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Research Paper

Enhancement of the Solubility and Efficacy of Poorly Water-Soluble Drugs by Hydrophobically-Modified Polysaccharide Derivatives

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Purpose. This work was intended to develop and evaluate a new polymeric system based on amphiphilic carboxymethylpullulans ($CMP_{49}C_8$ and $CMP_{12}C_8$) that can spontaneously self-assemble in aqueous solutions and efficiently solubilize hydrophobic drugs.
Methods. The self-assembling properties of $CMP_{49}C_8$ and $CMP_{12}C_8$ were characterized by fluorescence spectroscopy and surface tension measurements. The solubilization of benzophenone and docetaxel was assessed from surface tension measurements, UV spectrometry and HPLC assays. The *in vitro* cytoxicity of $CMP_{49}C_8$ solutions and the docetaxel commercial vehicle (Tween 80®/Ethanol–water) were evaluated in the absence and in the presence of docetaxel.
Results. Compared to $CMP_{12}C_8$, $CMP_{49}C_8$ in aqueous solutions appeared to self-organize into monomolecular aggregates containing hydrophobic nanodomains, and to significantly increase the apparent solubility of benzophenone. Docetaxel solubility could also be improved in the presence of $CMP_{49}C_8$ but to a lower extent due to the surface properties of the drug. Nevertheless, *in vitro*, the cytotoxicity studies revealed that against cancer cells, the $CMP_{49}C_8$-docetaxel formulation was equipotent to the commercial docetaxel one. Furthermore, in the absence of the drug, $CMP_{49}C_8$ appeared less cytotoxic against macrophages than the Tween® 80/Ethanol–water.
Conclusions. $CMP_{49}C_8$ is a good candidate for solubilizing hydrophobic drugs and could be applied to docetaxel formulations.

KEY WORDS: cytotoxicity studies; docetaxel; hydrophobically modified carboxymethylpullulan; solubilization; surface tension.

INTRODUCTION

In recent years, amphiphilic polymers have been the object of growing scientific attention because of their unique associative behaviour and their potential applications in pharmaceuticals. They are mainly constituted of hydrophilic and hydrophobic parts. In aqueous solutions, the hydrophilic segments are responsible for the polymer hydration, while the hydrophobic domains minimise their contact with water by self-assembling into aggregates (1–9). The first studies describing an associative behaviour were published in 1951 with a poly(4-vinylpyridine) derivative bearing ethyl and dodecyl groups (10). From this date, the interest for these original macromolecules increased as indicated by the numerous books and reviews on the subject (11–18). The structure of such polymers greatly varies from block amphiphilic copolymers to grafted amphiphilic polymers. The advantage of the latter compared to the former, is that the hydrophobic interaction can operate not solely between different polymeric chains (intermolecular interactions) but also within a same polymer chain (intramolecular interactions) (19). It is worth noting that the balance between inter- and intramolecular interactions may easily be controlled by adjusting the size and/or number of the hydrophobic grafted side-chains (7,20).

Among the amphiphilic polymers, hydrophobically modified polysaccharides have attracted a particular attention as promising vehicles for water-insoluble drugs because of their biocompatibility, biodegradability and low toxicity. The first studies on polysaccharide-based surfactants started at the beginning of the 1980s. The pioneering work of Landoll (21) dealt with viscometric, solubility, and surface-active properties of cellulose grafted with alkyl chains. The structure of amphiphilic polysaccharides was not only modified by grafting of various hydrophobic groups, but also by the use of different hydrophilic backbones (methylcellulose, hydroxyethylcellulose, etc.). This new class of polysaccharides possessed unique solution properties due to their inter and/

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or intra-chain associations. Although numerous studies were dedicated to the associative behaviour of these new compounds in aqueous solution and to their surface activities, only few of them dealt with their solubilization properties. However, it has been shown that the core-shell structure of these systems could be used as a vehicle for hydrophobic drugs. Indeed derivatives from dextran, cellulose, starch or alginate can encapsulate hydrophobic molecules such as cyclosporin (22), puerarin (23), vitamine B2 (24), paclitaxel (25) or even proteins (26,27).

The present work was aimed at evaluating the solubilizing properties of solutions of hydrophobically modified carboxymethylpullulan derivatives (HMCMPs). Pullulan is a water-soluble polysaccharide with a linear flexible chain formed from glucose and maltotriose units. Its hydrophobically modified derivatives were shown to develop spontaneous intra and/or intermolecular interactions depending on the content and length of grafted hydrophobic chains (7,8,20,28). The self-assembling properties of two hydrophobized carboxymethylpullulans ($CMP_{12}C_8$ and $CMP_{49}C_8$) varying in the substitution rate of grafted C_8 alkyl chains were characterized by fluorescence spectroscopy and by surface tension measurements. Their solubilizing properties were evaluated on two poorly water-soluble drugs differing in their molecular weight and their surface properties: Benzophenone has a simple chemical structure, a low molecular weight and is an utterly hydrophobic molecule used in sun-screen formulations, whereas docetaxel is a complex surface-active and high molecular weight anticancer drug. The solubilization of benzophenone and docetaxel was assessed from surface tension measurements, UV spectrometry and HPLC assays. The cytoxicity of the hydrophobically modified polysaccharide solution was compared to that of the Tween®/Ethanol–water vehicle, and the effective antitumoral activity of a Docetaxel-HMCMP formulation was verified on MCF-7 cells.

MATERIALS AND METHODS

Material

The hydrophobically modified carboxymethylpullulan derivatives ($CMP_{12}C_8$ and $CMP_{49}C_8$) (Fig. 1a) were synthesized in two steps according to the previously described procedure (20). The degree of modification (τ=12 or 49) is the number of alkyl chains per 100 anhydroglucose units. The charge density due to carboxylic groups decreases as the degree of hydrophobic modification (i.e. τ, the number of alkyl chains) increases. The water was de-ionized using a Milli-Ro 6+ system (Millipore) and then doubly distilled on acid $KMnO_4$ before experiments. This water had a surface tension of 71.8±0.1 mN/m at 25°C. 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) (Fig. 1b) and dimyristoylphosphatidylcholine (DMPC) were supplied from Sigma (France). Docetaxel (DXT, Mw: 807.88 g/mol) (Fig. 1c) was kindly provided by Dr Francoise Guéritte (ICSN-CNRS, France). Glutamax-I, RPMI 1640, 10% foetal

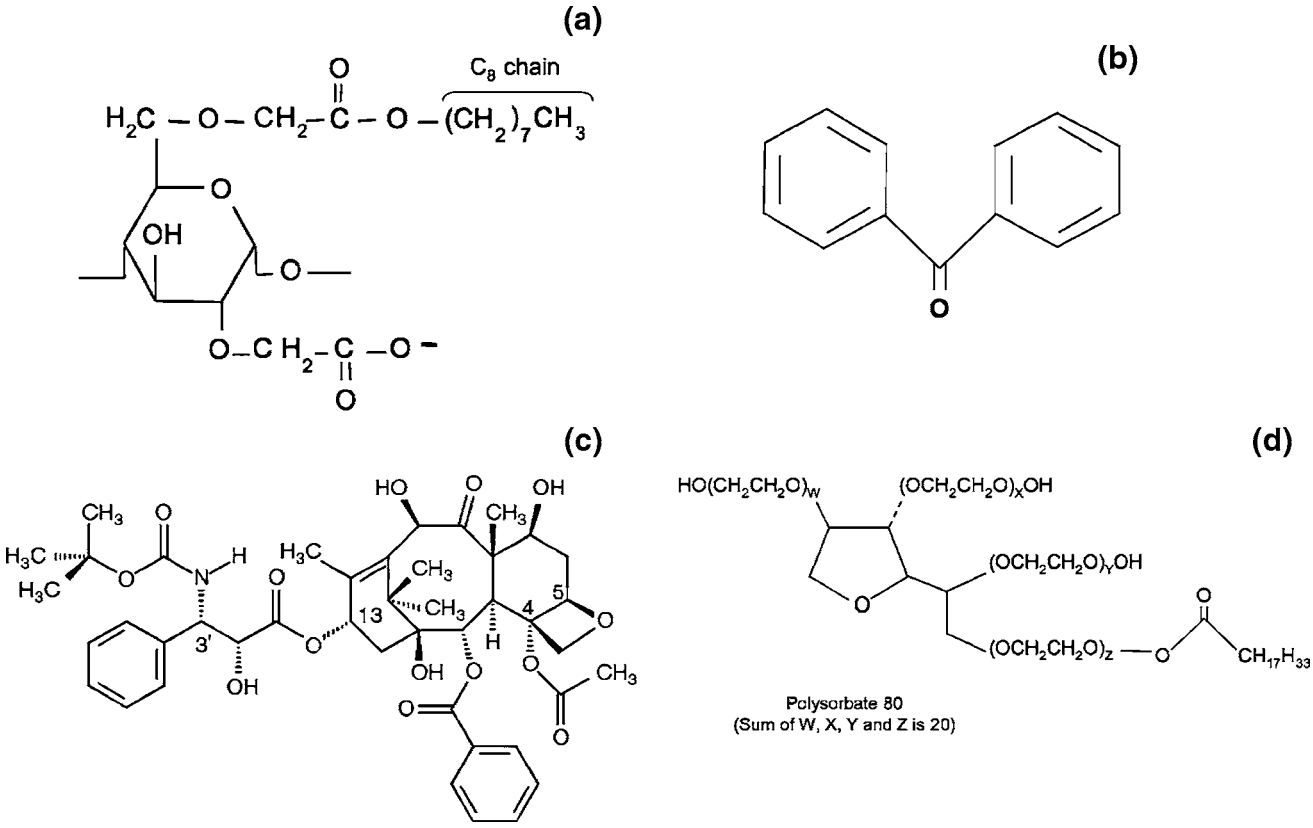

Fig. 1. Chemical structure of **a** CMP_7C_8 (τ=number of C_8 chains per 100 anhydrose units: τ is 12 and 49 for $CMP_{12}C_8$ and $CMP_{49}C_8$, respectively), **b** Benzophenone, **c** Docetaxel, and **d** Tween 80®.

calf serum, penicillin and streptomycin were purchased from Gibco (France). Sodium dodecylsulfate (SDS) at 20% (w/v) in ultrapure water was obtained from Laboratoires Eurobio (Courtaboeuf, France) and diluted in DMF (Sigma, France). 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ethanol, chloroform in analytical grade and acetonitrile in high performance liquid chromatography (HPLC) grade were obtained from Carlo Erba Reagents (France). Tween 80® (Fig. 1d) was provided by Prolabo-WVR (France). A Tween 80®/Ethanol–water mixture was obtained by adding 250 µL of Tween 80® to 750 µL of ethanol–water (13:87 v/v) (29). The glassware was cleaned in a freshly prepared sulfochromic solution and abundantly rinsed with the ultrapure water.

Preparation of Polymer Solutions

Polymer stock solutions were prepared by dissolving 14 g of polymer per litre of phosphate buffer (KH_2PO_4, KOH, 0.1M). Their pH was adjusted to 7.5, corresponding to the full ionization of carboxylate groups (20). To ensure complete solubilization of the polysaccharide derivatives, the solutions were stirred at room temperature for at least 48 h. Before the experiments, they were diluted to obtain samples with concentrations ranging from 14 to 1.4×10^{-6} mg/mL.

Drug Solubilization

3 ml of the polymer solutions varying in the [14×10^{-6}–14 mg/mL] range were incubated for 24 h at room temperature, under stirring, with 1 g benzophenone or 5 mg docetaxel. 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 in the polymer solutions was determined by UV spectroscopy for benzophenone (λ=259 nm) and by HPLC for docetaxel (29,30). For HPLC measurements, the samples were first diluted 280 fold in an acetonitrile-water (60:40) mixture, then 50 µL of the diluted sample were injected into a Nucleosil C18, 5 µm column (Merck) equipped with a C18 column guard. The column was eluted with acetonitrile-water (60:40) with a flow rate of 1 mL/min. UV detection was performed at 229 nm. Peak heights were recorded and the drug concentrations were calculated from standard curves.

Surface Tension Measurements

The interfacial behaviour of polymer solutions and polymer–drug mixtures was studied as a function of the polymer concentration. The surface tension (γ) was measured by the Wilhelmy plate method using a K10 tensiometer (Krüss, Germany). In order to maintain a constant level of the liquid and avoid any drift in the measured surface tensions, all experiments were performed under satured vapour pressure at 23±2°C. The surface tension was continuously recorded for 20 h. The reported surface tension values are mean values of at least three measurements. The experimental uncertainty was estimated to be 0.2 mN/m.

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 $CMP_{12}C_8$ or $CMP_{49}C_8$ solution and gently stirred for 18 h to ensure the incorporation of the molecular probe into polymer hydrophobic nanodomains. 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 (1,11,31). Such a low concentration was chosen to minimize the influence of pyrene on the formation and/or the stability of hydrophobic nanodomains. 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).

Cell Lines and Culture Conditions

Cytotoxicity Studies Against J774.A1 Murine Macrophages

The J774.A1 murine macrophage-like cell line (ATCC, USA) was maintained as an adherent culture in humidified atmosphere (95% air, 5% CO_2) at 37°C in RPMI-1640 with Glutamax-I supplemented with 10% (v/v) Fœtal Calf Serum (FCS), 100 IU/ml penicillin and 100 IU/ml streptomycin. Prior to the experiments, cells were mechanically detached and counted in a Neubauer plate. Their concentration was adjusted to 3×10^5 cells/mL with a complete cell medium and deposited in 96-well plates, each of them containing 100 µL of the cell medium. Cells were left for 3 h to allow their adhesion. Then, various dilutions of freshly prepared $CMP_{49}C_8$ solutions or the Tween 80®/Ethanol–water mixture were added into the wells and incubated with the cells for 1, 3 and 20 h at 37°C.

The cytotoxicity of the $CMP_{49}C_8$ solutions and Tween 80®/ethanol–water mixture was assessed using the MTT assay. This assay, first described by Mosmann in 1983 (32), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow soluble MTT and form dark blue insoluble formazan crystals that accumulate into the healthy cells. Solubilization of the cells membrane by addition of a detergent (SDS) results in the release of the crystals, which are collected and solubilized. The color of the formazan solution can be quantified using a simple colorimetric assay and a multiwell scanning spectrophotometer (32,33). The number of surviving cells is directly proportional to the level of the formazan product created. Thus, after incubation of the cells with a polymer solution or the Tween 80®/Ethanol–water mixture, 20 µL of a MTT solution (5 mg/mL) was added to each well and the plates were incubated for another 2.5 h. Then the plates were centrifugated (1,500×g, 5 min), the supernatants removed by suction and the purple colored precipitates of formazan were dissolved into 200 µL of a 10% SDS solution. The absorbance was read at 570 nm. Cells incubated in medium alone served as a control for cell viability. Each experiment was performed three times.

Toxicity of $CMP_{49}C_8$–Docetaxel Aggregates Against MCF-7 Human Breast Cancer Cells

MCF-7 cells were maintained in RPMI 1640 supplemented with Glutamax-I, 10% Fœtal Calf Serum, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. Before experiments, confluent cell monolayers were detached with trypsin, and counted in a Neubauer plate. MCF-7 cells were then seeded at 3×10^4 cells/well in 96-well plates and incubated for 3 h to allow cell adhesion. The blank polymer solutions, $CMP_{49}C_8$-docetaxel solutions, Tween 80®/Ethanol–water blank and docetaxel-containing Tween 80®/Ethanol–water mixtures were added in triplicate to each well and incubated for 1, 3 and 20 h. For the preparation of the Tween 80®/Ethanol–water mixture containing docetaxel, 10 mg of the drug were added to 250 µL of Tween 80®. Then 750 µL of a 13% ethanol in water solution were added to the Tween 80®/Docetaxel mixture according to the described protocol (29,34). The toxicity of each solution was assessed using the MTT assay as previously described. Each experiment was performed three times.

RESULTS AND DISCUSSION

Comparison of $CMP_{49}C_8$ and $CMP_{12}C_8$

The amphiphilic character of $CMP_{49}C_8$ and $CMP_{12}C_8$ and their ability to self-aggregate was evaluated by performing tensiometric and spectrofluorimetric studies in aqueous solution.

Interfacial Behaviour of the Hydrophobically Modified Carboxymethylpullulan Derivatives

The surface tension (γ) of hydrophobically modified carboxymethypullulan (HMCMP) solutions at the air/solution interface was measured as a function of the polymer concentration. The results are presented in Fig. 2. From the data, it is clear that the two modified compounds $CMP_{49}C_8$ and $CMP_{12}C_8$ exhibited surface properties in aqueous solutions. Up to a concentration of 0.014 mg/mL, the surface tension of both polymers remained close to that of water. Then, their surface tension decreased gradually and reached a clear break point, which would correspond to the saturation of the interface by the adsorbed HMCMP molecules. At the break points, the polymer concentrations were 3 mg/mL and 5 mg/mL for $CMP_{49}C_8$ and $CMP_{12}C_8$, respectively. Although these concentration values were significantly different for the two studied polymers, the γ values only slightly decreased when increasing the C_8 alkyl chains substitution rate from 12 to 49%. At the highest studied concentration, the surface tension was close to 50 mN/m for $CMP_{12}C_8$ but did not reach values below 45 mN/m for $CMP_{49}C_8$, despite its much higher substitution rate compared to $CMP_{12}C_8$. This phenomenon was observed in a previous study and was attributed to the formation of monomolecular aggregates of the associative polysaccharides that would adsorb at the air/solution interface in a more or less condensed conformation depending on the hydrophobic chains substitution rate. At a high substitution rate such as that of $CMP_{49}C_8$, it is believed that most of the hydrophobic grafted chains interact within the hydrophobic core of nanoaggregates and that only few of them are able to reach the interface (20).

Fluorescence Experiments

In an attempt to gain information on the aggregation process of the amphiphilic polymers, the I_1/I_3 ratio of the fluorescence spectra of pyrene was determined. Pyrene, a highly hydrophobic molecule, is one of the most widely used neutral fluorescence probes (35–38). Its emission spectra are dependent on the polarity of the microenvironment surrounding the probe. High values of the I_1/I_3 ratio indicate a polar environment whereas low values account for a non-polar environment. Fig. 3 illustrates the variation of the I_1/I_3 ratio with $CMP_{49}C_8$ and $CMP_{12}C_8$ concentrations. These experiments showed that at low polymer concentrations, the I_1/I_3 ratio was about 1.65, a value similar to that measured in an aqueous environment (10^{-6} M of pyrene in phosphate buffer). As the polymer concentration increased, a decrease in I_1/I_3 was observed, revealing an increasingly apolar local microenvironment of pyrene molecules. This phenomenon suggests that interactions between the HMCMPs alkyl groups induced the formation of self-aggregates with hydrophobic cores in which pyrene was solubilized. Apparently, as the polymer concentration increased, the pyrene was preferentially incorporated into the hydrophobic nanodomains until the concentration of the free probe in the surrounding water became negligible. A similar partition of this molecule has already been reported by Petit-Agnely *et al.* (1) and by us (20). The values of the break point concentrations as determined

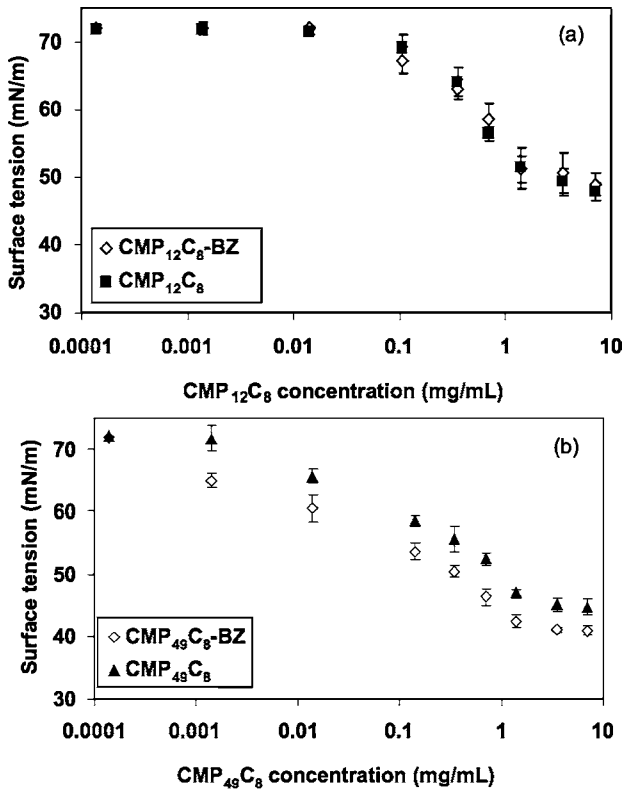

Fig. 2. Equilibrium surface tension of **a** $CMP_{12}C_8$ and $CMP_{12}C_8$–benzophenone mixtures and **b** $CMP_{49}C_8$ and $CMP_{49}C_8$–benzophenone mixtures, versus polymer concentrations.

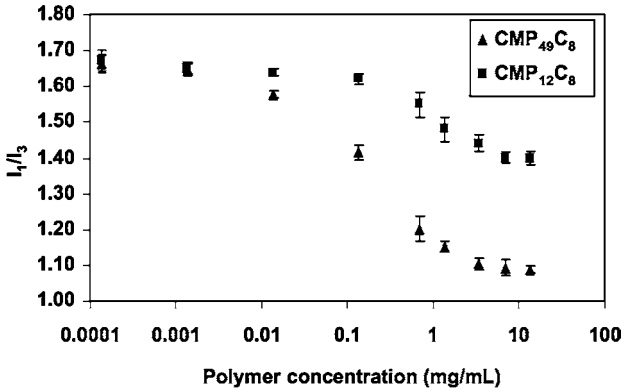

Fig. 3. Variation of pyrene fluorescence intensity ratios (I_1/I_3) versus polymer concentration for $CMP_{49}C_8$ and $CMP_{12}C_8$ solutions.

from the surface tension measurements were confirmed by the fluorescence experiments and corresponded to the stabilisation of the I_1/I_3 ratios. Apparently, the break point would not solely indicate the saturation of the interface but also a change in the solution. The concentration at this break point is often referred to as the critical aggregation concentration (or CAC) and could correspond for the studied polymers to the conformation of most macromolecules into monomolecular coils. Fig. 3 also shows the effect of the alkyl chain substitution rate on the I_1/I_3 ratio, which was lower for $CMP_{49}C_8$ (I_1/I_3=1.1) than for $CMP_{12}C_8$ (I_1/I_3=1.4). The higher I_1/I_3 ratio observed for $CMP_{12}C_8$ compared to $CMP_{49}C_8$ indicates that its hydrophobic core was less compact than that of $CMP_{49}C_8$. This was probably due to the low substitution rate of $CMP_{12}C_8$, insufficient to produce strong hydrophobic interactions between the chains and thus leading to the formation of loose monomolecular coils in which the pyrene could not be correctly incorporated.

The results of the surface tension and fluorescence measurements are in agreement with those obtained from Flow Field Flow Fractionation with on line MultiAngle Laser Light Scattering (F4/MALL) and viscosimetric measurements performed on polymer solutions (7). These measurements have shown that for 30% of C_8 chains and above, and for polymer concentrations higher than 1 mg/mL, intramolecular associations predominated and the conformation of the polymer was very compact. On the contrary, when the degree of grafting was lower than 20%, large isolated species were observed. From all these results, it appears that monomolecular coils were formed at lower concentrations than the CAC. For $CMP_{49}C_8$, above the CAC, at concentrations closer to another critical concentration C^{cr} (≈25 mg/mL), coils aggregates were probably formed (7). The hydrodynamic radii of $CMP_{49}C_8$ and $CMP_{12}C_8$ nanodomains could be determined by F4/MALL and were found to be lower than 20 nm (which is the minimum value that can be measured by multi-angle light diffusion), and as high as 43 nm, respectively.

Solubilization of Drugs in HMCMPs Solutions

The results described above clearly indicate that pyrene molecules incorporated into the $CMP_{49}C_8$ nanodomains were well protected from the bulk water, suggesting the possible role of the alkyl chains in the formation of hydrophobic solubilization sites for non-polar molecules. In order to evaluate the applicability of the HMCMPs derivatives as solubility enhancers, solubilization studies were undertaken. Two model molecules were chosen for their well-known poor water solubility. Although both of them are hydrophobic, benzophenone is unable to lower the surface tension of water, whereas docetaxel showed significant surface properties when spread at the air–water interface from a chloroform solution (Fig. 4). The calculated molecular area of docetaxel at the interface was found equal to 44.0 $Å^2$. When one considers the molecular weight of the drug and its complex chemical structure, it is obvious that only a small portion of the molecule actually protruded at the interface, and that most of it was actually immersed into the subphase.

Assessment of the Surface Properties of Benzophenone–HMCMP Mixtures and Relation with Drug Solubilization

As a hydrophobic compound, benzophenone was expected to interact with the alkyl groups of the HMCMPs as previously observed with a dextran derivative (39). Fig. 2 shows the change in the surface tension of pure HMCMP and HMCMP-benzophenone mixed solutions as a function of polymer concentration. The pure $CMP_{12}C_8$ solutions and the $CMP_{12}C_8$–benzophenone mixtures showed a similar interfacial behaviour (Fig. 2a). Obviously, benzophenone was unable to modify the surface tension of the polymer solution. Conversely, when benzophenone was added to $CMP_{49}C_8$ solutions (Fig. 2b), the surface tension of the solution was lowered to a higher extent than that of the pure polymer solutions. It is worth noting that although benzophenone contributed to the surface tension lowering, it did not affect the isotherm profile, i.e. the slope of the γ-log C plot (Fig. 2b). The interaction between the drug and $CMP_{49}C_8$ could proceed from two coexisting mechanisms: (1) the incorporation of benzophenone into the hydrophobic core of the polymeric nanoaggregates leading to the reinforcement of hydrophobic interactions between the polymeric alkyl chains. The tighter packing and consequent shrinking of the nanodomains would allow the adsorption of a greater number of them at the interface, resulting in a reduction of γ; (2) the association of benzophenone molecules to all alkyl chains of the modified carboxymethylpullulan, in and out of polymeric nanodomains. The decrease in surface tension would thus be attributed to the specific organisation of the alkyl chains–benzophenone complexes adsorbed at the interface.

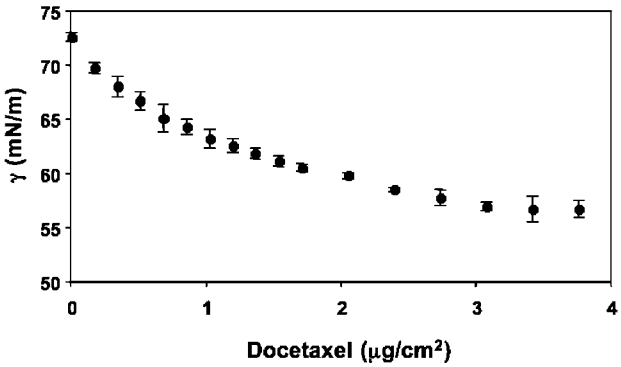

Fig. 4. Surface tension-surface density isotherm for docetaxel spread at the air–water interface from a chloroform solution.

The solubility profiles reported in Fig. 5 demonstrate the influence of the substitution rate of a polysaccharide derivative on its solubilizing property. Whereas, the solubility of benzophenone in $CMP_{12}C_8$ was equivalent to that in water (≈ 61 μg/mL), it was significantly enhanced in the $CMP_{49}C_8$ solution. An increase in solubility up to 6 fold could be achieved using high $CMP_{49}C_8$ concentrations (14 mg/mL). The difference in the apparent solubility of the drug in the two polymer ($CMP_{12}C_8$ and $CMP_{49}C_8$) solutions is in agreement with the results obtained from surface tension measurements (Fig. 2). Therefore, the higher solubility of benzophenone in $CMP_{49}C_8$ solutions compared to $CMP_{12}C_8$ ones could be directly related to the higher $CMP_{49}C_8$ substitution rate. This has been previously reported for poly (lactones), which difference in hydrophobicity affected the loading efficiency of indomethacin (40). The solubilization of the drug would be directly dependent upon the occurrence of strong intramolecular interactions between alkyl chains yielding a shrunken polymer conformation (drug entrapping structure). The inner hydrophobic core would interact with the drug, while the polysaccharide outer shell would guarantee the solubility of the whole system. The use of the $CMP_{49}C_8$ aggregates could thus be envisioned for the solubilization of hydrophobic poorly water-soluble molecules.

Solubilization of Docetaxel in HMCMPs Solutions

Docetaxel (DTX) is a promising drug against cancer but its clinical applications have been hindered by its poor water solubility (41). The only presently available formulation for clinical use consists in an ethanol–water solution containing a high concentration of Tween 80®. This surfactant is known for inducing several hypersensitivity reactions and is incompatible with common PVC intravenous administration sets (42,43). The self-assembled nano-aggregates formed by amphiphilic pullulans appeared as promising drug carriers since they self-assemble spontaneously in aqueous solution, without resorting to organic solvents. These unique properties were expected to reduce the toxicity and enhance the biocompatibility of docetaxel preparations as compared to other dosage forms (29,44,45).

Since $CMP_{12}C_8$ proved its inability to form hydrophobic domains and to solubilize benzophenone, the interaction of docetaxel with the HMCMPs was studied with $CMP_{49}C_8$, only. The surface tension–concentration measurements showed that, at low concentrations, the presence of docetaxel in mixtures with the hydrophobized polysaccharide lowered the surface tension of water to a much higher extent than the polymer alone (Fig. 6). However, contrarily to benzophenone–$CMP_{49}C_8$ mixtures, the profile of the docetaxel-$CMP_{49}C_8$ isotherm was very dissimilar to that of $CMP_{49}C_8$ alone. The slope of the γ-log C relationship and the critical aggregation concentration of the mixture were lower. Also, the maximum surface tension lowering was more limited for $CMP_{49}C_8$–DXT (24.5 mN/m) than that for the pure polysaccharide derivative (28.5 mN/m). These dissimilarities may be explained by the surface properties of the drug (Fig. 4): Unlike benzophenone, docetaxel can adsorb at the interface by itself and may interact with hydrophobized carboxymethylpullulans at the level of both the alkyl chains and polysaccharide backbone.

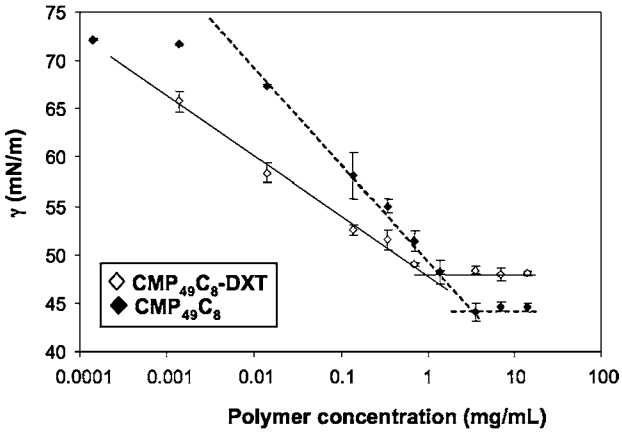

Fig. 6. Equilibrium surface tension of $CMP_{49}C_8$ and $CMP_{49}C_8$–docetaxel mixtures versus polymer concentrations.

Docetaxel solubilization by $CMP_{49}C_8$ solutions was evaluated and the results are plotted in Fig. 7. The interaction of the drug with the polysaccharide derivative allowed increasing the drug apparent solubility. The curve was linear over the [1.4×10^{-3}–1.4 mg/mL] polymer concentration range (inset to Fig. 7). However, at high polymer concentrations, the relationship between the drug solubility and polymer concentration was no longer linear demonstrating the limited solubilizing properties of the polymer above a critical concentration (C=1.4 mg/mL). It is very likely that due to

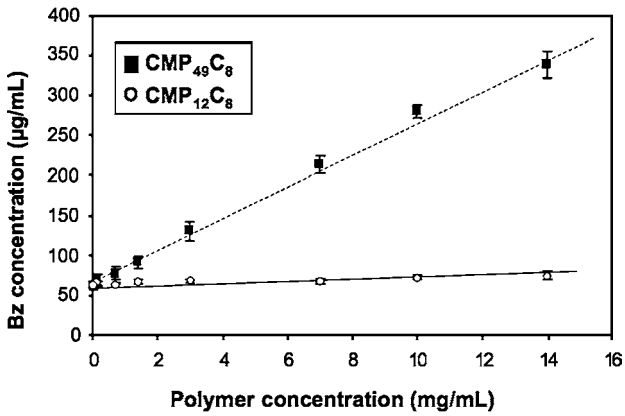

Fig. 5. Apparent solubility of benzophenone in $CMP_{12}C_8$ and $CMP_{49}C_8$ aqueous solutions.

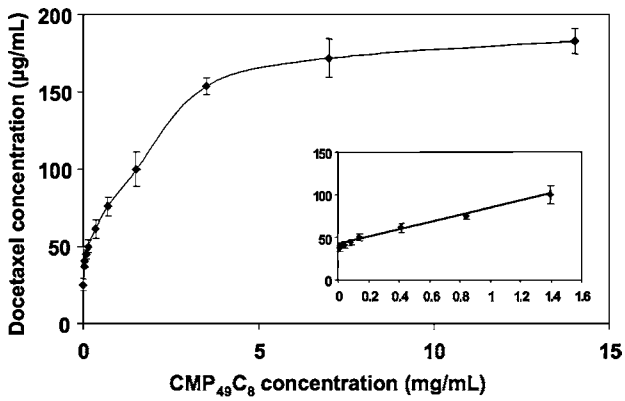

Fig. 7. Apparent solubility of docetaxel in $CMP_{49}C_8$ hydrophobic domains.

its amphiphilic structure docetaxel was not only incorporated into the hydrophobic core of the $CMP_{49}C_8$ aggregates but also interacted with its outer shell. These findings are in agreement with previous reports on the solubilization of another amphiphilic drug (amphotericin B) by polymeric micelles (46). It is possible that docetaxel molecules acted as bridges between the numerous monomolecular coils, reinforcing intermolecular interactions. The resulting decrease in the critical concentration (C^{cr}) at which these intermolecular interactions appeared, eventually led to a decrease in polymer solubility.

Biological Evaluation of the $CMP_{49}C_8$–docetaxel System

In order to evaluate the biocompatibility of the $CMP_{49}C_8$ aggregates, and the cytotoxic activity of docetaxel when associated to them, we performed a series of cytotoxicity studies using J774.A1 murine macrophages and MCF-7 human breast cancer cells.

Cytotoxicity of $CMP_{49}C_8$ and Tween 80®/Ethanol–water Mixture Against J774.A1 Murine Macrophages

The cytotoxicity of the $CMP_{49}C_8$ solutions in the absence of docetaxel was analysed using J774.A1 murine macrophages, as it was done in comparable studies (47–49). As shown in Fig. 8a, the $CMP_{49}C_8$ solutions reduced the number of viable cells in a time and dose-dependent manner. If the phagocytic capacity of the J774.A1 cells was involved in this cytotoxicity of the $CMP_{49}C_8$ solutions, then this effect should not be observed with the MCF7 cells studied later. Indeed, similar results were reported for poly(ε-caprolactone)-dextran nanoparticles for which a significant decrease in J774 viability was observed in the presence of the nanoparticles, whereas 80% viability of MCF7 cells was maintained (49). We also analysed the cytotoxicity of the vehicle used in the Taxotere® formulation (Tween 80®/ethanol–water) (Fig. 8b). Apparently, at similar concentrations Tween 80® appeared less cytotoxic than the polysaccharide derivative. However, the $CMP_{49}C_8$ and Tween 80® concentrations allowing solubilization of a same amount of docetaxel are different. Therefore, we compared in Fig. 8c the cytotoxic effect of the two systems at concentrations necessary for solubilizing the *same* docetaxel amounts. In this figure, the % survival is expressed as the potential docetaxel concentration in the culture cell medium. However, one must keep in mind that no drug was present in the studied formulations. The results demonstrate that in these conditions, $CMP_{49}C_8$ was less cytotoxic against macrophages than the Tween 80®/ethanol–water mixture.

Tween 80® is known to strongly interact with biological membranes inducing severe damages to cells (50). In order to get an insight into the mechanism of interaction of both docetaxel vehicles with a cell membrane, a phospholipid monolayer (DMPC) was spread at the air/water interface and solutions of the two excipients (at the concentrations used in the cytotoxicity experiments) were injected into the subphase. The changes in the surface pressure of the monolayer ($\Delta\pi=\pi-\pi_i$, with $\pi_i=\gamma_{water}-\gamma_{DMPC}$, and $\pi=\gamma_{water}-\gamma_{DMPC\text{-}excipient}$) were monitored as a function of time. The results are plotted in Fig. 9. They show a significant

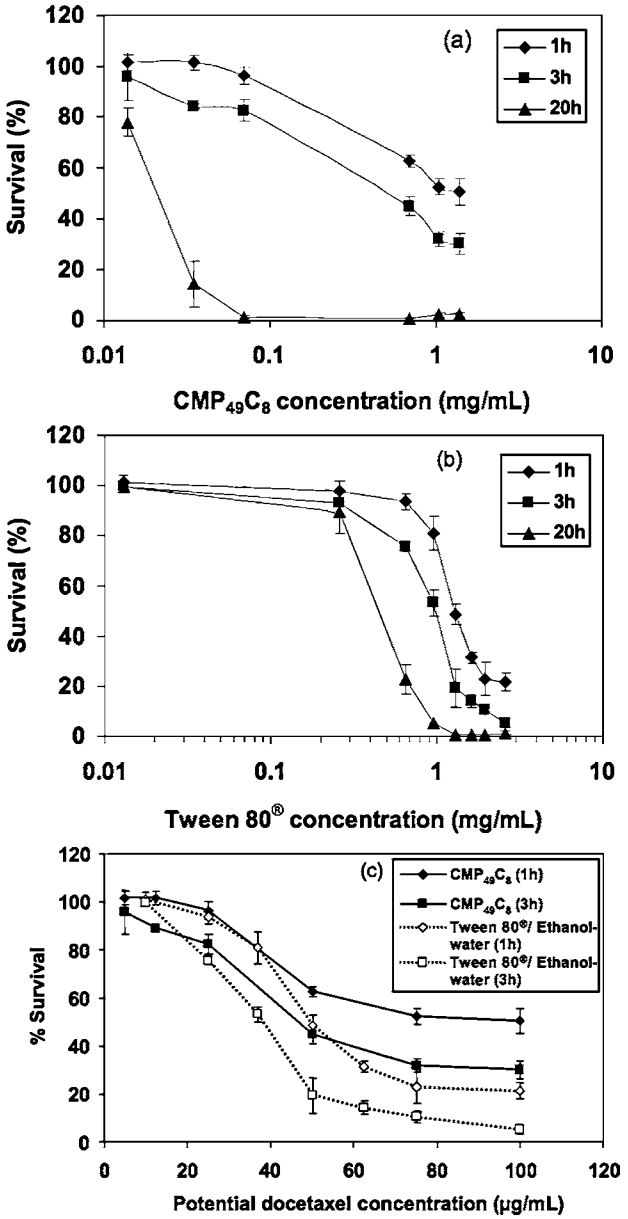

Fig. 8. Effect of **a** $CMP_{49}C_8$ and **b** Tween 80® used at the same concentrations, on the viability of J774.A1 cell lines after 1, 3 and 20 h of exposure. **c** Effect of the two excipients on the viability of the cells (1 and 3 h of exposure) when used at concentrations allowing solubilization of the same amounts of docetaxel.

adsorption-penetration of Tween 80® molecules into the already condensed DMPC monolayer (π_i=25 mN/m). Conversely, for $CMP_{49}C_8$, the penetration of the polysaccharide derivative is hindered by the phospholipid monolayer. This would account for a milder toxicity of $CMP_{49}C_8$ compared to Tween 80®.

Toxicity of $CMP_{49}C_8$–Docetaxel Mixed Solutions and Tween 80®/Ethanol–water/Docetaxel Mixtures Against MCF-7 Human Breast Cancer Cells

The cytotoxic activity of docetaxel associated to $CMP_{49}C_8$ aggregates was tested against MCF-7 human breast cancer

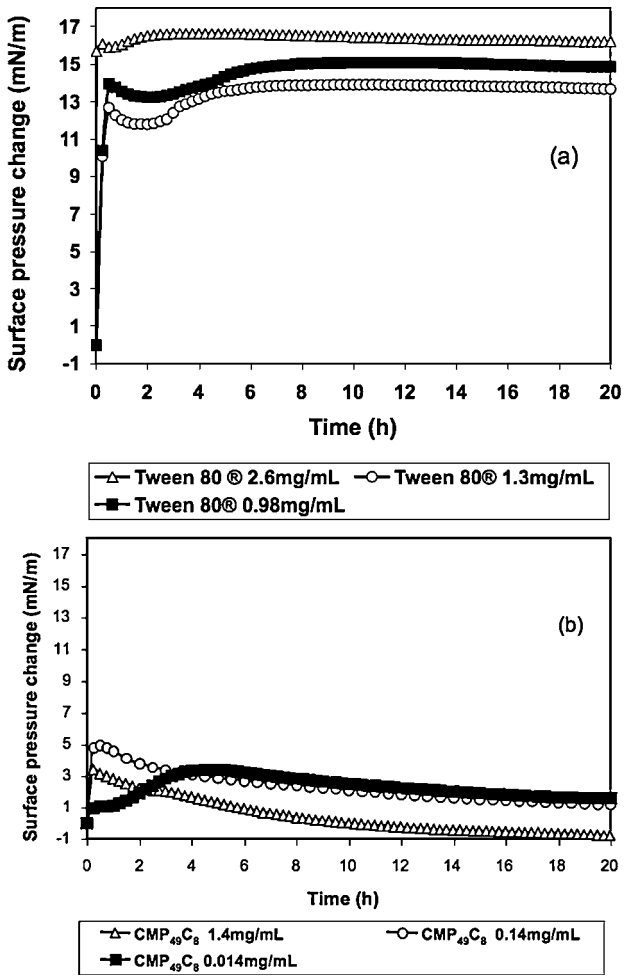

Fig. 9. Surface pressure change upon adsorption of **a** Tween 80® and **b** $CMP_{49}C_8$ into DMPC monolayers (initial DMPC surface pressure: π_i=25 mN/m).

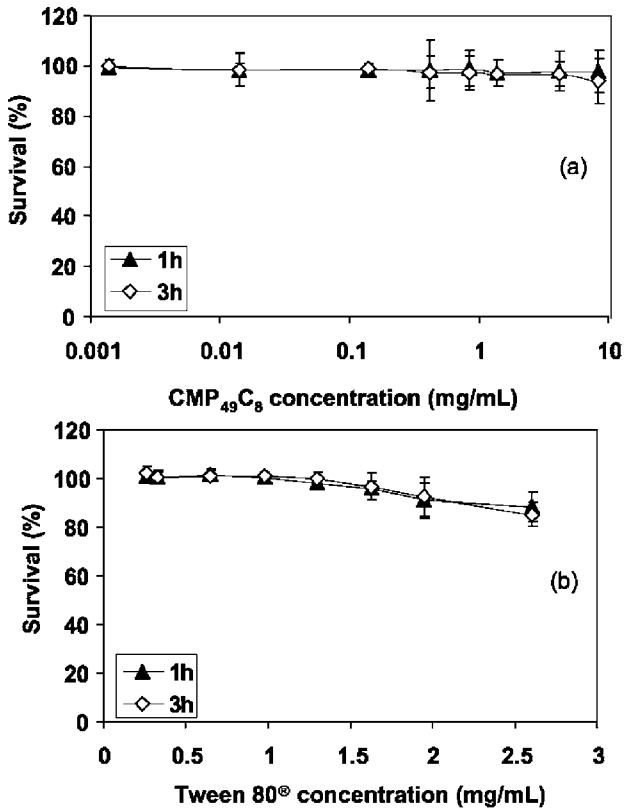

Fig. 10. Effect of docetaxel-free $CMP_{49}C_8$ solutions **a** and Tween 80® mixtures **b** on MCF-7 breast cancer cells after 1 and 3 h exposure.

cells. Tween 80®/ethanol–water/docetaxel mixture and docetaxel-free formulations were used as controls (Figs. 10 and 11).

As it can be seen in Fig. 10a, docetaxel-free $CMP_{49}C_8$ solutions did not affect MCF-7 cell growth at all concentrations tested. Conversely, the Tween 80®/ethanol–water mixture induced 15% cell death at the highest Tween 80® concentration in the cell culture medium (2.6 mg/mL) (Fig. 10b). This was not surprising because it is well documented that Tween 80® has intrinsic both biological and pharmacological activities. Drori *et al.* (51) and Crispens and Sorenson (52) have demonstrated that Tween 80® alters membrane fluidity and increases its permeability. This is in agreement with our own results (Fig. 9a).

Fig. 11 shows the survival percentage of MCF-7 cancer cells after 1 and 3 h exposure to docetaxel solubilized into the $CMP_{49}C_8$ solutions or the Tween 80®/ethanol–water mixture. The two formulations showed a similar dose-dependent cytotoxic activity, which caused a strong decrease in cell survival. These results suggest that the $CMP_{49}C_8$–docetaxel solutions and Taxotere® have a similar toxicity against MCF-7 human breast cancer cells, at the same drug concentration. Furthermore, docetaxel incorporated into $CMP_{49}C_8$ nanodomains is as potent as Taxotere® when tested on MCF-7

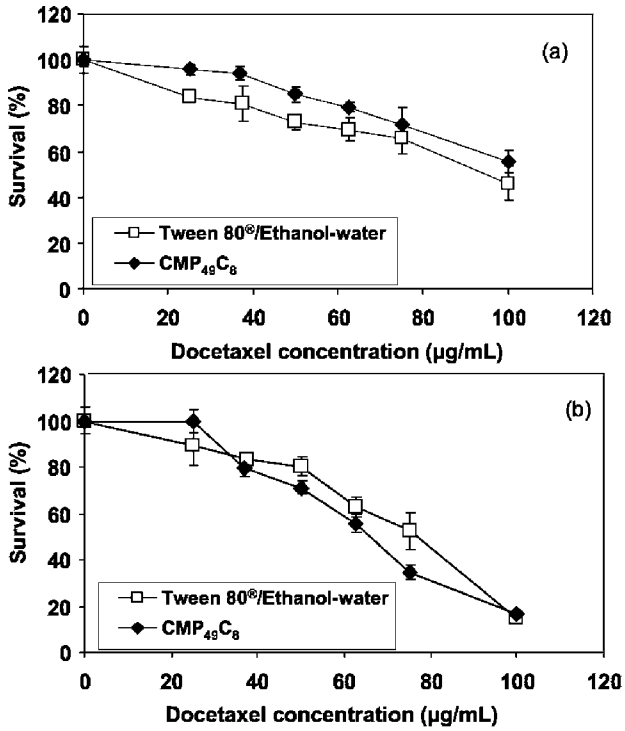

Fig. 11. Comparison of *in vitro* cytotoxicity of $CMP_{49}C_8$ aggregates vs Tween 80®/Ethanol–water formulations after 1 h (**a**) and 3 h (**b**) exposure.

cells indicating that the aggregates do not impair drug efficacy *in vitro*.

CONCLUSION

Hydrophobically modified carboxymethylpullulan derivatives spontaneously associate in aqueous media inducing the formation of soluble nanoaggregates with a hydrophobic core and hydrophilic outer shell. These polymers were tested as solubility enhancers and the results showed that the most hydrophobic of them, $CMP_{49}C_8$, could solubilize insoluble drugs such as benzophenone and docetaxel. The results obtained from surface tension measurements showed that benzophenone and docetaxel displayed different behaviors when added to polymer solutions. Benzophenone, a highly hydrophobic and low molecular weight molecule interacted strongly with the C_8 chains grafted onto the polymer backbone, inducing a reinforcement of the hydrophobic interactions. Conversely, the surface active docetaxel interacted not solely with the alkyl chains but also with the polysaccharidic backbone thus probably reducing polymer solubility. The drug free-$CMP_{49}C_8$ solutions proved to be less toxic against macrophages than Tween 80® (the excipient used in the commercialized formulation of docetaxel), and in the presence of the drug, the solutions showed a similar *in vitro* cytotoxic activity against MCF 7 cells.

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