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ORIGINAL CONTRIBUTION

Influence of alkyl chains length on the conformation and solubilization properties of amphiphilic carboxymethylpullulans

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Abstract The existence of inter- and/or intramolecular interactions in aqueous solutions of hydrophobically modified polysaccharides induces specific macromolecular conformations at the air–solution interface and in the bulk, where hydrophobic microdomains may be formed. The present work focuses on the interfacial and solubilizing properties of amphiphilic pullulans ($CMP_{12}C_8$, CMP_7C_{14}, and CMP_4C_{16}) differing in the length and percentage of alkyl chains grafted to the anhydroglucose units. The surface tension studies of these polymers evidence a marked difference between the interfacial properties of $CMP_{12}C_8$ on one hand and those of CMP_7C_{14} and CMP_4C_{16} on the other hand. Pyrene fluorescence spectroscopy and benzophenone solubilization experiments demonstrate a similar partition. Increasing alkyl chains length from eight up to 14 or 16 carbons improves the solubilization properties of nonpolar molecules, even though at the same time, the average number of grafted chains per 100 anhydroglucose units is decreased from 12 down to seven and four for the three compounds, respectively.

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Introduction

Over the past two decades, hydrophobically modified water soluble polymers have found an increasing number of applications. As a result of their remarkable thickening properties, they are widely used in cosmetics, paints, or for enhanced oil recovery, etc. [1, 2]. These materials are amphiphilic water soluble polymers mainly constituted of a hydrophilic backbone and hydrophobic side or end groups as, for instance, alkyl chains [3–5]. They show unique associative properties in aqueous media. These properties arise from the inter- and/or intramolecular interactions, which occur between hydrophobic groups, inducing the formation of hydrophobic microdomains. The mode of association depends on polymer concentration but also on structural parameters such as the content, the length, and the nature of the hydrophobic groups [6–8]. In highly diluted aqueous solutions, intrapolymer hydrophobic associations are generally favored, but as polymer concentration increases, interpolymer hydrophobic interactions also take place [9–11]. Polymers with a low hydrophobic group substitution rate show a strong tendency to interpolymer associations even at very low polymer concentrations [12]. In contrast, highly substituted polymers show a strong propensity to intrapolymer associations and may lead to the formation of polymer aggregates made up of a single macromolecular chain ("monomolecular aggregate" or "unimolecular micelle") independent of polymer concen-

tration [13]. The solution properties of these polymers may be studied by fluorescence spectroscopy using a luminescent hydrophobic probe, such as pyrene. This probe preferentially localizes in the hydrophobic domains and the changes in its fluorescence spectrum provide information on the conformation of the macromolecules in solution [3, 14, 15].

Among well-known associative polymers, hydrophobically modified polysaccharides are particularly attractive due to their biocompatibility, biodegradability, and low toxicity, which are advantageous for pharmaceutical applications. Since Landoll's pioneering work [16], dealing with alkyl chains-modified cellulose, many other polysaccharides have been hydrophobically modified [17–23]. Although the thickening properties of their aqueous solutions have attracted much attention [24–26], their surface properties have been considered to a much lower extent [19, 27–30]. In this work, we focus on the behavior of amphiphilic derivatives of pullulan, a flexible polysaccharide consisting of α-1,4-linked glucose units included in α-1,6-linked maltotriose ones [31]. In a previous article, we showed that increasing the substitution rate from 10% to 35% of two carboxymethylpullulan modified with C_8 alkyl chains induced a reinforcement of the hydrophobic interactions and, thus, the formation of more compact and stable monomolecular aggregates. Both hydrophobized polymers adsorbed at the air–solution interface in a coil conformation occupying large interfacial molecular areas [30]. Due to the existence of these apparently stable nanodomains, these polysaccharide derivatives appeared of interest for solubilizing hydrophobic insoluble drugs. To get a better insight into the parameters controlling the formation of the nanodomains and the consequences on drug solubilization, we analyzed the effect of increasing hydrophobic chain lengths versus substitution rates of these polysaccharide derivatives both on their behavior at air–solution interface and on their solubilization properties using pyrene and benzophenone as model hydrophobic molecules.

Experimental

Material

The hydrophobically modified carboxymethylpullulan (HMCMP) derivatives (CMP_4C_{16}, CMP_7C_{14}, and $CMP_{12}C_8$) were synthesized in two steps according to the previously described procedure [30]. The degree of modification (τ=4, 7, or 12) of these polymers is the number of alkyl chains per 100 anhydroglucose units. These chains contain 16, 14, and 8 carbons, respectively. Table 1 summarizes the average molar weights of the pullulan precursor, carboxymethylpullulan (determined by size-exclusion chromatography measurement coupled with multi-angle laser light scattering detector, MALLS) and the studied HMCMP derivatives (determined by F4/MALLS) [23].

Table 1 Number ($\overline{M}_n$) and weight ($\overline{M}_w$) average molar masses of pullulan, carboxymethylpullulan, and hydrophobically modified carboxymethylpullulan derivatives

	$\overline{M}_n$ (g/mol)±10%	$\overline{M}_w$ (g/mol)±10%
Pullulan	170,000	345,000
CMP	160,000	300,000
CMP_4C_{16}	140,000	300,000
CMP_7C_{14}	140,000	300,000
$CMP_{12}C_8$	140,000	400,000

The ultrapure water (γ=72.4 mN/m at 22 °C) was produced by a Millipore Synergy 185 apparatus coupled with a RiOs5™, with a resistivity of 18.2 MΩ/cm. KH_2PO_4 and KOH were purchased from Merck and $KMnO_4$ from Aldrich. Highly purified pyrene (>99%) was obtained from Fluka and used as received. Benzophenone (BZ; Mw, 182.22 g/mol) was supplied from Sigma (France). The glassware was cleaned in a freshly prepared sulfochromic solution and abundantly rinsed with the ultrapure water.

Preparation of polymer solutions

Polymer solution samples were prepared by dissolving the appropriate amount of polymer in water or in phosphate buffer (KH_2PO_4, KOH). Buffer concentration was 0.5 or 0.1 M and its pH was chosen equal to 7.5, corresponding to the full ionization of carboxylate groups [32]. The pH of polymer solutions prepared in phosphate buffer remained constant as the polymer concentration increased. The polymer solutions were then stirred at room temperature for at least 48 h. Before the experiments, polymer solutions were diluted with buffer. Concentrations were varied from 1.4×10^{-6} mg/ml up to 7 mg/ml.

Fluorescence spectroscopy

The experiments were performed using a Spex-Fluorog 1681–0.22 m spectrometer (Hitachi, Jobin Yvon). In these experiments, a pyrene stock solution (10^{-3} M) was prepared in acetone. A 10-μl aliquot of this solution was introduced into empty vials and the solvent was evaporated under vacuum. After evaporation, the vials were filled with 10 ml of a $CMP_{12}C_8$, CMP_7C_{14}, or CMP_4C_{16} solution and gently stirred for 18 h to ensure the incorporation of the molecular probe into possibly existing polymer hydrophobic domains. The final pyrene concentration was 10^{-6} M. At this low concentration, no excimer band due to the interaction of an excited state pyrene with a ground state pyrene was observed [3, 15, 33]. Such a low concentration was chosen to minimize the influence of pyrene on the formation and/or

the stability of HMCMPs hydrophobic domains. All samples were excited at 335 nm and the emission spectra of pyrene showed vibronic peaks at λ_1=372 nm (intensity I_1) and λ_3=382 nm (intensity I_3).

Surface tension measurements

The interfacial behavior of polymer solutions was studied as a function of the polymer concentration. Surface tension was measured by the Wilhelmy plate method using a K10 tensiometer (Krüss, Germany). The measurements were made without detaching the plate from the interface and the data were continuously plotted on a chart recorder for 24 h. In order to maintain a constant level of the liquid and avoid any drift in the measured surface tensions, all experiments were performed at 23±2 °C under saturated vapor pressure to limit water evaporation during the experiments. The reported surface tension values are mean values of at least two measurements. The experimental uncertainty was estimated to be 0.2 mN/m.

Drug solubilization

Benzophenone (1 g) was added to 3 ml of the polymer solutions varying in the [14×10^{-6} −7 mg/ml] range. The mixtures were left under stirring for 24 h at room temperature. The excess drug that could not be dissolved in the polymer solutions was removed by centrifugation (20,000×*g*, 30 min). The amount of drug solubilized into polymer solutions was determined by ultraviolet spectroscopy at λ=259 nm. Absorbance values were recorded and the drug concentrations were calculated from standard curves.

Results

Incorporation of pyrene into polymer aggregates in solution

Figure 1 shows the variation of the I_1/I_3 ratio with the HMCMP concentration in water or in phosphate buffer (at 0.1 and 0.5 M). In the very dilute regime (C=1.4×10^{-5} mg/ml), the three studied systems exhibit I_1/I_3 values in the range 1.64–1.67, similar to the value of 1.67 measured for pyrene molecules in an aqueous environment [3]. However, at concentrations higher than 10^{-3} mg/ml in water and 10^{-2} mg/ml in phosphate buffer (0.1 M and 0.5 M), the I_1/I_3 values decrease, indicating an increasing nonpolar environment surrounding pyrene molecules. Apparently, the decrease in I_1/I_3 in water solutions is of low extent compared to that in buffer solutions.

Obviously, the presence of salts in the aqueous medium influences the general behavior of HMCMPs. For a given

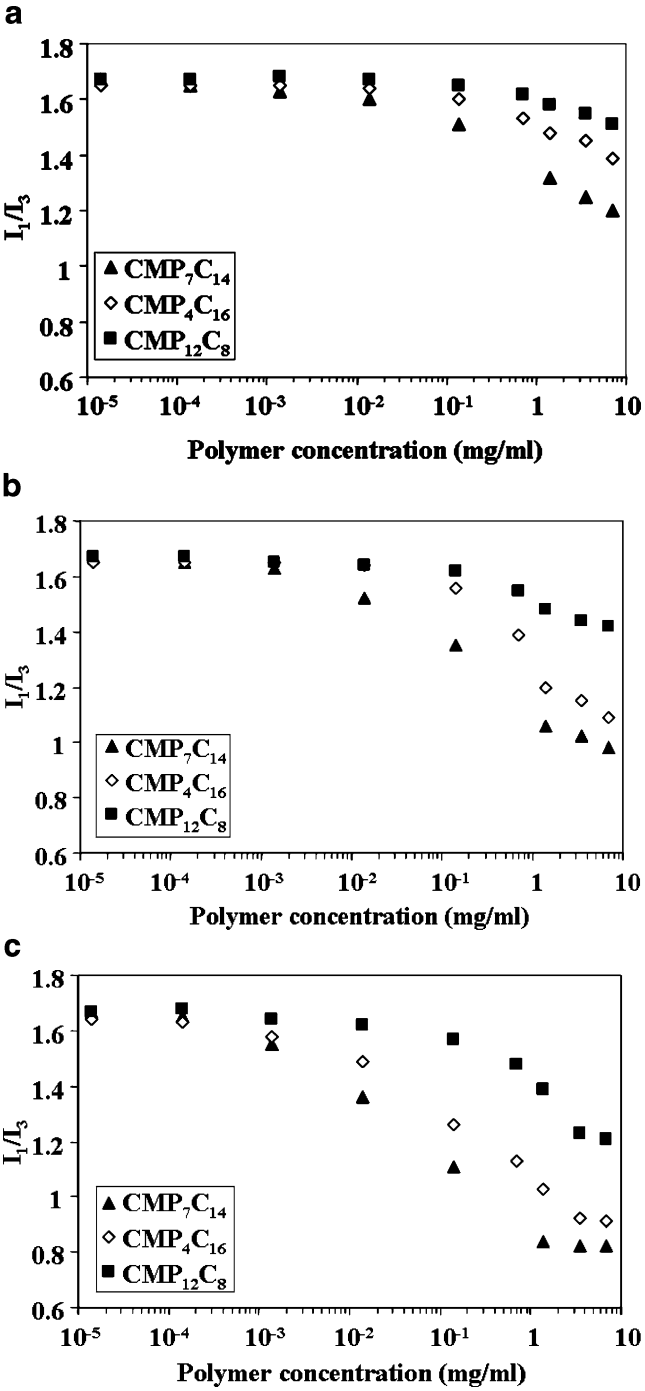

Fig. 1 Variation of pyrene fluorescence intensity ratios (I_1/I_3) versus polymer concentration for $CMP_{12}C_8$, CMP_7C_{14}, and CMP_4C_{16} solutions in **a** pure water, **b** phosphate buffer 0.1 M, and **c** phosphate buffer 0.5 M

polymer, the minimum concentration at which a diminution of the I_1/I_3 ratio is observed, $[CMPC]_{min}$ and the minimum I_1/I_3 value $(I_1/I_3)_{min}$ decrease as the ionic strength increases. Interestingly, in the studied concentrations range, a shift of the I_1/I_3 curves towards lower concentration values is observed and a plateau (at the minimum I_1/I_3 values) is

reached at the highest ionic strength. This plateau starts at a polymer concentration $[CMPC]_{plateau}$, depending upon the nature of the polymer. The various $[CMPC]_{min}$, $(I_1/I_3)_{min}$, and $[CMPC]_{plateau}$ values for the three studied polysaccharide derivatives are summarized in Table 2.

Interfacial behavior of the hydrophobized polymers

Figure 2 shows the evolution of the surface tension γ versus polymer concentration for the three modified carboxymethylpullulans, in water or phosphate buffer. As in Fig. 1, in the studied concentration range and as the ionic strength of the aqueous medium increases, a shift of the surface tension plots towards lower polymer concentration and lower surface tension values is observed. From the data in this figure, it is clear that CMP_4C_{16} and CMP_7C_{14} induced a more pronounced surface tension lowering than $CMP_{12}C_8$ and that for the latter one, minimum surface tensions were attained at higher concentrations. Compared to CMP_4C_{16}, CMP_7C_{14} lowered the surface tension to a much higher extent. The data in Fig. 2 also show that the surface tension of the three polysaccharide derivatives decreased gradually and for some of them reached a clear break, which would correspond to the saturation of the interface by the adsorbed alkyl chains, at the concentration C_{sat}. At higher polymer concentrations, the surface tension remained constant, equal to γ_{sat}. The application of Gibbs equation to the linearly decreasing portion of the γ–logC relationships in Fig. 2b and c (in conditions where carboxylate charges are screened by buffer ions) allows calculation of the areas occupied by the adsorbing polymer species at the interface. In these calculations, it was assumed that, at low HMCMP solution concentrations, activities could be replaced by polymer concentrations, since the second virial coefficient as determined from F4/MALLS measurements, was negative [25]. The relationship between the slope of the curve, $d\gamma/d\ln C$, and the apparent interfacial excess concentration Γ may be written:

$$\Gamma = 1/RT(d\gamma/d\ln C) \quad (1)$$

and the corresponding molecular areas can then be deduced from the equation:

$$A = 1/N\Gamma \quad (2)$$

where N is the Avogadro's number. The calculated molecular areas A, C_{sat}, and γ_{sat} values are summarized in Table 3.

As generally observed for the surface tension values in Fig. 2, γ_{sat}, C_{sat}, and A are lower for CMP_7C_{14} than for CMP_4C_{16}. This would be due to the higher substitution rate and consequently lower number of charges in CMP_7C_{14} compared to CMP_4C_{16}. Conversely, despite a comparatively larger number of alkyl chains per 100 anhydroglucose units and, thus, a reduced number of charges, $CMP_{12}C_8$ exhibits the largest molecular area and highest γ_{sat} values. It is obvious that this compound adsorbs in a different conformation relative to the polysaccharide derivatives with longer alkyl chains. It is worth to note that the molecular area calculated for $CMP_{12}C_8$ in 0.1 M phosphate buffer (103 Å^2) is close to that previously reported for a $CMP_{10}C_8$ (107 Å^2) [30].

Solubilization of a hydrophobic drug in HMCMPs solutions

The results described above clearly indicate that pyrene molecules are associated with HMCMPs hydrophobic nanodomains, suggesting the possible role of the alkyl chains in the formation of hydrophobic solubilization sites for nonpolar molecules. In order to evaluate the possible use of the HMCMPs derivatives as solubility enhancers, solubilization studies were undertaken with benzophenone, a hydrophobic model molecule, chosen for its well-known poor water solubility. The solubility profiles reported in Fig. 3 show a linear increase of benzophenone concentration in the solution with polymer concentration. CMP_7C_{14} and CMP_4C_{16} are apparently four times more efficient than $CMP_{12}C_8$ as solubilizing agents.

Table 2 Characteristic $[CMPC]_{min}$, $(I_1/I_3)_{min}$, and $[CMPC]_{plateau}$ values obtained from pyrene fluorescence measurements for the three studied polysaccharide derivatives in water and buffer solutions (0.1 and 0.5 M)

Aqueous medium	HMCMP derivative	$[CMPC]_{min}$ (mg/ml)	$(I_1/I_3)_{min}$	$[CMPC]_{plateau}$ (mg/ml)
Water	$CMP_{12}C_8$	0.70	1.51	None
	CMP_7C_{14}	0.014	1.20	None
	CMP_4C_{16}	0.14	1.39	None
Buffer 0.1 M	$CMP_{12}C_8$	0.14	1.40	3.5
	CMP_7C_{14}	0.014	0.98	None
	CMP_4C_{16}	0.14	1.09	None
Buffer 0.5 M	$CMP_{12}C_8$	0.014	1.21	3.5
	CMP_7C_{14}	0.0014	0.82	1.4
	CMP_4C_{16}	0.0014	0.91	3.5

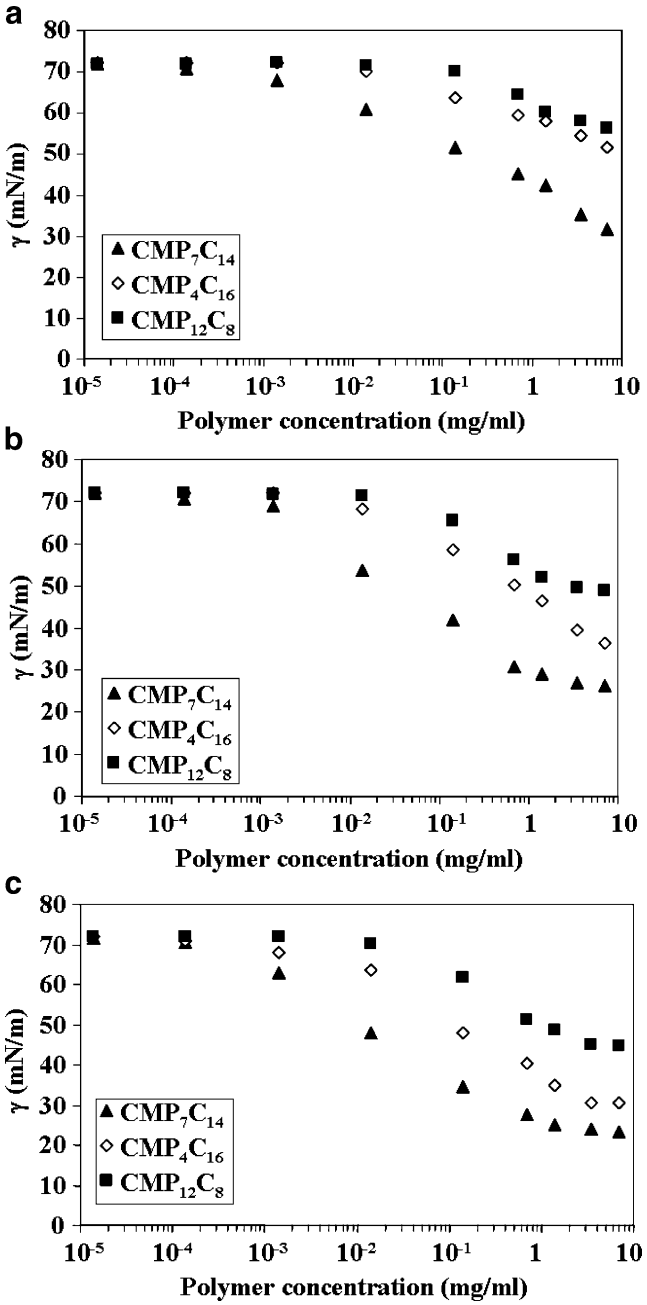

Fig. 2 Surface tension (γ) dependence on the concentration of $CMP_{12}C_8$, CMP_7C_{14}, and CMP_4C_{16} in **a** pure water, **b** phosphate buffer 0.1 M, and **c** phosphate buffer 0.5 M

Discussion

From the curves in Fig. 1 and the values in Table 2 and as previously observed for two CMPτC_8 derivatives [30], it is apparent that an increase in the ionic strength of HMCMP solutions results in an increase in pyrene association to polymers hydrophobic nanodomains. Indeed, even though they do not reach the reported I_1/I_3 value for pyrene solubilized in a pure aliphatic solvent (0.56 in n-hexane [34]), the low $(I_1/I_3)_{min}$ values measured indicate the existence of a more apolar environment in buffer than in water polymer solutions. For a given polysaccharide derivative, $[CMPC]_{min}$ values decrease from water to 0.5 M phosphate buffer, revealing the higher affinity of pyrene for polymer hydrophobic domains. Two factors may explain these observations. The first one would be the global decrease in pyrene water solubility due to the increase in ionic strength in the aqueous medium, as the activity coefficient of water molecules is lowered by the dissolution of salts [35]. The second one is the expected screening effect of salts on ionized sites of the carboxymethylpullulan derivatives, the diminution of electrostatic repulsions between nonsubstituted carboxylate groups resulting in a higher flexibility of polysaccharide chains. This enhancement of their conformational freedom makes easier the formation of hydrophobic nanodomains by alkyl groups able to come closer to each other and would maximize their interactions with pyrene.

Table 3 Surface properties of HMCMPs in 0.1 and 0.5 M phosphate buffer

Aqueous medium	Polymer	Molecular area (Å^2/molecule)	C_{sat} (mg/ml)	γ_{sat} (mN/m)
Buffer 0.1 M	$CMP_{12}C_8$	103	3	49
	CMP_7C_{14}	65	2	27
	CMP_4C_{16}	72	None	None
Buffer 0.5 M	$CMP_{12}C_8$	90	3.5	45
	CMP_7C_{14}	63	1.4	24
	CMP_4C_{16}	63.5	3	30

As mentioned before, the results in Fig. 2 evidence a more pronounced surface tension lowering for CMP_7C_{14} than for CMP_4C_{16}. Apparently, the length of the hydrophobic chains would have a lesser effect on the surface tension than the number of grafted chains. This is not the case for $CMP_{12}C_8$, which obviously adsorbs in a different conformation. The surface tension values reached for CMP_4C_{16} and

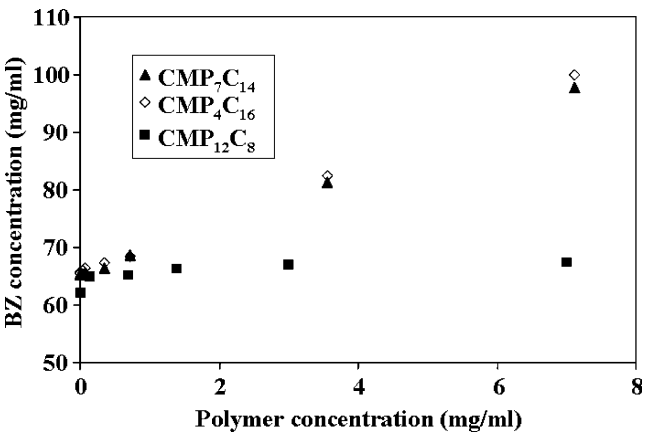

Fig. 3 Apparent solubility of benzophenone in $CMP_{12}C_8$, CMP_7C_{14}, and CMP_4C_{16} aqueous solutions

CMP_7C_{14} indicate that these HMCMPs easily adsorb at the interface by anchoring their tetradecyl and hexadecyl chains, whereas their hydrophilic sugar moieties remain completely immersed into the solution. The distance between the neighboring alkyl chains anchored at the interface is large enough to enable the formation of so-called "loops" in the aqueous phase and, thus, a dense packing of the alkyl chains at the interface. Similar results were obtained with neutral pullulan derivatives bearing cholesteryl groups [29]. The quite large molecular areas reported in Table 3 do not solely correspond to the area of the hydrophobic anchors of these two polysaccharide derivatives. They account for the effect of the conformation of the macromolecules in the subphase, which in turn is dependent upon the substitution rate of alkyl chains and the ionic strength of the surrounding medium. The calculated molecular areas are much larger than the cross-section of a methyl group of the alkyl chains (25 $Å^2$). In the literature, the molecular areas reported for C_{14} and C_{16} chains bound to one sugar moiety are evaluated to 42–45 $Å^2$ [36]. When a polysaccharide is grafted with hydrophobic groups at a sufficiently low substitution rate to allow formation of loops into the subphase, then the value of the calculated molecular area may be close to that of the anchor alone [27]. However, it is most often larger [37–39]. In phosphate buffer solutions, molecular areas of CMP_4C_{16} and CMP_7C_{14} range from 63 to 72 $Å^2$. They are 1.6 times larger than that of an alkyl chain bound to a sugar. As the ionic strength increases, polymer charges are screened by salt ions, allowing a denser packing of adsorbed alkyl chains and the area occupied by adsorbed groups significantly diminishes. Probably due to its flexible structure, the polysaccharide backbone only barely hampers the tightening of the adsorbed alkyl chains. It is, thus, reasonable to think that the polysaccharidic chains of these CMPs are stretched rather than coiled into the aqueous phase, forming loops in the solution whereas the alkyl chains protrude at the interface. For $CMP_{12}C_8$, the low surface tension values and large occupied molecular areas account for the same shrunken conformation at the interface as previously described for $CMP_{10}C_8$ [30], which would correspond to the adsorption of monomolecular aggregates formed in the solution. The smaller molecular area occupied by the former compound relative to the latter one is coherent with its slightly higher substitution rate, which favors connections within a same polysaccharidic chain. This shrinking effect is enhanced by the neutralization of charges at high ionic strength.

The surface tension plots of CMP_4C_{16} and CMP_7C_{14} present a clear breakpoint, which would correspond to the saturation of the interface by the adsorbed HMCMP molecules. The values of breakpoint concentrations as determined from the surface tension measurements were confirmed by the fluorescence experiments in 0.5 M phosphate buffer and correspond to polymer concentrations necessary to stabilize the I_1/I_3 ratios. Thus, the breakpoint in surface tension would not solely indicate the saturation of the interface but also the formation of intermolecular aggregates as confirmed by viscosimetric studies performed on HMCMP modified by 1.3% to 6.8% of C_{16} chains [21]. For these polymers, intermolecular interactions exist even in dilute solutions, below the critical concentration C^{Cr} at which the viscosity of these solutions sharply increases.

Pyrene fluorescence spectroscopy measurements reinforce the argument in favor of the existence of polymer aggregates with hydrophobic regions generated by intra- and/or intermolecular interactions between apolar groups. The formation and structure of these aggregates depend to a large extent on the substitution rate of carboxylic groups [40] and are, thus, influenced by the ionic strength of the media. From the data reported in Fig. 1, it may be assumed that the charges borne by CMP_7C_{14} and CMP_4C_{16} are progressively screened upon addition of electrolytes. There is a balance between two effects: (i) the electrostatic repulsions between the charges of the polymer chain, which would contribute to the formation of expanded aggregates, and (ii) attractive hydrophobic interactions between alkyl groups that would account for the formation of aggregates with well-defined hydrophobic microdomains. These results are in agreement with general principles predicting the enhancement of hydrophobic interactions when the ionic strength increases [41].

Since aggregates with hydrophobic cores can be formed in aqueous solutions, it was assumed that they could solubilize a hydrophobic drug such as benzophenone. The solubility profiles of the drug in Fig. 3 show that the presence of CMP_7C_{14} and CMP_4C_{16} only increased the apparent solubility of benzophenone by a factor of 4.56 μg of BZ per milligram polymer. Apparently for these two polysaccharide derivatives, the extent of drug solubilization depends on polymer concentration to a higher extent than on its structure. The similarity in the apparent solubility of the drug in the two polymer solutions is in agreement with the results obtained from pyrene fluorescence experiments, which showed very comparable I_1/I_3 plots for both polymers in 0.1 M phosphate buffer. As reported in the literature [42], the solubilizing properties of polymer aggregates would not be affected by alkyl chains lengths differing by two carbons only. The organization of these polymers at the interface and in solution is probably similar even if CMP_7C_{14} seems to present the balance between the length of its hydrophobic chains and their substitution rate that is the most favorable to the formation of hydrophobic domains. The low hydrophobic substitution rate of the studied modified CMPs, however, would explain their moderate ability to solubilize benzophenone.

Compared to these two polymers, $CMP_{12}C_8$ appeared unable to improve benzophenone solubility. Indeed, it only increased from 61 μg/ml in the buffer solution to 70 μg/ml

in the most concentrated $CMP_{12}C_8$ solutions. Obviously for the HMCMPs with longer chains, the combination of intramolecular interactions and intermolecular interactions even in the dilute regime would favor solubilization of a hydrophobic drug to a higher extent than the sole intramolecular interactions in $CMP_{12}C_8$.

Conclusion

The studied hydrophobically modified carboxymethylpullulan derivatives exhibit good associative and interfacial properties, which strongly depend on their structural parameters and the ionic strength of the media. As the modification of one of the hydrophibization parameters, such as the substitution rate or alkyl chain length, may change the aqueous solution conformation of these polymers and their associative behavior, the solubilizing properties of the three studied HMCMPs may vary in a rather complex way. It appears, thus, necessary to combine physicochemical studies of the polymer solutions and solubilization experiments to get a clear information on the most effective modification of carboxymethylpullulans to achieve in order to increase the aqueous solutions concentrations of poorly soluble drugs.

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