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Research paper

Characterization of self-assembling copolymers in aqueous solutions using Electron Paramagnetic Resonance and Fluorescence spectroscopy

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Abstract

Electron Paramagnetic Resonance and fluorescence spectroscopy have been used to determine the micropolarity and microviscosity of self-assembling systems based on mmePEG-p(CL-co-TMC) having different PEG chain lengths and different CL/TMC ratios and PEG/MOG/SA (45/5/50) polymers with different PEG chain lengths. Four reporter probes have been used: two spin probes, 16-doxyl stearic acid and 5-doxylstearic acid, and two fluorescent probes, pyrene and 1,3-bis(1-pyrenyl) propane (P3P). We found that the micelles based on mmePEG-p(CL-co-TMC) polymers are of a biphasic nature. The micelles are made of a hydrophilic corona with low viscosity while the core of the micelle is more hydrophobic and more viscous. The outer shell is made up of PEG chains, the hydrophobic part of the chains making the core. The partial hydration of the shell seems to lead to a looser chain network than that associated with deeper domains in the micelles. By contrast, in micelles composed of PEG/MOG/SA, there is no clear domain separation. This is consistent with a spatial configuration of random polymeric chains forming a loose network. In these micelles, the microviscosity is low and the hydrophobicity is high.

Keywords: Self-assembling polymers; Electron Paramagnetic Resonance (EPR, ESR); Fluorescence; Microviscosity; Micropolarity

1. Introduction

Polymeric micelles are formed by the self-assembly of block amphiphilic copolymers. The hydrophobic block of the copolymer forms the core which can solubilize poorly soluble drugs whereas the hydrophilic block, usually PEG, forms the corona. Their small size, typically in the range of 10 to 100 nm, and their structure prevent their uptake by the mononuclear phagocyte system. They can be used as a drug carrier for passive targeting of anticancer drugs as well as for gene therapy [1–11].

Copolymers made of monomethyl polyethylene glycol (mmePEG), caprolactone (CL) and trimethylene carbonate (TMC), named mmePEG-p(CL-co-TMC) copolymers, and copolymers made of 45 mol% of polyethylene glycol (PEG), 5 mol% of monooleyl glycerol (MOG) and 50 mol% of succinic anhydride (SA), namely PEG/MOG/SA (45/5/50) copolymers, have been identified as families of biodegradable polymers which self-assemble by gentle mixing in aqueous solutions without solvent, surfactant or an external source of energy. These polymers present interesting solubilization properties regarding poorly water-soluble drugs and stability features in physiological-like conditions. The first class of polymer would be a potential candidate for oral as well as for intravenous administration whereas the second family might be restricted to oral administration [12–15].

Electron Paramagnetic Resonance (EPR) is widely used for the characterization of the microenvironment of colloidal

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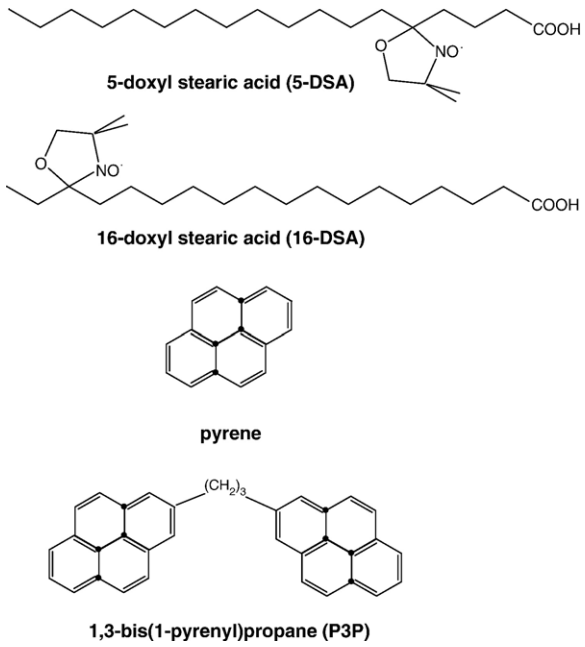

Fig. 1. Chemical structures of the probes used in the study.

systems and especially of micellar systems [16–22]. The principle is based on the entrapment of spin probes in various regions of the system. In particular, the doxyl stearic amphiphilic probes with the nitroxide group located at different positions of the carbon chain can bring information on the local polarity and viscosity at different depths of the self-assembled systems [18,23–25]. In this work, two nitroxide spin probes from the doxyl stearic acid family, 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA) (Fig. 1), have been used to characterize the local micropolarity and microviscosity of self-assembled systems. The microviscosity was determined from the correlation time τ_c and the micropolarity was estimated by means of the hyperfine splitting constant a_N.

Fluorescence probe techniques have also been developed for the structural study of colloidal solutions [26]. Pyrene (Fig. 1) has been reported to be an interesting probe since its absorption spectrum, and more precisely the ratio of the intensity of the first absorption band over the intensity of the third absorption band varies as a function of the surrounding micropolarity [27]. The fluorescence intensity ratio of the excimer over the monomer emission of P3P, 1,3-bis(1-pyrenyl)propane (Fig. 1), allows the determination of the microviscosity in the surrounding area of this probe solubilized in the self-assembled systems [28].

In order to optimize the use of self-assembling copolymers, there is a clear need for a better characterization of the local microviscosity and micropolarity gradient. This information could eventually lead to a better representation of their structure. In this study, we used EPR and fluorescence spectroscopy to characterize the micropolarity and the microviscosity of self-assembled systems based on mmePEG-p(CL-co-TMC) having different PEG chain lengths and different CL/TMC ratios and PEG/MOG/SA (45/5/50) polymers with different PEG chain lengths.

2. Materials and methods

2.1. Chemicals

The monooleyl glycerol (MOG) monomers were purchased from Quest International, and all the other chemicals were purchased from Aldrich.

2.2. Polymers

The polymers of interest were prepared from monomethoxylated polyethylene glycol (mmePEG), caprolactone (CL) and trimethylene carbonate (TMC), mmePEG-p(CL-co-TMC), with different CL/TMC ratios (30/70, 50/50, 70/30) and PEG chain lengths (750 and 2000 Da). The PEG/MOG/SA polymers contained 45 mol% polyethylene glycol (PEG), 5 mol% monooleyl glycerol (MOG) and 50 mol% succinic anhydride (SA) as well as PEG/MOG/SA (5/45/50), with different PEG chain lengths (400, 600, 1000 Da). The first family of polymers has been synthesized by ring-opening polymerisation using the mmePEG as the initiator [12] and the second by polycondensation [13] at the Johnson & Johnson Center of Biomaterials and Advanced Technologies (Somerville, NJ, USA). The polymer composition and residual monomer content were analysed by proton NMR. Gel permeation chromatography was employed to determine the molecular weight and the polydispersity (PD) of the polymers. The size of the particles was determined by photon correlation spectroscopy using a Malvern autosizer 4700 or Nanosizer ZS (Malvern) at 20 °C. Data on the characterization are given in Table 1.

2.3. Electron paramagnetic resonance

EPR spectra were measured with a 9.3 GHz Bruker EMX EPR X-band spectrometer equipped with a variable temperature controller BVT-3000 employing 100 kHz modulation frequency and 0.1 G modulation of amplitude. The center field was 3346 G, the sweep width of the magnetic field was 75 G. In order to avoid saturation of the signal, a power of 2 mW was selected. Measurements were done at least in triplicate. The samples were prepared as follows: 25 μl of a solution of the probe (16-doxyl stearic acid or 5-doxyl stearic acid, 40 mM in isopropanol) was placed in vials and the solvent evaporated under a nitrogen flux.

Table 1
Polymer characterization

Polymer	Mw	PD	Size (nm)	CMC (g/ml)
mmePEG 750-p(CL-co-TMC) 30/70	5448	1.5	20	$2.5*10^{-5}$
mmePEG 750-p(CL-co-TMC) 50/50	5100	1.7	20	$1.4*10^{-5}$
mmePEG 750-p(CL-co-TMC) 70/30	6185	1.6	20	$1.4*10^{-5}$
mmePEG 2000-p(CL-co-TMC) 50/50	6500	1.9	20+10/200	$3.0*10^{-5}$
PEG400/MOG/SA (45/5/50)	3770	2.3	10+ aggregates	$3.4*10^{-4}$
PEG600/MOG/SA (45/5/50)	3020	1.9	10+ aggregates	$5.0*10^{-4}$
PEG1000/MOG/SA (45/5/50)	4640	2	10+ aggregates	$4.0*10^{-3}$

mmePEG=monomethoxylated glycol, CL=caprolactone, TMC=trimethylene carbonate, MOG=monooleyl glycerol, SA=succinic anhydride.

Aqueous solutions of polymers in PBS buffer (pH=7.2) were added and mixed for 24 h at room temperature. The final concentrations are 50 mM for the polymer, and 1 mM for the nitroxide. For comparison, EPR spectra were also recorded for 5-DSA and 16-DSA (1 mM) dissolved in NaOH 0.1 M. The microviscosity in proximity of the nitroxide free-radical probe was determined by calculating the correlation time τ_c (in seconds), related to the rotational reorientation of the probe:

$$\tau_c = 6.5e^{-10} * \Delta H_0 * \left\{ \sqrt{\frac{I_0}{I_{+1}}} + \sqrt{\frac{I_0}{I_{-1}}} - 2 \right\} \text{ if the signal is isotropic} \quad (1)$$

$$\tau_c = 6.5e^{-10} * \Delta H_0 * \left\{ \sqrt{\frac{I_0}{I_{+1}}} - 1 \right\} \text{ if } \tau_c < 3\text{ns} \quad (2)$$

where ΔH_0 is the line width (in gauss) of the zero transition

I_0, I_{+1}, I_{-1} are the peak-to-peak heights of the 0, +1 and −1 transitions in the first-derivative spectrum (see Fig. 2) [21,23].

A calibration curve (viscosity as a function of correlation time) was established by determining the correlation time of the probes in solvents of increasing viscosities. The method described above was used to prepare the samples, the polymeric solutions being replaced by a solvent.

The micropolarity of the environment in the vicinity of a nitroxide radical has been determined by means of the hyperfine splitting constant a_N. For 16-DSA, a typical fast motional spectrum is obtained, and a_N was determined graphically as half of the distance between the 2 extremes. For 5-DSA, which gives a complex spectra, the a_N parameter was calculated using the formula $a_N=(A_{max}+2A_{min})/3$. The parameters A_{max} and A_{min} were determined graphically [24] (see Fig. 2).

2.4. Fluorescence

Pyrene: Steady-state fluorescence spectra were recorded with a SLN 48000 S spectrometer (Aminco) at an excitation wavelength of 334 nm. Using pyrene, the micropolarity was determined from the ratio I_1 (373 nm)/I_3 (383 nm) of the pyrene

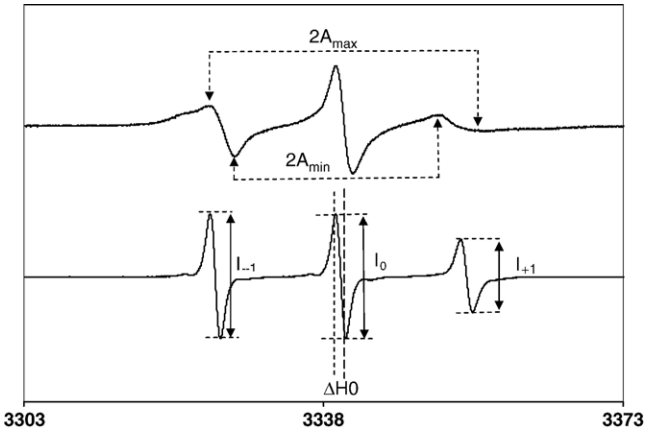

Fig. 2. X-Band EPR spectra of 16-DSA at 350 K in polymeric solutions. Top: 16-DSA in PEG 400/MOG/SA (45/5/50). Bottom: 16-DSA in mmePEG-p(CL-co-TMC) (50/50).

Table 2
Hyperfine splitting constant a_N (G) of 16-DSA ESR spectra and I_1/I_3 of pyrene fluorescence spectra in 50 mM aqueous solutions of mmePEG-p(CL-co-TMC) and PEG/MOG/SA (45/5/50) polymers at room temperature

Polymer	a_N		I_1/I_3
	5-DSA	16-DSA	
mmePEG 750-p(CL-co-TMC) 30/70	15.1±0.3	12.8±0.1	1.5
mmePEG 750-p(CL-co-TMC) 50/50	15.2±0.0	12.9±0.1	1.5
mmePEG 750-p(CL-co-TMC) 70/30	14.9±0.0	13.1±0.0	1.5
mmePEG 2000-p(CL-co-TMC) 50/50	15.0±0.1	13.2±0.1	1.5
PEG400/MOG/SA (45/5/50)	14.6±0.5	14.2±0.0	1.3
PEG600/MOG/SA (45/5/50)	14.3±0.5	14.2±0.1	
PEG1000/MOG/SA (45/5/50)	14.1±0.1	14.3±0.0	1.3

spectra (emission wavelength). In the procedure, a given amount of pyrene stock solution (10^{-6} M) in acetone was evaporated. An aqueous solution of the copolymer was then added to residual pyrene. The pyrene concentration was fixed at $6*10^{-7}$ M.

P3P: P3P (1,3-bis(1-pyrenyl)propane) was also chosen to determine the microviscosity of the aggregates core [35]. The I_1/I_3 values were averaged over 3 determinations and plotted versus the polymer concentration. The critical micellar concentration and core microenvironment were determined [12–14]. The pyrene concentration was fixed at $6*10^{-7}$ M. P3P (1,3-bis(1-pyrenyl)propane) was also chosen to determine the microviscosity of the aggregate cores. Due to the presence of two pyrenyl moieties in the molecule, an excimer forms upon excitation. The excimer formation is more important when the medium surrounding the probe is less viscous. A calibration of the ratio IE/IM (intensity of the excimer (505 nm)/intensity of the monomer (395 nm)) versus the viscosity was performed with P3P solutions in solvents of known viscosity. The requisite amount of a solution of P3P in acetone was placed in vials and the solvent evaporated under a stream of nitrogen. The appropriate volumes of solvent or aqueous polymeric solution were added and mixed for 24 h at room temperature in order to obtain $1*10^{-3}$ and $5*10^{-3}$ M polymer concentrations and $1*10^{-5}$ M probe concentration. The aqueous samples were deaerated by purging with nitrogen and the nonaqueous samples were deaerated using freeze–thaw cycling. As the oxygen quenches the excimer to a greater extent than the monomer, its presence influences the IE/IM ratio and it has to be carefully eliminated [28].

3. Results

The characteristics of the self-assembling polymers tested are described in Table 1. All amphiphilic polymers had low molecular weight and were liquid or semi-solid at room temperature. They self-assembled spontaneously in water. The particles formed by mmePEG-p(CL-co-TMC) were spherical and had a size of 20 nm [12–14]. The size of PEG/MOG/SA particles was 10 nm with some aggregates. To confirm that polymeric micelles were formed, CMC of the different self-assembling polymers have been determined using a fluorimetric method with a pyrene probe. The progressive decrease of I_1/I_3

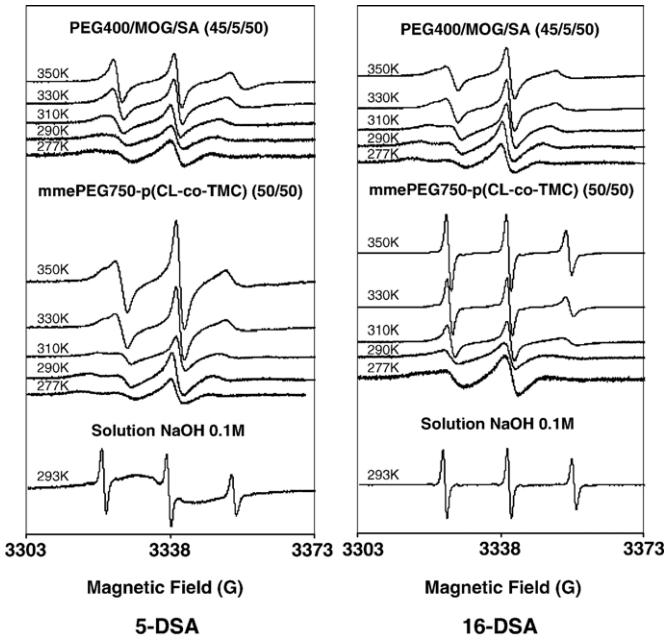

Fig. 3. X-Band EPR spectra of 16-DSA and 5-DSA in polymeric solutions of mmePEG750-p(CL-co-TMC) 50/50 and PEG/MOG/SA (45/5/50) at different temperatures.

ratio of the pyrene spectrum up to a value of approximately 1.5 for mmePEG-p(CL-co-TMC) and 1.3 for PEG/MOG/SA (45/5/50) confirms that a CMC could be determined (Table 2). The CMC were in the range of $2*10^{-5}$ g/ml for mmePEG-p(CL-co-TMC) copolymers and $3*10^{-4}$ g/ml for PEG400/MOG/SA (45/5/50) copolymers (Table 1).

3.1. Characteristics of the EPR spectra

Due to their solution properties, the doxyl stearic acids tested are expected to be incorporated inside the aggregates, with nitroxide moieties located at different depths inside the polymeric structures. In Fig. 2, typical EPR spectra are presented. They were recorded at 350 K using 16-DSA entrapped in mmePEG750-p(CL-co-TMC) 50/50 and PEG400/MOG/SA (45/5/50). The influence of the temperature on the EPR spectra is shown in Fig. 3.

EPR spectra using 16-DSA. The EPR spectrum of 16-DSA in NaOH solution was isotropic which is typical of fast motional averaging, with three lines of similar amplitude. When 16-DSA was incubated in buffer solutions of polymers, the EPR spectrum was broadened revealing the presence of motionally hindered species. The temperature had a dramatic effect on the shape of the EPR spectrum of 16-DSA in mmePEG750-p(CL-co-TMC) 50/50. At 277 K, the spectrum was anisotropic, which is typical of slowly moving species. There was a sharpening of the EPR spectra when the temperature was increased from 290 K to 350 K (Fig. 3). The EPR spectrum of 16-DSA in PEG400/MOG/SA at 277 K was also anisotropic, but the temperature had a smaller influence on the signal of the probe encapsulated in PEG400/MOG/SA (Fig. 3).

EPR spectra using 5-DSA. The EPR spectra of 5-DSA at 293 K in NaOH (0.1 M) was composed of an isotropic triplet and of an additional broad signal which has already been observed by other authors [21] and which was assigned to partial self-aggregation, i.e., the concentration of 5-DSA was above its critical micellar concentration. At a 50 mM in mmePEG750-p(CL-co-TMC) 50/50 in PBS buffer, the EPR spectrum was completely anisotropic with features similar to a powder spectrum (Fig. 2). The motion was less hindered in the presence of 50 mM PEG400/MOG/SA (45/5/50). Under 330 K, the 5-DSA spectra in mmePEG750-p(CL-co-TMC) 50/50 50 mM solutions were typical of immobilized systems. When 5-DSA was in the presence of PEG400/MOG/SA (45/5/50), a sharpening of the spectra was observed when the temperature increased.

3.2. Micropolarity

The micropolarity has been evaluated from EPR and fluorescence spectroscopy for solutions of mmePEG-p(CL-co-TMC) and PEG/MOG/SA (45/5/50) polymers of different compositions. The hyperfine splitting constant a_N obtained using 5-DSA and 16-DSA are reported in Table 2 as well the ratio I_1/I_3 obtained using fluorescence spectra of pyrene. The evolution of the hyperfine splitting constant as a function of temperature is shown in Fig. 4.

16-DSA. The a_N values of 16-DSA solubilized in NaOH solution were not dependent on the temperature (Fig. 4, values around 15.75±0.03 G), consistent with the literature [22,29]). The a_N values were dramatically lower in the presence of the polymers. In the presence of mmePEG750-p(CL-co-TMC) 50/50, a_N increased from 12.8 G at 290 K to reach a plateau value around 14.6 G at higher temperature (Fig. 4). When the temperature returned to room temperature, the a_N value returned to its initial value; this phenomenon was reversible. The temperature had a lower effect on the a_N values recorded in the presence of PEG400/MOG/SA (45/5/50) (between 14.20 and 15.0 G) compared to mmePEG750-p(CL-co-TMC) 50/50. The largest difference in a_N values was observed at 290 K: 12.9 G and 14.2 G for mmePEG750-p(CL-co-TMC) 50/50 and PEG400/MOG/SA (45/5/50), respectively. In both self-assembling systems, the PEG chain length and/or the CL/TMC ratio had no influence on the a_N values (Table 2).

5-DSA. As in the case of 16-DSA, the a_N of the probe solubilized in NaOH solution was around 15.75±0.05 G at all

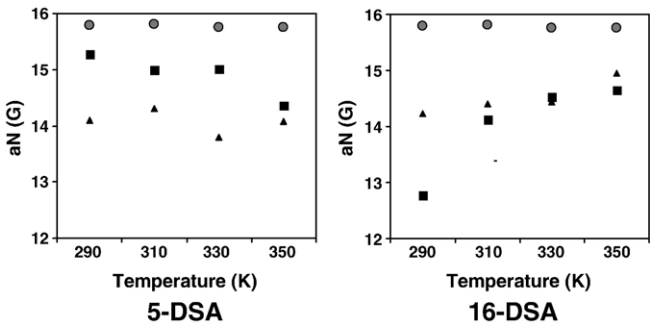

Fig. 4. Evolution of the hyperfine splitting constant a_N (G) as a function of temperature (K) of 16-DSA and 5-DSA in mmePEG750-p(CL-co-TMC) 50/50 (squares) or PEG400/MOG/SA (45/5/50) (triangles) 50 mM in PBS buffer and of 16-DSA and 5-DSA in NaOH 0.1 M (circles).

Table 3
Microviscosity (cP) in PEG/MOG/SA (45/5/50) and mmePEG-p(CL-co-TMC-polymeric solutions measures by ESR with the 16-DSA and 5-DSA probes and calculated from formula (1) and (2) in 50 mM aqueous solutions and by fluorescence with P3P in 1 and 5 mM solutions at room temperature

	ESR				Fluorescence	
	5DSA(1)	5DSA(2)	16DSA(1)	16DSA(2)	1 mM	5 mM
mmePEG 750-p(CL-co-TMC) 30/70	57.1±2.4	40.6±2.4	183.8±11.4	153.2±5.4	76	74
mmePEG 750-p(CL-co-TMC) 50/50	57.5±0.5	39.0±1.9	127.9±6.7	110.3±6.4	72	69
mmePEG 750-p(CL-co-TMC) 70/30	48.2±3.1	31.5±2.0	90.5±4.7	78.7±4.1	67	64
mmePEG 2000-p(CL-co-TMC) 50/50	49.1±5.2	33.7±3.7	108.4±16.4	102.3±0.8	71	67
PEG400/MOG/SA (45/5/50)	41.2±4.0	32.4±4.7	32.8±0.0	30.6±0.5	33	28
PEG600/MOG/SA (45/5/50)	41.8±1.0	32.4±0.0	25.3±4.6	25.8±3.5	26	31
PEG1000/MOG/SA (45/5/50)	41.5±3.3	31.0±0.7	31.7±0.0	28.7±0.4	28	29

temperatures. The hyperfine splitting constant of 5-DSA decreased significantly in the presence of both polymers. a_N values were constant with temperature using PEG400/MOG/SA (45/5/50), but slightly decreased with temperature for mmePEG750-p(CL-co-TMC) 50/50. Between 290 and 330 K, the hyperfine splitting constant values of mmePEG750-p(CL-co-TMC) 50/50 were superior to those obtained for PEG400/MOG/SA, but at 350 K a_N values were similar. At room temperature, the hyperfine splitting constant values determined with the 5-DSA spectra in mmePEG-p(CL-co-TMC) solutions (15.05 G±0.13) were higher than those calculated from PEG400/MOG/SA (14.3 G±0.25) (Table 2). Using PEG400/MOG/SA, the a_N values slightly decreased when PEG chain length increased (Table 2), whereas there was no significant change in a_N values using mmePEG750-p(CL-co-TMC). The proportion of caprolactone monomer also did not influence the hyperfine splitting constants.

Pyrene. At room temperature, the I_1/I_3 ratio of pyrene fluorescence spectra in the different PEG-p(CL-co-TMC) copolymer solutions was 1.5 whereas the I_1/I_3 ratio in the different PEG/MOG/SA copolymer solutions was 1.3.

3.3. Microviscosity

The microviscosities (cP) at room temperature were calculated from the EPR spectra with Eqs. (1) and (2). Eq. (1) gave slightly higher values than Eq. (2). The values are reported in Table 3 for the two probes (50 mM in polymer solutions). The microviscosities calculated from the fluorescence spectra of P3P (1 and 5 mM in polymer solutions) are also given in Table 3. The evolution of the correlation time of 16-DSA and 5-DSA probes as a function of temperature is given in Fig. 5 (τ_c obtained with Eq. (1)).

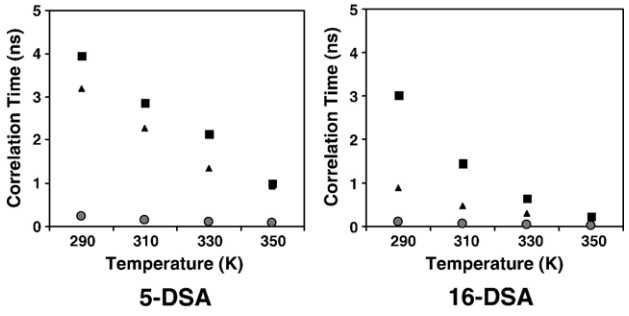

Fig. 5. Evolution of the correlation time τ_c (ns) calculated from Eq. (1) as a function of the temperature (K) of 16-DSA and 5-DSA in mmePEG750-p(CL-co-TMC) 50/50 (squares) or PEG400/MOG/SA (45/5/50) (triangles) 50 mM solutions and of 16-DSA and 5-DSA in NaOH 0.1 M (circles).

16-DSA. At room temperature, the correlation times calculated for the mmePEG-p(CL-co-TMC) polymers were between 0.8 and 5.5 ns, corresponding to microviscosities values between 90.5 and 183.8 cP. These microviscosities for the mmePEG-p(CL-co-TMC) polymers values were much higher than in the PEG400/MOG/SA system (from 25.3 to 32.8 cP). Among the polymers initiated with mmePEG750, the viscosity decreased by 50% when the proportion of caprolactone monomer increased from 30 mol% to 70 mol%. In mmePEG-p(CL-co-TMC) polymeric system, the increase in PEG chain length (from 750 Da to 2000 Da) resulted in a decreased microviscosity by about 15%. By contrast, the PEG chain length did not influence the microviscosity in PEG400/MOG/SA system. As expected from the details of the EPR spectra, the correlation time τ_c was significantly higher in polymer buffer solutions than in NaOH solution. The correlation time, τ_c of the probe was dependent on temperature as shown in Fig. 5. The difference in the microviscosity between both classes of polymer decreased when the temperature increased (Fig. 5). The microviscosity values recorded in mmePEG-p(CL-co-TMC) were always higher than in those observed for PEG400/MOG/SA, except at the highest temperature measured (350 K). The changes were reversible for each polymer.

5-DSA. The microviscosities obtained from 5-DSA results at room temperature were lower for the mmePEG-p(CL-co-TMC) polymers than those calculated from the 16-DSA (Table 3). For the PEG/MOG/SA (45/5/50), there was practically no difference between the 2 probes. The difference in microviscosities observed between both classes of polymer was smaller using 5-DSA than using 16-DSA (Table 3). The microviscosity was to a slight extent dependent on the PEG chain length for mmePEG-p(CL-co-TMC), but not for PEG400/MOG/SA system. Among the polymers initiated with mmePEG750, the viscosity decreased by 15% when the proportion of caprolactone monomer increased from 30 mol% to 70 mol%. In both polymeric systems, the correlation time value decreased when temperature increased. At all temperatures, the τ_c values obtained for mmePEG-p(CL-co-TMC) were higher than for PEG400/MOG/SA, except at 350 K.

The changes in correlation time τ_c of 5-DSA in 50 mM polymer buffer solutions were more pronounced than for the NaOH solution (Fig. 5). All changes were reversible (identical values when heated and cooled back to room temperature).

P3P. The microviscosities calculated from fluorescence experiments were between those calculated with the two EPR equations (70±4 cP) for the mmePEG-p(CL-co-TMC) polymers, and in the same range (around 35 cP) for the PEG/MOG/SA (45/5/50) polymers (Table 3). The high values in microviscosity have to be taken with caution because the calibration curve has been validated only up to viscosities of 40 cP.

4. Discussion

It is well established that different values of microviscosities and micropolarities can be obtained when using a given method and probe, because each probe may be located in a different micellar site on a time average basis [30]. In this work, four different probes, namely 16-DSA, 5-DSA, pyrene and P3P, have been used to characterize the microviscosity and micropolarity in self-assembling systems in order to get a better representation of their structure. Due to differences in their structure properties, these probes will be located at different places in the polymeric aggregates. Pyrene and P3P, which are the most hydrophobic probes, will migrate to the core of the aggregates. The nitroxide groups of the 16-DSA and 5-DSA probes will be closer to the interface due to the charge on the carboxylate, and the nitroxide in 5-DSA will be closer to the surface than the nitroxide of the 16-DSA.

All probes confirmed the formation of aggregates: above a given concentration, the spectra drastically changed indicating that the probes migrated from the bulk aqueous medium to a more hydrophobic environment. Indeed, the ratio of the intensity of peak 1 over the intensity of peak 3 of the pyrene spectra significantly decreased over a range of polymer concentration. The ratio of the intensity of peak 1 over the intensity of the excimer of the P3P spectra decreased in the presence of polymer. In EPR spectrometry, the probe fraction present in the aggregates increased with the polymer concentrations (data not shown). At a given concentration all the probes were located in the polymeric aggregates.

4.1. Characteristics of micelles made up of mmePEG-p(CL-co-TMC) polymers

As already mentioned in the literature [14], these polymers form micelles. The comparison of the EPR results using the 2 different probes at room temperature clearly indicates the biphasic nature of the micelles environment. The hyperfine splitting constant and the microviscosity determined from the spectra of 5-DSA probes were very different than those obtained using 16-DSA. The a_N values ranged from 14.9 G to 15.2 G using 5-DSA, and from 12.8 G to 13.2 G using 16-DSA. The measured microviscosities varied ranging from 48.2 cP and 57.5 cP for 5-DSA, and from 90.5 cP to 183.8 cP for 16-DSA. The micelles are made of a more hydrophilic and less viscous corona and a more hydrophobic and more viscous core. The diblock structure of the copolymer is in accordance with these findings. The hydrophilic part of the polymer is made up of PEG and the hydrophobic part is a random copolymer between the two hydrophobic monomer, poly-ε-caprolactone and trimethylene carbonate. The outer shell could be made of PEG chains, the hydrophobic part of the chains making the core. Partial hydration of the shell could lead to a looser chains network than that associated with deeper domains in the micelles. The CL/TMC ratio modified the microviscosity of the 16-DSA (50%) and less for the 5-DSA (22%) environment: an increase of this ratio decreased the microviscosity deeper in the aggregates and had a lower influence on the outer shell. The PEG chain length slightly modified the microviscosity and did not influence the micropolarity of any region of the micelles.

The influence of temperature on the hyperfine splitting constant and correlation time determined using both spin probes entrapped in mmePEG750-p(CL-co-TMC) (50/50) micelles reinforced the structure hypothesis. As expected, the correlation time decreased as temperature increased. The results showed that the polarity in the vicinity of the 5-DSA probe was rather stable whereas the polarity in the vicinity of the 16-DSA probe significantly increased with temperature. It is likely that some 'swelling' took place with the increase in temperature: the increase in temperature increased the Brownian movement of the polymeric chains, allowing water molecules to go deeper in the aggregates structure. This phenomenon was reversible.

The polarity of the core of the micelles determined by fluorescence from the I_1/I_3 ratio of the pyrene spectra was similar no matter the nature of the polymeric structure ($I_1/I_3=1.5$) [12]. It was very close to values reported in the literature for other polymeric micellar systems [28,31,32] and corresponds to the value in ethyl acetate [33].

An attempt of representing of the structure of the micelle using this polymer is given in Fig. 6.

4.2. Characteristics of micelles using PEG/MOG/SA (45/5/50) polymers

As the micropolarities and microviscosities obtained with the different probes in presence of the PEG/MOG/SA polymers were very similar, it can be assumed that there are not two well-

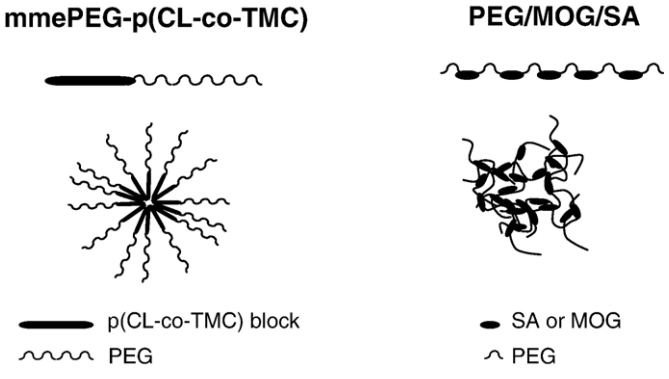

Fig. 6. Schematic representation of the structure of mmePEG750-p(CL-co-TMC) 50/50 micelles and PEG/MOG/SA (45/5/50) aggregates.

defined domains in contrast to what was reported for the mmePEG-p(CL-co-TMC) polymers. The polymers are most probably random copolymers, the succinic anhydride units alternating with either the PEG or the MOG monomers. The PEG units would thus not be localized specifically on one side of the polymeric chain but along the entire chain. The spatial configuration of the polymeric chains would be quite tortuous allowing the exposure of as many PEG units as possible to the aqueous phase. The low microviscosity values are an indication of a loose polymeric chains arrangement corroborating the hypothesis of a tortuous spatial configuration of the chains. The polymer structure had no direct influence on the microviscosity and micropolarity.

The evolution of the micropolarity and microviscosity with temperature is similar for both probes. The micropolarity was not changed by variation in temperature as the hyperfine splitting constant was constant. As expected, the correlation time decreased when the temperature increased.

The interior of the PEG/MOG/SA (45/5/50) aggregates is quite hydrophobic. The a_N values measured by ESR are similar to those of DSA probes in polypropylene oxide [18,24,34]. The I_1/I_3 ratio of pyrene measured by fluorescence was 1.3 and independent of the PEG chain length. This value is very close to the values in polypropylene oxide [32]. It is lower than the values measured for other polymeric aggregates [31,32] and than of the mmePEG-p(CL-co-TMC) micelles. The core of the aggregates is thus more hydrophobic than the conventional polymeric systems. Its solubilization potential for poorly water-soluble drugs has already been demonstrated [14].

An attempt at representing the structure of the micelle based on this polymer is presented in Fig. 6.

5. Conclusion

The combination of EPR and fluorescence analysis confirmed that both polymers studied form micelles by gentle mixing in water. Moreover, this analysis gave information on the microenvironment within these micelles. The micelles with mmePEG-p(CL-co-TMC) polymers are biphasic in nature. The micelles are composed of a more hydrophilic and less viscous corona and a more hydrophobic and more viscous core. The outer shell is likely made up of PEG chains, the hydrophobic part of the chains making the core. The partial hydration of the shell seems to lead to a looser chains network. In micelles with PEG/MOG/SA, there is no clear domain separation. This is consistent with a spatial configuration of random polymeric chains forming a loose network. In these micelles, the microviscosity is low and the hydrophobicity is high.

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References

[1] M.-C. Jones, J.-C. Leroux, Polymeric micelles — a new generation of colloidal drug carriers, Eur. J. Pharm. Biopharm. 48 (1999) 101–111.
[2] V. Torchilin, Structure and design of polymeric surfactant-based drug delivery systems, J. Control. Release 73 (2001) 137–172.
[3] K. Kataoka, A. Harada, Y. Nagasaki, Block copolymer micelles for drug delivery: design, characterization and biological significance, Adv. Drug Deliv. Rev. 47 (2001) 113–131.
[4] M. Adams, A. Lavasanifar, G. Kwon, Minireview Amphiphilic block copolymers for drug delivery, J. Pharm. Sci. 92 (2003) 1343–1355.
[5] C. Allen, D. Maysinger, A. Eisenberg, Nano-engineering block copolymer aggregates for drug delivery, Colloids Surf., B Biointerfaces 16 (1999) 3–27.
[6] A. Lavasanifar, J. Samuel, G. Kwon, Poly(ethylene oxide)-block-poly(L-amino acid) micelles for drug delivery, Adv. Drug Deliv. Rev. 54 (2002) 169–190.
[7] G. Gaucher, M.H. Dufresne, V.P. Sant, N. Kang, D. Maysinger, J.C. Leroux, Block copolymer micelles: preparation, characterization and application in drug delivery, J. Control. Release 109 (2005) 169–188.
[8] W. Charman, Lipids, lipophilic drugs and oral drug delivery — some emerging concepts, J. Pharm. Sci. 89 (2000) 967–978.
[9] C. Pouton, Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and self-microemulsifying drug delivery systems, Eur. J. Pharm. Biopharm. 11 (2000) S93–S98.
[10] A. Lukyanov, V. Torchilin, Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs, Adv. Drug Deliv. Rev. 56 (2004) 1273–1289.
[11] N. Shah, M. Carvajal, C. Patel, M. Infeld, A. Malick, Self-emulsifying drug delivery systems (SEDDS) with polyglycolyzed glycerides for improved in vitro dissolution and oral absorption of lipophilic drugs, Int. J. Pharm. 106 (1994) 15–23.
[12] L. Ould-Ouali, A. Ariën, J. Rosenblatt, A. Nathan, P. Twaddle, T. Matalenas, M. Borgia, S. Arnold, D. Leroy, M. Dinguizli, L. Rouxhet, M. Brewster, V. Préat, Biodegradable self-assembling copolymers a vehicle for poorly water-soluble drugs, Pharm. Res. 21 (2004) 1581–1590.
[13] A. Nathan, Amphiphilic polymers for medical applications, European Patent Application EP1 369 136 A1 (2003).
[14] L. Ould-Ouali, M. Noppe, X. Langlois, B. Willems, P. Te Riele, P. Timmerman, M.E. Brewster, A. Arien, V. Preat, Self-assembling PEG-p (CL-co-TMC) copolymers for oral delivery of poorly soluble drugs: a case study with risperidone, J. Control. Release 102 (2005) 657–668.
[15] F. Mathot, L. van Beijsterveldt, V. Preat, M. Brewster, A. Arien, Intestinal uptake and biodistribution of novel polymeric micelles after oral administration, J. Control. Release 111 (2006) 47–55.
[16] K. Mäder, B. Gallez, Pharmaceutical applications of in vivo EPR, in: L. Berliner (Ed.), In Vivo EPR (ESR), Biological Magnetic Resonance, Kluwer Academic/Plenum Publishers, New York, 2003, pp. 515–545.
[17] K. Persson, B. Bales, EPR study of an associative polymer in solution: determination of aggregation number and interactions with surfactants, J. Chem. Soc., Faraday Trans. 91 (1995) 2863–2870.
[18] A. Caragheorgeopol, J. Pilar, S. Schlick, Hydration and dynamics in reverse micelles of the triblock copolymer EO13PO30EO13 in water/o-xylene mixtures: a spin probe technique, Macromolecules 30 (1997) 2923–2933.
[19] M. Vasilescu, A. Caragheorgeopol, H. Caldararu, Aggregation numbers and microstructure characterization of self-assembled aggregates of poly (ethylene oxide) surfactants and related block-copolymers, studied by spectroscopic methods, Adv. Colloid Interface Sci. 89–90 (2001) 169–194.
[20] R. Bartucci, S. Belsito, L. Sportelli, Spin-label electron spin resonance studies of micellar dispersions of PEGs-PEs polymer-lipids, Chem. Phys. Lipids 124 (2003) 111–122.
[21] H. Caldararu, Structural aspects in self-assembled systems of polyoxyethylene surfactants, as studied by the spin probe technique, Spectrochim. Acta, Part A: Mol. Biomol. Spectrosc. 54A (1998) 2309–2336.
[22] L. Zhou, S. Schlick, Electron spin resonance (ESR) spectra of amphiphilic spin probes in the triblock copolymer EO13PO30EO13 (Pluronic L64): hydration, dynamics and order in the polymer aggregates, Polymer 41 (2000) 4679–4689.
[23] G. Mendz, W. Moore, I. Kaplin, B. Cornell, F. Separovic, D. Miller, L. Brown, Characterization of dodecylphophocholine/myelin basic protein complexes, Biochemistry 27 (1998) 379–386.

[24] A. Caragheorgeopol, H. Caldararu, I. Dragutan, H. Joela, W. Brown, Micellization and micellar structure of a poly(ethylene oxide)/poly (propylene oxide)/poly(ethylene oxide) triblock copolymer in water solution, as studied by the spin probe technique, Langmuir 13 (1997) 6912–6921.
[25] L. Almeida, I. Borisevitch, V. Yushmanov, M. Tabak, Different micellar packing and hydrophobicity of the membrane probes TEMPO and TEMPOL influence their partition between aqueous and micellar phases rather than location in the micellar interior, J. Colloid Interface Sci. 203 (1998) 456–463.
[26] F. Winnik, A. Davidson, G. Hamer, H. Kitano, Amphiphilic poly(N-isopropylacrylamides) prepared by using a lipophilic radical initiator: synthesis and solution properties in water, Macromolecules 25 (2002) 1876–1880.
[27] M. Wilhelm, C. Zhao, Y. Wang, R. Xu, M. Winnik, J.-L. Mura, G. Riess, M. Croucher, Poly(styrene-ethylene oxide) block copolymer micelle formation in water: a fluorescence probe study, Macromolecules 24 (1991) 1033–1040.
[28] E. Szajdzinska-Pietek, M. Wolszczak, A. Plonka, S. Schlick, Fluorescence studies of self-assembly in aqueous solutions of poly(ethylene-co-methacrylic acid) ionomers, J. Am. Chem. Soc. 120 (1998) 4215–4221.
[29] N. Rapoport, K. Caldwell, Structural transactions in micellar solutions of pluronic P 105 and their effect on the conformation of dissolved cytochrome C: an electron paramagnetic resonance investigation, Colloids Surf., B Biointerfaces 3 (1994) 217–228.
[30] R. Zana, M. In, H. Levy, G. Duportail, Alkanediyl-a,w-bis(dimethylalkylammonium bromide). 7. Fluorescence probing studies of micelle micropolarity and microviscosity, Langmuir 13 (2002) 5552–5557.
[31] C. Allen, J. Han, Y. Yu, D. Maysinger, A. Eisenberg, Polycaprolactone-b-poly(ethylene oxide) copolymer micelles as a delivery vehicle for dihydrotestosterone, J. Control. Release 63 (2000) 275–286.
[32] K. Yasugi, Y. Nagasaki, Y. Kato, K. Kataoka, Preparation and characterization of polymer micelles from poly(ethylene glycol)-poly(D,L-lactide) block copolymers as potential drug carrier, J. Control. Release 62 (1999) 89–100.
[33] T. Nivaggioli, P. Alexandridis, T. Hatton, A. Yekta, M. Winnik, Fluorescence probe studies of pluronic copolymer solutions as a function of temperature, Langmuir 11 (2002) 730–737.
[34] C. Teixeira, R. Itri, F. Casallanovo, S. Schreier, Local anesthetic-induced microscopic and mesoscopic effects in micelles. A fluorescence, spin label and SAXS study, Biochim. Biophys. Acta 1510 (2001) 93–105.
[35] C. Zhao, M. Winnik, Fluorescence probe techniques used to study micelle formation in water-soluble block copolymers, Langmuir 6 (1990) 514–516.