I_3 ratio of the vibronic band intensities of pyrene (1×10^{-6} M) as a function of substituted dextran concentration: (a) (DX1-T10) and (b) (DX1-T40).

spectrum of pyrene remains about 1.8–1.9 for concentration up to 2 g/l. This value is consistent with an aqueous polar environment for pyrene, as has been reported by studies of similar hydrophobically modified dextrans (Zhang et al., 1998; Nichifor et al., 1999). For the modified dextrans the ratio I_1/I_3 will depend on the degree of substitution α. Fig. 2 shows a plot of the monitored parameter I_1/I_3 as a function of DXT1 concentration ($M_w = 40.000$ and $M_w = 11.000$). The two dextrans present the same behavior, i.e. initially the I_1/I_3 decreases slightly as the concentration is increased and an inflection point can be identified when the polymer concentration reaches a value which is dependent on the degree of substitution α. CAC values were determined from curves of I_1/I_3 versus polymer concentration. As shown in Fig. 2 the CAC was defined as the intercept of the tangents to the curve before and after the point of inflection (Fischer et al., 1998).

The CAC values were also determined using the probe 1,8-ANS, by performing experiments with the probe in different dextran concentrations. ANS has been used for a long time to study proteins due to its photophysical properties. The fluorescence quantum yield as well as the fluorescence spectra are changed when the probe is displaced from a hydrophilic to a hydrophobic microenvironment (Slavík, 1982). Fig. 3 shows the fluorescence spectra of 1,8 ANS in the presence of increasing dextran concentrations, where a maximum fluorescence can be observed at 518 nm in water. As the polymer concentration

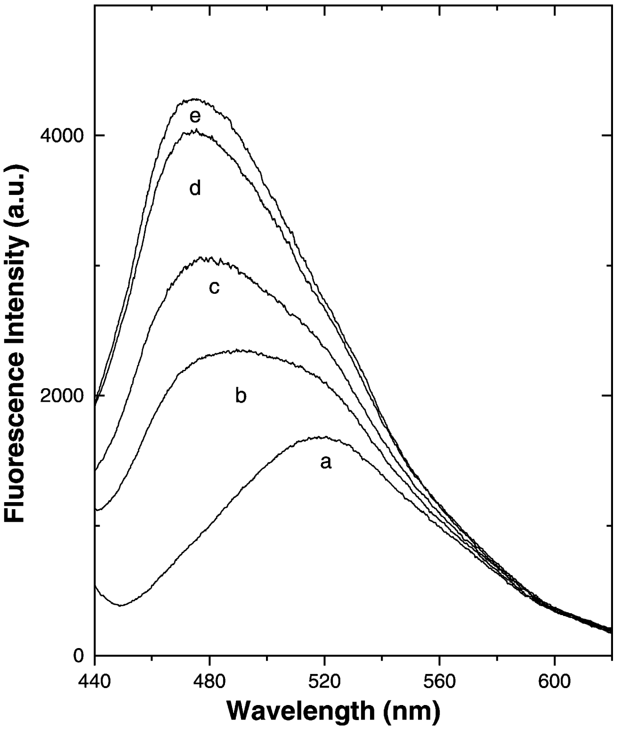

Fig. 3. Fluorescence spectra of 1,8-ANS (1×10^{-6} M) as a function of DX2-T40 concentration: (a) water; (b) 1.7×10^{-2}; (c) 2.5×10^{-1}; (d) 3.6×10^{-1}; (e) -4.9×10^{-1} g/l.

increased a blue shift in the fluorescence spectra was observed. The new band exhibits a maximum emission at 474 nm and corresponds to probe solubilized in a hydrophobic microenvironment. The ratio I_{518}/I_{474} was plotted as a function of the polymer concentration and the curves obtained are very similar to those obtained for pyrene (Fig. 4). The CAC values obtained using both probes are collected in Table 2.

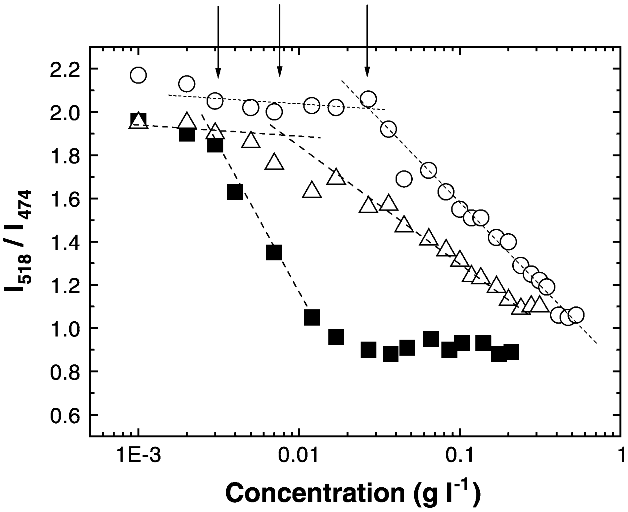

Fig. 4. I_{523}/I_{474} ratio of the fluorescence intensities of 1,8-ANS (1×10^{-5} M) as a function of DX1-T40 (■) DX2-T40 (△) and DX3-T40 (○) concentration.

Table 2
CAC of modified dextrans in aqueous solutions determined by pyrene (Py) and ANS fluorescence

Dextran	Dextran-T40 ($M_w = 40.000$)		Dextran-T10 ($M_w = 11.000$)	
	CAC × 10^{-3} (g/l) (Py/ANS)	I_1/I_3 (0.2 g/l)	CAC × 10^{-3} (g/l) (Py)	I_1/I_3 (4.0 g/l)
DXT-1	3.7/3.3	1.11	740	1.29
DXT-2	7.0/8.0	1.14	1000	1.35
DXT-3	16/22	1.16	1980	1.45
DXT-4	71/69	1.26	2420	1.55
DXT-5	144/149	1.42	3490	1.75

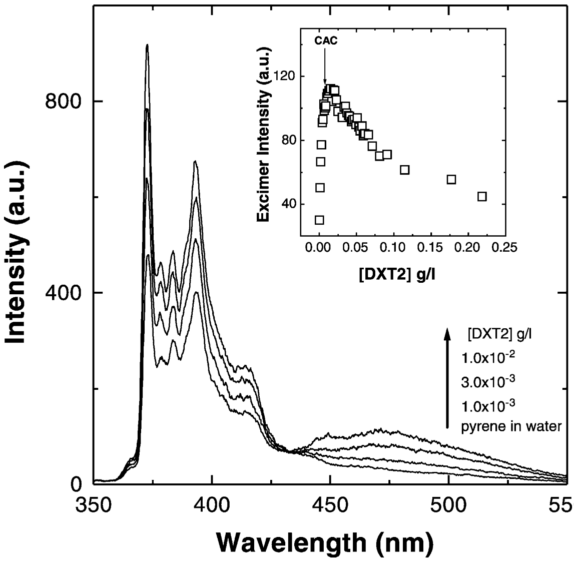

Fig. 5. Fluorescence spectra of pyrene (1×10^{-6} M) in the presence of increasing concentrations of DX2-T40. Insert: Excimer emission of pyrene as a function of DX2-T40 concentration.

An interesting aspect of the photophysical studies is the variation of the excimer emission of pyrene with increases of polymer concentration. Initially at low concentrations the excimer emission (470 nm) was more pronounced (although no I_1/I_3 variation was observed) disappearing as the polymer concentration was increased above the CAC (Fig. 5 and insert). This behavior can be interpreted as a migration of free pyrene molecules to pre-aggregates of the hydrophobized dextran, which leads to increase the excimer emission. When the polymer concentration is higher, the pyrene molecules can be redistributed amongst the polymer aggregates and the excimer emission is diminished. A scheme describing the polymer–polymer and polymer–probe interactions is shown in Fig. 6.

Table 2 shows that the CAC values determined by pyrene and ANS are in good agreement despite the very different characteristics of the two probes. Clearly the CAC decreases with the degree of substitution and Fig. 7 shows that the same behavior was exhibited for both the dextrans studied (T40 and T10). In general the increase of the dextran molecular weight from 11.000 to 40.000 decreased the CAC by about a magnitude of 100, which is an expected trend for aggregation of amphiphilic polymers in aqueous solution. The increase of the molecular weight leads to the formation of hydrophobic microenvironments that occur at lower polymer concentrations (Amiji, 1995). Intra and/or intermolecular interactions have been reported as responsible for the formation of aggregates in native polysaccharides such as pullulan (Akioshi et al., 1993) as well as in more flexible polymers, including polyelectrolytes having hydrophobic residues grafted to the main chain (Wang & Engberts, 1996; Tiera et al., 2000). The solution behavior observed here by the substituted dextrans might be explained by

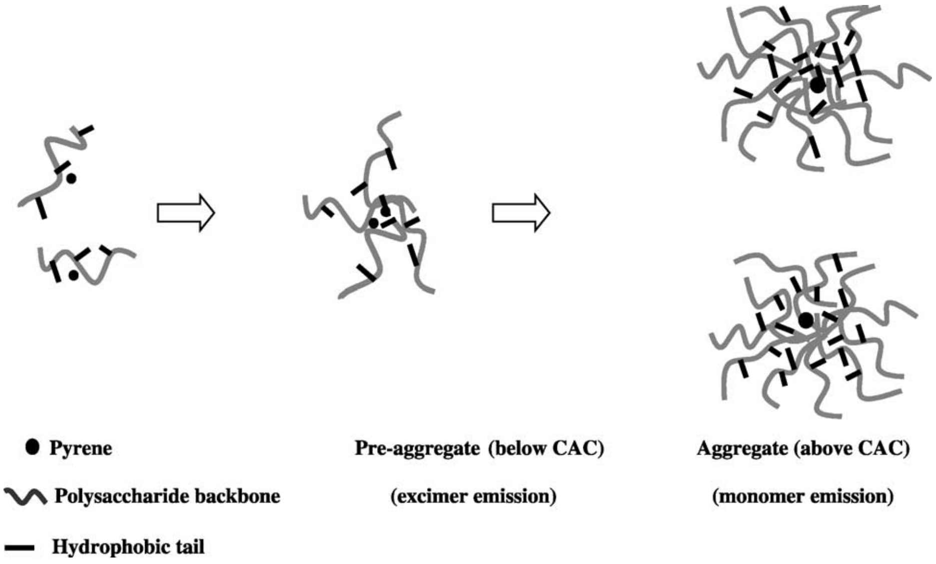

Fig. 6. Scheme describing the aggregation of the modified polysaccharides as the polymer concentration is increased.

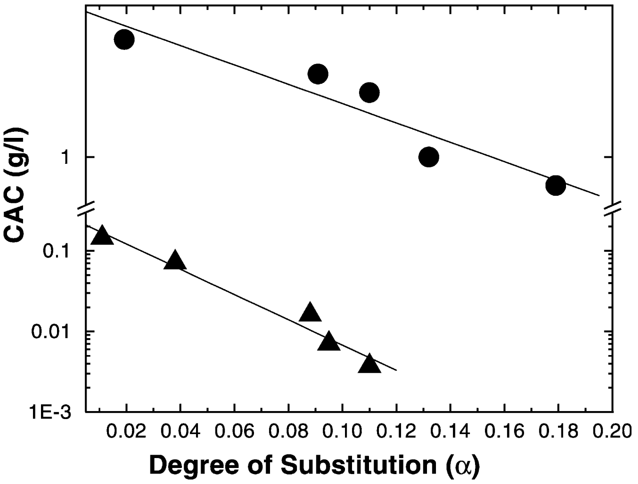

Fig. 7. CACs determined by pyrene I_1/I_3 ratios as a function of degree of substitution (α) for dextrans T10 (●) and T40 (▲).

postulating the existence of intermolecular associations. Although dextran is considered to be a flexible chain due to 1 → 6 glycosidic linkage it is believed that the hydrophobe content are not sufficient to promote intramolecular interactions, since it can be estimated that for the most substituted dextran (DEX-1) there is only one substituent for five D-glucose residues. On the other hand the formation of intramolecular aggregates must be indicated by the pyrene spectrum at low polymer concentration. This was not observed because, before reaching the CAC, the I_1/I_3 ratio remains at 1.8–1.9. As can be seen from Fig. 6 linear relationships can be obtained for the logarithm of CAC as a function of the degree of substitution (α). This type of relationship is normally found for the critical micelle concentration (CMC) of nonionic surfactants. Therefore it is believed that the polymer backbone tends to undergo intermolecular hydrophobic association and the observed behavior is similar to that of common surfactants.

At higher polymer concentrations (above CAC), when the presence of aggregates can be postulated, the polarity indicated by the ratio I_1/I_3 of pyrene showed to be dependent on both α and the molecular weight of dextran. For the more substituted dextran the I_1/I_3 ratio reached smaller values than for the less substituted dextran, indicating that for higher α values more hydrophobic aggregates are formed. However for dextran T40 smaller I_1/I_3 values were obtained indicating that for this system water is more effectively excluded from the inner region of the aggregate. Comparing the ratios I_1/I_3 for the different modified dextrans (Table 2) it can be seen that the I_1/I_3 values for dextran T40 are about 15–20 percent smaller than those observed for T10. The ANS fluorescence data also supported this explanation and the ratio I_{518}/I_{474} decreased with increase of both α and molecular weight.

4. Conclusions

The investigation of two hydrophobically modified dextrans in aqueous solution revealed that for polymers having low molecular weights an aggregation process takes place depending on the degree of substitution (α). The pyrene and ANS fluorescence studies showed that for dextrans having low degrees of substitution (α) CACs can be identified as the polymer concentration is increased. The aggregation is more efficient when α is increased and interchain interactions are responsible for the formation of hydrophobic aggregates. The increase of molecular weight from 10 to 40 kDa leads to CAC values that decrease by about two orders of magnitude. The results provide evidence that small and/or pre-aggregates might precede the aggregation. The properties of the aggregates are influenced by increases in both the degree of substitution and the molecular weight of the sample, leading to formation of more hydrophobic aggregates.

Acknowledgements

N.A.B.V. thanks CAPES for a graduate Fellowship and M.S.M. (grant 98/01572-6) wishes to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for a scholarship.

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