Polycaprolactone-*b*-Poly(ethylene oxide) Biocompatible Micelles as Drug Delivery Nanocarriers: Dynamic Light Scattering and Fluorescence Experiments

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Summary: Dynamic light scattering (DLS) and fluorescence experiments were carried out to study PCL_{44}-*b*-PEO_{114} biocompatible micelles used as nanocarriers in drug delivery. Micelles prepared by a simple procedure (THF removal under nitrogen flow) exhibited a narrow size distribution with an average diameter of 100 nm. For micelles containing a hydrophobic model compound (pyrene) within the PCL core, a smaller average micellar size of 80 nm was observed, with a simultaneous broadening in the size distribution profile. In parallel to DLS results, fluorescence experiments showed evidence of pyrene encapsulation, and that the onset of the micellization process occurs at approximately 10/90 (v/v) THF/water mixtures in the case of PCL_{44}-*b*-PEO_{114} polymer.

Keywords: drug delivery systems; dynamic light scattering (DLS); fluorescence; micelles; PCL-*b*-PEO

Introduction

An amphiphilic AB type block copolymer is made of two segments of different chemical structures with hydrophilic and hydrophobic components. One of the most interesting and fascinating solution properties of these AB block copolymers is their ability to self-assemble into micelles (and other ordered structures such as lamellar aggregates, vesicles, etc.) when dissolved in a selective solvent, i.e., a solvent thermodynamically good for one block and poor for the other.[1–8] Micellar aggregates are characterized by their unique core-shell architecture, where in an aqueous environment the hydrophobic blocks of the copolymer are segregated from the aqueous exterior to form the inner core, and the hydrophilic blocks form the corona or the outer shell.[4]

Recently, micelles made from copolymer chains have been increasingly used as drug delivery vehicles due to the fact that their hydrophobic core serves as a microreservoir for the incorporation of small molecules.[1] The properties that make micellar systems advantageous for drug delivery applications include the facts that they (i) can be made of biocompatible and/or biodegradable block copolymers, (ii) have a small size (10–100 nm) and narrow distribution allowing intravenous injection, (iii) are able to incorporate and release poorly water soluble, hydrophobic, and/or highly toxic compounds, (iv) minimize drug degradation and loss and (v) increase bioavailability.[1,3,4,9,10] From this standpoint, there has been a great interest in biocompatible block copolymer micelles whose core-forming block is polycaprolactone (PCL), and the corona-forming block is poly(ethylene oxide) (PEO). PCL-*b*-PEO micellar nano-containers were

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shown, notably by Eisenberg's pioneer group,[1,9,11,12] to be suitable carries for lipophilic steroids,[11] neurotrophic (FK506 and l-685,818),[12] anti-inflammatory,[13,14] anticancer,[15] and hypertension agents.[16] Besides, these micelles have been found to distribute in defined cytoplasmic organelles of PC12 cells.[17]

Despite the increasing number of studies reported on PCL-*b*-PEO and other biocompatible block copolymer micelles, the methodology of micelles preparation that is the key parameter controlling the mechanism (kinetics and thermodynamics) of micelle formation is neither fully described in the literature nor unique. Such methods have direct consequences on drug loading as well as on the structure and dynamics of noncovalently drug-loaded and unloaded micellar aggregates. Basically, two principal methods have been employed: direct dissolution or dialysis. The choice of which method to use depends mostly on the copolymer solubility in water. In the case of PCL-*b*-PEO, the dialysis method has applied due to the copolymer insolubility in water.[1–4,9,11,12,17] It consists in dissolving the PCL-*b*-PEO copolymer in a common organic solvent (i.e., good for both blocks such as THF, DMF, acetone) that is miscible with water. Subsequently, the copolymer/organic solvent/water mixture is dialyzed against water to remove the organic solvent, so that during this process the micellization is induced owing to amphiphilic nature of the polymeric chain. Although this procedure is satisfactory and gives excellent results, it shows some handling difficulties especially when working with small quantities.[2] In an different approach, Yoo et al.[16] prepared PCL-*b*-PEO micelles using acetone as organic solvent, which was distilled off from the solution under reduced pressure.

The structure and dynamics of biocompatible block copolymer micelles when used as drug delivery systems is also of great interest not only before but also after drug loading. The incorporation of hydrophobic molecules implies in remarkable changes in micellar properties such as size distribution and diffusion coefficient. These characteristics have implications on primary aspects in drug delivery such as drug loading efficiency, partition coefficient, release kinetics and micelle stability.[3]

We report here on dynamic light scattering (DLS) and fluorescence experiments carried out on PCL_{44}-*b*-PEO_{114} (the subscripts refers to the number of repeat monomeric units) biocompatible micelles obtained by a simplified method of preparation and drug loading by taking advantage of the high volatility of the organic solvent. Pyrene (Py) was used as a model hydrophobic drug due to its well-known photo-physical properties.

Experimental Part

Chemicals

The block copolymer used was polycaprolactone-*b*-poly(ethylene oxide) (PCL_{44}-*b*-PEO_{114}) containing 44 caprolactone units and 114 ethylene oxide repeat units, $M_w/M_n = 1.06$ (Polymer Source Inc.). The volume fraction of PCL (ϕ_{PCL}) in this copolymer is 0.503, calculated as $\phi_{PCL} = (m_{CL} \cdot N_{PCL}/d_{PCL})/[(m_{CL} \cdot N_{PCL}/d_{PCL}) + (m_{EO} \cdot N_{PEO}/d_{PEO})]$, where m is the molar weight of the monomer, N is the degree of polymerization, and d is the density of the polymer. Pyrene (Sigma-Aldrich) was recrystallized from ethanol. Distilled and deionized water was employed for all solution preparations. Tetrahydrofuran (THF) (Merck) was distilled under reduced pressure prior to use.

Sample Preparation

As previously outlined, the preparation method of block copolymer micelles depends on the copolymer solubility in water. Direct dissolution was initially tested for the PCL_{44}-*b*-PEO_{114} polymer, which has a large degree of polymerization N as compared to other systems (40–50 repeat units) studied elsewhere.[9,11,12] A polymer solution was prepared by direct addition of PCL_{44}-*b*-PEO_{114} to water and overnight stirring at room temperature. Such a naive

method, however, did not show a narrow size distribution profile characteristic of micelles (see discussion below).

As an alternative approach to obtain PCL-*b*-PEO micelles in water, the copolymer was first dissolved in common organic good solvents (THF or acetone) and left to stir overnight at room temperature in a closed vial. Micellization was achieved by slow addition of water (selective solvent for PEO) at a rate of approximately 0.05 $g \cdot s^{-1}$. Subsequently, the organic solvent (THF or acetone) was removed from the aqueous/organic mixture by stirring under a gentle nitrogen flow, which was maintained until the total mass of the copolymer solution was approximately the sum of added water and polymer masses. The copolymer concentrations in the resulting aqueous micellar solutions were 0.01, 0.05 and 0.10 $mg \cdot mL^{-1}$. Py-loaded micelles (1.0×10^{-7} $mol \cdot L^{-1}$ Py) were prepared by adding an appropriated amount of Py to the copolymer organic solution prior to induce micellization to insure the Py encapsulation.

Dynamic Light Scattering (DLS) Experiments

Dynamic scattering measurements were performed using an ALV laser goniometer, which consists of a 22 mW HeNe linear polarized laser with 632.8 nm wavelength and an ALV-5000/EPP multiple τ digital correlator with 125 ns initial sampling time. The samples were kept at an exact and constant temperature of 25.0 °C during all the experiments. The accessible scattering angle range is from 15° up to 150°. However, all the scattering measurements were done at 40°, 60°, 90° and 120°. The solutions were put in 10 mm diameter glass cells. The minimum sample volume required for the experiment was 1 mL. The data acquisition was done with the ALV Correlator Control software, and the counting time varied for each sample from 300 up to 600 s.

For diffusive scattering particles, the relaxation frequency (Γ) is q^2-dependent ($\Gamma = Dq^2$, where D is the diffusion coefficient).[18] Such a behavior was observed in the PCL-*b*-PEO micellar system, and is illustrated in Figure 1 for a 0.10 $mg \cdot mL^{-1}$ solution. Accordingly, some micellar solutions were studied at a fixed scattering angle (90°) using a Malvern Zetasizer light scattering apparatus, and the micellar size was obtained from the Stokes-Einstein relation [$R_H = k_B T/(6\pi\eta D)$, where R_H is the hydrodynamic radius, k_B is Boltzmann constant, T is the temperature of the sample, and η is the viscosity of the medium].[18]

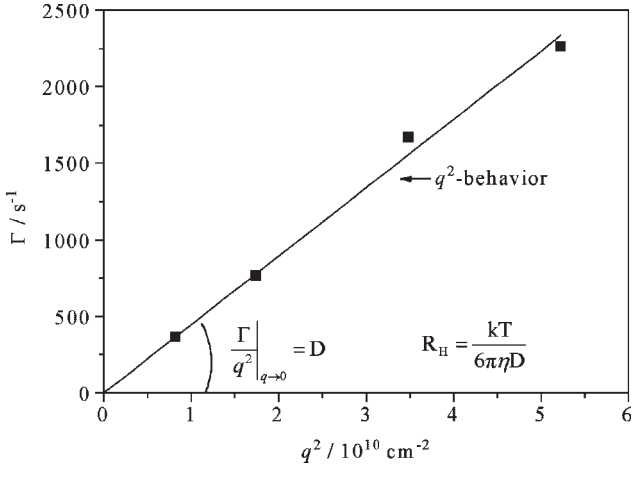

Figure 1.
Variation of the relaxation frequency (Γ) as a function of q^2 for a 0.10 $mg \cdot mL^{-1}$ PCL-*b*-PEO aqueous micellar solution.

Fluorescence Experiments

Steady-state fluorescent spectra were measured using a Hitachi F-4500 spectrometer in the right-angle geometry (90° collecting optics). For the fluorescence measurements, 2 mL of solution was placed in a 10-mm square quartz cell. All spectra were run on air-equilibrated solutions. For fluorescence emission spectra, λ_{ex} was 339 nm. Spectra were accumulated with an integration time of 1 s per 0.5 nm.

Results and Discussion

Method of Preparation

DLS experiments were carried out to measure the effective diameter of the micelles and CONTIN analysis[19] was employed to determine their distribution in terms of their size. Figure 2 shows the size distribution of 0.10 mg · mL^{-1} PCL-*b*-PEO solutions prepared by different methods: direct copolymer dissolution in water (A), and dissolution in acetone (B) and THF (C) followed by water addition and evaporation of the organic solvent. Copolymer solutions prepared by direct dissolution in water [Figure 2(A)] revealed the presence of two relaxation modes corresponding to effective diameters of about 150 and 1 500 nm. The overall size distribution profile is clearly not characteristic of PCL-*b*-PEO micelles, exhibiting large and broadly dispersed macromolecular aggregates. This is indeed due to the low solubility of the PCL block in water.

In order to obtain PCL-*b*-PEO micelles in water without using the dialysis method, highly volatile organic solvents as compared to water were chosen. Figure 2(B) and 2(C) show the effective diameter of micelles in water prepared by dissolution first in acetone and THF, respectively. In Figure 2(B), two broad size distributions were evidenced at ca. 50 and 150 nm, and successive measurements performed on these solutions gave irreproducible results as illustrated in the inset of Figure 2(B). On the contrary, narrow distribution in size (average diameter = 102 nm) and excellent reproducibility were observed for micelles prepared by dissolution first in THF [Figure 2(C)]. Therefore, aqueous micellar solutions were thereafter prepared by copolymer dissolution in THF prior to induce micellization with a selective solvent (water) and THF evaporation under nitrogen flow to speed up the process.

Unloaded and Py-loaded PCL_{44}-b-PEO_{114} Micelles: DLS Experiments

The size of colloidal nanoparticles used as drug delivery vehicles is one of the properties that largely influence, for instance, the circulation time, organ distribution, loading efficiency and release kinetics. Ideally, drug carries whose size are less than 200 nm in diameter are less susceptible to clearance by the reticuloendothelial system, and those less than 5 μm have access to small capillaries.[20] Likewise, the size of the carrier may influence its mechanism of entry into cells, and may, in turn, affect the kinetics and extent of cell uptake.

DLS experiments were performed on PCL-*b*-PEO micelles with the objective of studying the dependence of the micellar size and size distribution on the copolymer concentration and Py-loading. Figure 3 shows average diameters and diffusion coefficients of unloaded and Py-loaded micelles as a function of the copolymer concentration. It has been observed that unloaded micelles following the method of preparation described in this work present nearly the same size with an average diameter of ca. 100 nm regardless of the polymer content in solution. For micelles containing pyrene molecules within the hydrophobic PCL core, one observes a smaller micellar size down to 80 nm. In this case, however, the micellar diameter slightly decreases as the copolymer concentration is increased. The extrapolation at infinite dilution ($c \rightarrow 0$) allows us to determine the single micelle particle size.[18] Therefore, in absence of Py, the single particle size of a PCL_{44}-*b*-PEO_{114} micelle is 101 nm, whereas in presence of the hydrophobic probe is 87 nm. The reciprocal behavior is evidenced in terms

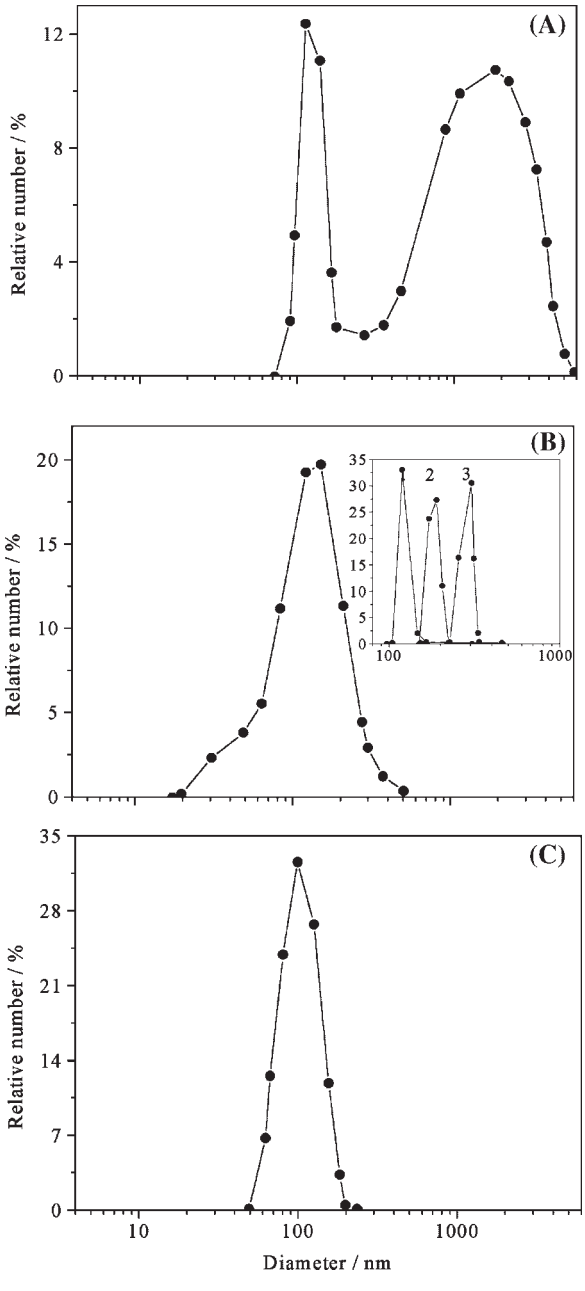

Figure 2.
Size distribution of 0.10 mg · mL^{-1} PCL-*b*-PEO aqueous solutions prepared by different methods: direct copolymer dissolution in water (A), and dissolution in acetone (B) and THF (C) followed by water addition and solvent evaporation.

of the diffusion coefficients and it is also illustrated in Figure 3 (smaller scattering particles diffuse faster as defined by the Stokes-Einstein relationship).

Figure 4 shows typical autocorrelation functions $C(q,t)$ measured at different scattering angles and distributions of the relaxation times $A(t)$ at 90° as revealed by

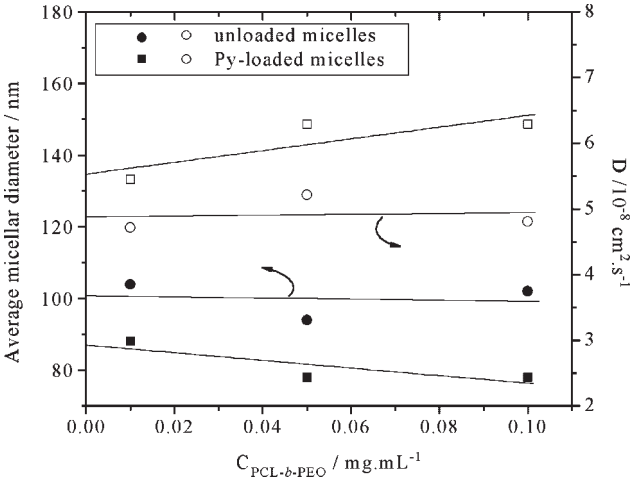

Figure 3.
Average diameter and diffusion coefficient of unloaded and Py-loaded PCL-*b*-PEO micelles in water prepared using THF as organic solvent as a function of the copolymer concentration.

CONTIN analysis at the concentrations 0.05 mg · mL^{-1} (A,C) and 0.10 mg · mL^{-1} (B,D) PCL-*b*-PEO in absence (A,B) and in presence (C,D) of Py within the micelle core. In general, the distributions of relaxation times exhibit a dominant mode, which

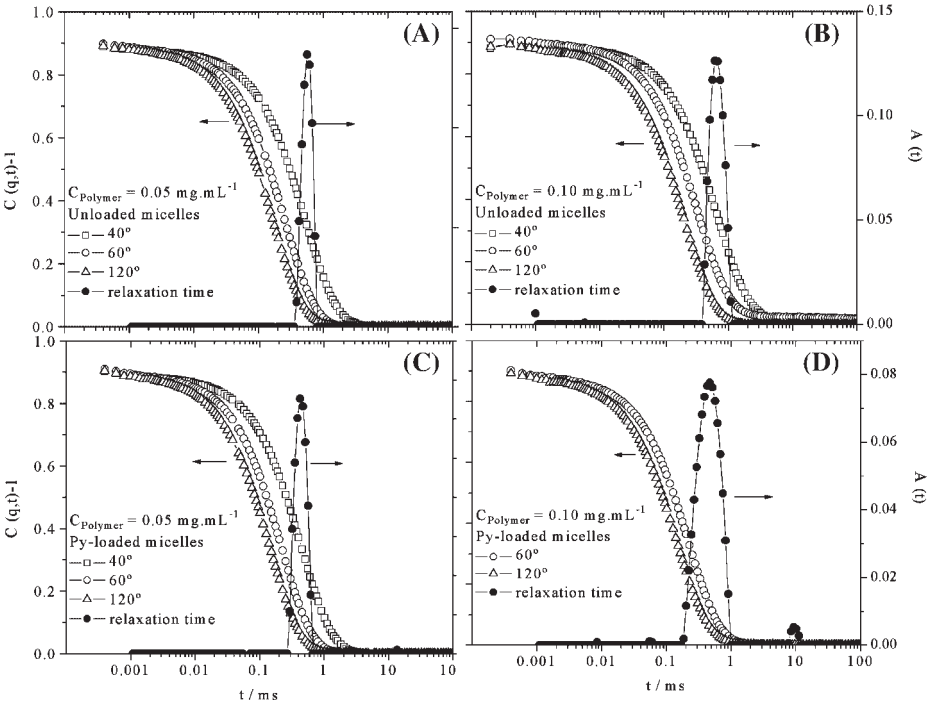

Figure 4.
Autocorrelation functions $C(q,t)$ measured at different shown scattering angles (θ) and distributions of the relaxation times $A(t)$ as revealed by CONTIN analysis for different PCL-*b*-PEO concentrations in absence and in presence of Py within the micelle core, as indicated.

is related to the diffusive motion of the micelles. The relaxation frequency associated with these processes increases (relaxation time decreases) as Py molecules are incorporated into the hydrophobic PCL core, evidencing a change in the average micellar size.

The hydrophobic compound, as illustrated in Figure 4(D), also induces a broadening in the distribution of relaxation times as compared to the same solution in absence of pyrene [Figure 4(B)]. Furthermore, the autocorrelation functions measured at low scattering angles (such as 40°) for this particular system revealed the presence of a slower relaxation mode, as shown in Figure 5. This is due to the formation large dynamical macromolecular aggregates (500–700 nm), likely of small individual micelles as earlier proposed by Allen et al.[12] for PCL_{20}-*b*-PEO_{44} micelles. It is worth noting that this slow mode is even observed (very small amplitude) even at 90° scattering angle [see Figure 4(D)].

The micellar size is controlled by several parameters, among which are the lengths forming the core and the corona.[3] Allen et al.[12] prepared PCL_{20}-*b*-PEO_{44} and PCL_{14}-*b*-PEO_{44} micelles by dialyzing a polymer/DMF/water mixture against pure water and found a narrow size distribution with an effective diameter of 62 and 55 nm, respectively. In our approach, larger PCL_{44}-*b*-PEO_{114} micelles (diameter $\sim$ 100 nm) were formed as compared to earlier results,[9,12,16] primarily because of the block lengths. In contrast, Yoo et al.[16] prepared the same PCL_{44}-*b*-PEO_{114} micelles system using acetone as common organic good solvent, which was distilled off from the polymer/acetone/water mixture under reduced pressure. These authors observed micellar aggregates smaller (diameter = 42 nm) than those obtained by Allen et al.[12] (55–62 nm diameter) and different from the ones presented in this work (diameter $\sim$ 100 nm). Accordingly, the 42 nm in diameter is incoherent with Allen et al.[12] 's results and different from ours. This is certainly due to the use of acetone as co-solvent leading to irreproducible results as we have stated above and illustrated in Figure 2(B).

The decrease in the micelle diameter from ca. 100 nm to ca. 80 nm upon incorporation of a hydrophobic molecule is likely related to changes in the core assembly structure. If the number of chains within a micelle is constant, the hydrophobic nature of both PCL block and Py enhances intermolecular interactions within the core, which may cause shrinking

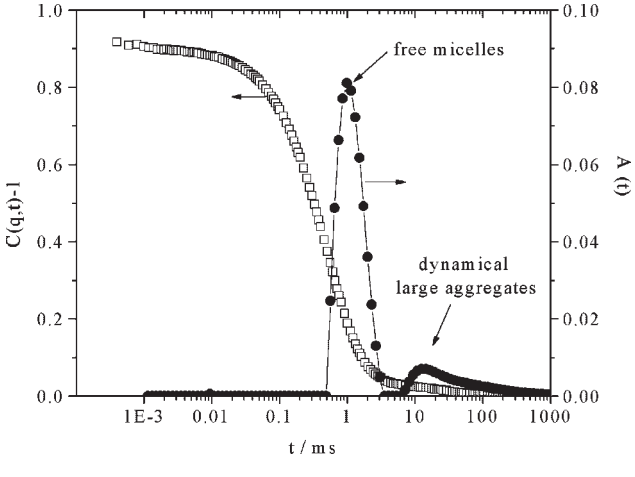

Figure 5.
Autocorrelation function $C(q,t)$ measured at $\theta = 40°$ and distribution of relaxation times $A(t)$ as revealed by CONTIN analysis at concentration of 0.10 mg · mL^{-1} PCL-*b*-PEO in presence of Py.

of PCL chains, in agreement with the findings observed by Shuai et al.[15], who concluded from NMR experiments that the core of doxorubicin-loaded PCL-*b*-PEG micelles exhibited lower chain motion. On the other hand, the presence of a highly hydrophobic probe in solution might also induce a decrease in the micellar aggregation number and critical micellar concentration (CMC) leading to a decrease in the effective diameter.

From a drug delivery point of view, these findings are very important to achieve a better understanding of the diffusive behavior of the drug-carrier (intrinsically related to its size) within the body, and accordingly of its circulation time, bio-distribution, bioavailability and loading efficiency. To date, however, a limited extent of data is available on this issue, namely after drug incorporation.

Pyrene-loaded PCL_{44}-b-PEO_{114} Micelles: Fluorescence Experiments

Pyrene has commonly been used as a hydrophobic fluorescence probe, to evaluate the polarity of various environments. The fluorescence emission spectrum of Py shows vibrational bands, which are affected by the polarity of the surrounding environment of the probe molecules. Specifically, changes in the relative intensity of the first (I_1 at 372 nm) and the third (I_3 at 383 nm) vibrational bands in the Py emission spectrum have proven to be reliable tools in examining the polarity of a microenvironment.[21] In this way, it is possible to follow the transfer of Py molecules from the solvent (polar) to inside micellar core (apolar). Incorporated pyrene molecules will be, therefore, probing the core polarity. Besides, for a given solvent mixture such as THF/water, the hydrophobic probe is distributed between the solvent and the micellar core, so that incorporated molecules are protected from the external environment. Consequently, one can have access to the incorporated Py concentration by adding small aliquots of a hydrophilic fluorescence quencher.[22]

Figure 6 shows the variation of the I_3/I_1 ratio as a function of the solvent composition in absence and in presence of 0.10 mg · mL^{-1} PCL-*b*-PEO. In absence of the block copolymer the I_3/I_1 ratio gradually decreased from ca. 0.83 (pure THF) to ca. 0.55 (pure water). The values observed for pure solvents are in very good

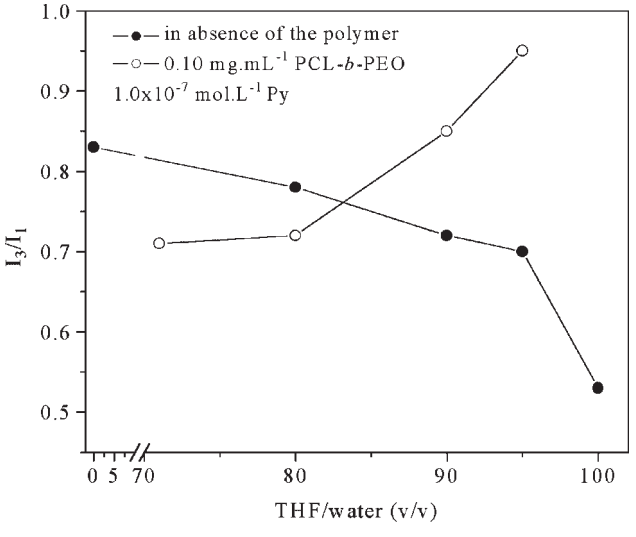

Figure 6.
Variation of the I_3/I_1 ratio as a function of the solvent composition in absence and in presence of 0.10 mg · mL^{-1} PCL-*b*-PEO.

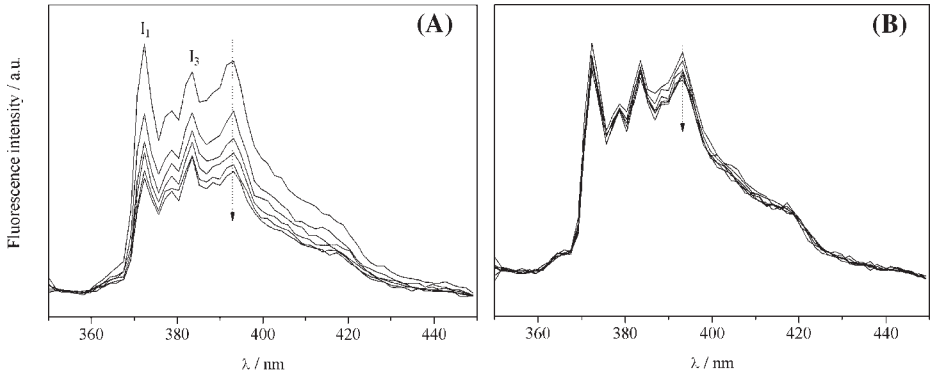

Figure 7.
Pyrene fluorescence quenching by N-ethylpyridinium bromide (NEPB) in absence (A) and in presence (B) of PCL-*b*-PEO micelles [solvent composition: 05/95 (v/v) THF/water]. The arrows indicate increasing NEPB concentration.

agreement with those obtained by Kalyanasundaran and Thomas.[21] When the PCL-*b*-PEO copolymer was added to the solution, however, a clear increase in the I_3/I_1 ratio was noticed for water contents higher than 90%, reflecting the incorporation of Py within the core of the micelle. Py-loading into PCL-*b*-PEO micelles was also confirmed by fluorescence quenching experiments as illustrated in Figure 7. The addition of small aliquots of *N*-ethylpyridinium bromide (NEPB) (0.0–6.0 m mol $\cdot$ L^{-1}) notably quenched the Py fluorescence [Figure 7(A)], whereas in presence of the block copolymer the spectra remained virtually unchanged [Figure 7(B)] because the probe in the core is not susceptible to quenching.

In order to gain insight into the loading process during the micellization of PCL-*b*-PEO as a function of both water content in the solvent mixture and copolymer concentration, fluorescence quenching results were analyzed as described by Auger et al.[22] Figure 8 exhibits the variation of the I/I_0 ratio (where I and I_0 are the fluorescence intensities taken at 395 nm in presence of different quencher concentrations and prior its addition, respectively) as a function of $1/C_{NEPB}$, where C_{NEPB} is the concentration of the quencher (NEPB). The intercept of a linear fit of these data is proportional to the amount of Py in the micellar core.

It is verified that for 10/90 (v/v) THF/water solutions [Figure 8(B)] the probe accessibility for quenching is drastically decreased as compared to 20/80 (v/v) THF/water solutions [Figure 8(A)] and evidenced by the increasing in the I/I_0 ratio at high quencher concentrations. This result suggests that the onset of the micellization processes occurs at ca. 10/90 (v/v) THF/water in the case of PCL_{44}-*b*-PEO_{114}, in excellent agreement with data presented in Figure 6. Furthermore, the amount of pyrene loaded into the micelles increases with the block copolymer concentration as shown in the variation of I/I_0.

Conclusion

In this work, we have studied PCL_{44}-*b*-PEO_{114} biocompatible micelles prepared by a simple method, which comprised polymer dissolution in THF (good solvent for both blocks) followed by water addition (selective solvent for the PEO block) and THF evaporation under nitrogen flow. It was found that unloaded PCL_{44}-*b*-PEO_{114} micelles present nearly the same size (average diameter $\sim$ 100 nm) and a relatively narrow size distribution in the range of studied polymer concentrations (0.01–0.10 mg $\cdot$ mL^{-1}). However, upon loading pyrene molecules (a hydrophobic model compound), a decrease in the average

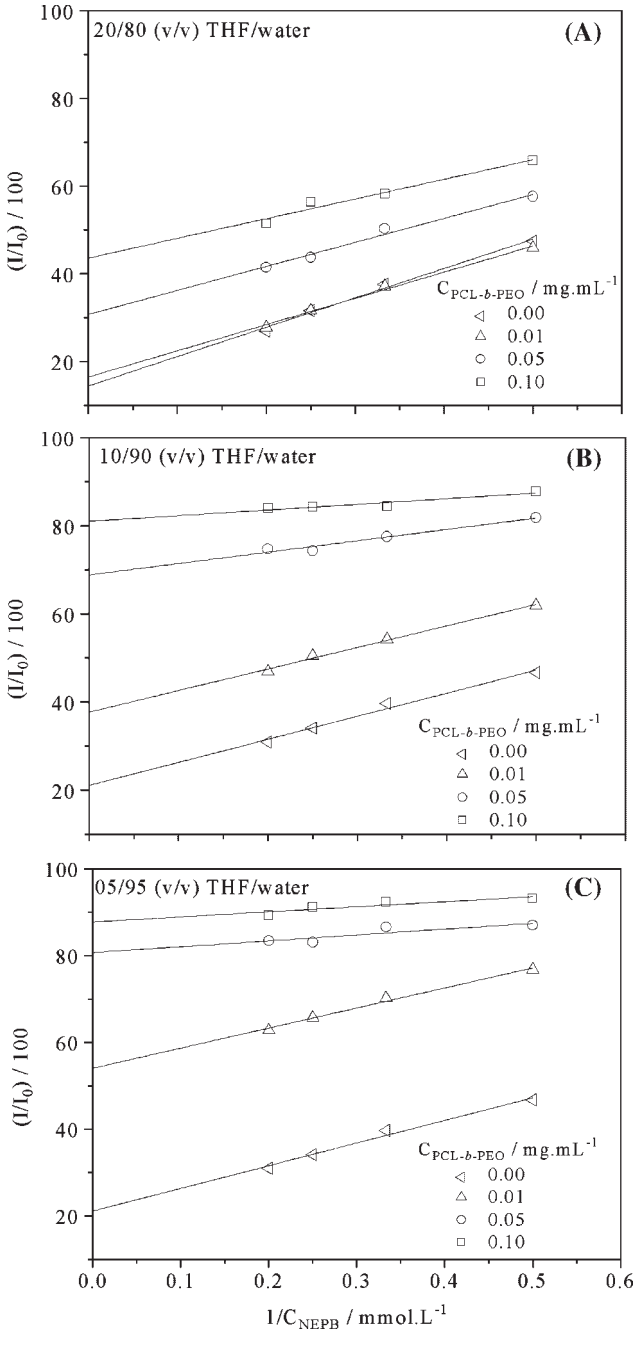

Figure 8.
Pyrene fluorescence quenching by *N*-ethylpyridinium bromide (NEPB) in different solvent mixtures: (A) 20/80, (B) 10/90 and (C) 05/95 (v/v) THF/water.

micelle size (diameter ~ 80 nm) was systematically observed, with a simultaneous broadening in the size distribution profile. This decrease may be due to a change in the aggregation number or to a shrink of the core caused by the interaction

of Py and PCL. In parallel to DLS results, fluorescence experiments showed evidence of Py encapsulation within the micelle (core), and that the onset of the micellization processes occurs at about 10/90 (v/v) THF/water solvent composition.

Acknowledgements: R. B. acknowledges financial support from the *CNRS*, *Région Aquitaine* and *FEDER*. C. G. acknowledges *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil)* for the PhD fellowship. The authors are grateful to Dr. V. Soldi and Dr. D. Zanette (Universidade Federal de Santa Catarina, Brazil) for helpful discussions and to J. Buysse for her participation in preliminary experiments in this work.

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