Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutics and Biopharmaceutics 56 (2003) 337–346
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Solubilization of cyclosporin A in dextran-*g*-polyethyleneglycolalkyl ether polymeric micelles

Mira F. Francis[a], Luc Lavoie[b], Françoise M. Winnik[a,b], Jean-Christophe Leroux[a,c,*]

[a]*Faculty of Pharmacy, University of Montreal, Montreal, QC, Canada*
[b]*Department of Chemistry, University of Montreal, Montreal, QC, Canada*
[c]*Canada Research Chair in Drug Delivery, University of Montreal, Montreal, QC, Canada*

Received 5 February 2003; accepted in revised form 19 May 2003

Abstract

Solubilization of the poorly water-soluble drug, Cyclosporin A (CsA), in aqueous dispersions of dextran-grafted-polyethyleneglycolalkyl ether (DEX-*g*-PEG-C*n*) polymeric micelles was examined as a function of copolymer structure. In aqueous solution, DEX-*g*-PEG-C*n* form polymeric micelles of low critical association concentrations (CAC) and small micelle sizes as determined by fluorescence spectroscopy and dynamic light scattering (DLS). Copolymers with longer polysaccharide chain showed larger CAC and mean diameter. The percentage of CsA loading into micelles was determined by high performance liquid chromatography. It was significantly larger in polymeric micelles compared to unmodified dextrans. It increased with increasing number of PEG-C*n* units grafted per dextran chain and decreasing dextran molecular weight. The cytotoxicity of DEX-*g*-PEG-C_{16} polymeric micelles towards Caco-2 cells, tested by MTT cytotoxicity assay, was significantly lower than that of free PEG-C_{16} molecules. It can be concluded that the length of the hydrophilic part as well as the content and chemical nature of the hydrophobic substituents have an important effect on the ability of polymeric micelles to solubilize poorly-water soluble drugs.

Keywords: Polymeric micelle; Poorly water soluble drug; Oral delivery; Cyclosporin A; Dextran; Polyethyleneglycolalkyl ether; Solubilization; Cytotoxicity

1. Introduction

Peroral drug administration represents by far the easiest, most common and most convenient route of drug delivery, especially when repeated or routine administration is necessary [1]. For effective delivery *via* the oral route, a therapeutic agent must first dissolve in the gastrointestinal lumen [2]. This can pose major challenges in the case of poorly-water soluble drugs [3]. A drug may be defined as 'poorly soluble' if its dissolution rate is slower than the transit time to its absorptive sites [4]. The dissolution of a poorly water-soluble drug in the gastrointestinal contents is quite often the rate-limiting step that, ultimately, controls the bioavailability of the drug at its site of action [5].

One approach to enhance the solubility and bioavailability of a highly lipophilic drug is to dissolve it on the molecular level in the hydrophobic core of a delivery system, itself soluble or dispersible in the aqueous environment. In the late 1960s, surfactant micelles drew much attention as drug delivery carriers, due to their good pharmacological characteristics [6]. They are widely used as adjuvants and drug carrier systems in many areas of pharmaceutical technology and controlled drug delivery [7]. Surfactant micelles form only above a critical concentration, the critical micelle concentration (CMC) and rapidly break apart upon dilution which could result in a premature leakage of the drug and its precipitation in situ.

These limitations of surfactant micelles as drug delivery carriers triggered the search for micelles of significantly enhanced stability and solubilizing power. Like their low molecular weight counterpart, amphiphilic polymers associate in water to form 'polymeric micelles' [8] consisting of a hydrophobic core stabilized by a corona of hydrophilic polymeric chains exposed to the aqueous environment [9]. The size of polymeric micelles ranges from ~10 nm to ~100 nm and usually the size distribution is narrow [9].

* Corresponding author. Faculty of Pharmacy, University of Montreal, C.P. 6128 Succ. Centre-ville, Montreal, QC, Canada H3C 3J7. Tel.: +1-514-343-6455; fax: +1-514-343-7738.
E-mail address: jean-christophe.leroux@umontreal.ca (J.-C. Leroux).

0939-6411/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0939-6411(03)00111-5

This topology is similar to that of surfactant micelles, hence polymeric micelles can be expected to solubilize hydrophobic drugs within their core. However, there are significant differences between the two types of assemblies from the physico chemical viewpoint. The polymer concentration at which the association first takes place, sometimes known as the critical association concentration (CAC), is lower by several orders of magnitudes than typical surfactant CMC values. Thus, polymeric micelles are more stable towards dilution. From the pharmaceutical point of view, these amphiphilic carriers can solubilize more poorly-water soluble drugs within their hydrophobic core than most surfactant micelles. They can increase drug bioavailability and retention, since the drug is well protected from possible inactivation under the effect of their biological surroundings [8].

Cyclosporin A (CsA) is a highly effective immunosuppressive agent that is used for prevention of graft rejection following organ transplantation [10]. CsA is a neutral cyclic undecapeptide with a molecular weight of 1202 g/mol [11], consisting of hydrophobic amino acids linked via 11 amide bonds, seven of which are *N*-methylated. Four intramolecular hydrogen bonds [12] (Fig. 1) contribute to the rigidity of its skeleton. This chemical composition is responsible for the extremely low solubility of CsA in water (23 μg/ml at 20 °C). The oral administration of CsA is complicated by the presence of several metabolizing enzymes: cytochrome P-450 3A4 (CYP3A4), the multi-drug transporter P-glycoprotein (PGP) in the small intestine, and hepatic CYP3A4 [13]. These factors, together with the poor solubility of CsA in the aqueous fluids of the gastrointestinal tract, severely limit the absorption of CsA through the gastrointestinal mucosa. They account for the low bioavailability of the drug and significantly increase the risks of both acute and chronic rejection [14]. In view of the clinical importance of CsA, much effort has been directed towards designing oral formulations leading to acceptable bioavailability. A number of innovative drug delivery approaches including mixed micelles [15], charged nanoparticles [16], liposomes [17], lipids [18], surfactants [19], microspheres [20], and microemulsions [21] have been investigated in order to improve the unfavorable absorption characteristics of CsA.

We set out to investigate the suitability of polymeric micelles as carriers in the oral delivery of CsA. This drug was selected, not only because of its clinical importance, but also since it is representative of a number of new, highly water-insoluble drugs in current development. The chemical composition and architecture of the amphiphilic polymers forming the polymeric micelle drug carriers was selected with care, taking into account the following requirements. The hydrophilic sections of the polymer should have high water solubility and be non-toxic, and ideally should enhance the bioavailability of the drug transported within the micelle. We selected dextrans, which are branched polysaccharides introduced in medicine in the early 1950s as non-toxic plasma substitutes [22]. They are readily available in a range of molecular weights [23] and their chemical modification has been studied extensively [24–26]. In order to impart dextran with an amphiphilic character, we linked to its backbone a small number of polyethylene glycol *n*-alkyl ether (PEG-C*n*; C*n*EO$_{10}$) chains (Fig. 2). Nonionic surfactants such as polyethyleneglycol alkyl ethers are known to enhance drug absorption. In fact, the effect of PEG-C*n* has been reported in several studies to be superior to those of fatty acid esters and sorbitan derivatives [27,28]. However, several PEG-C*n*

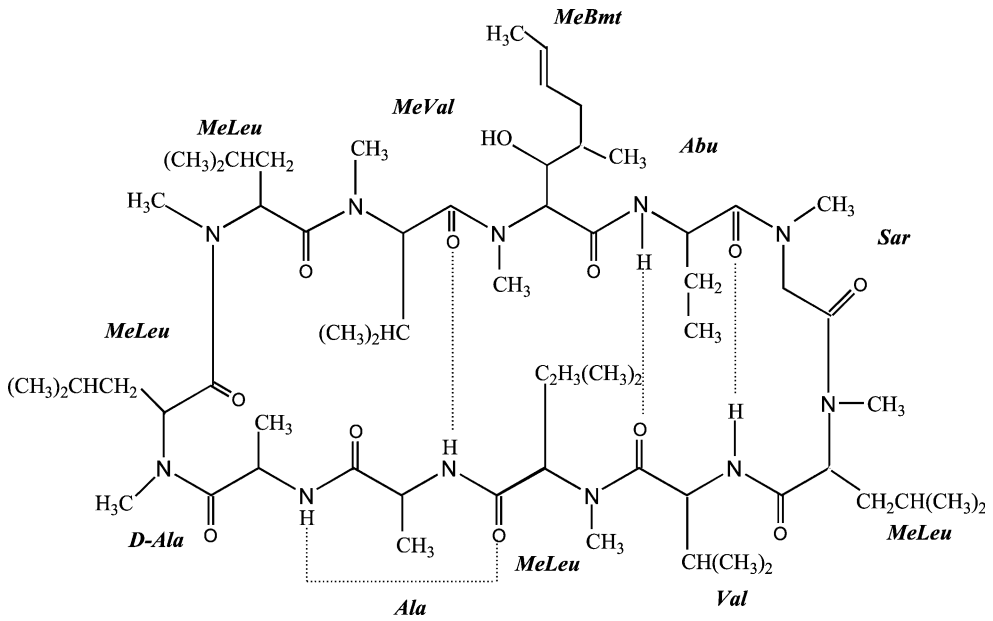

Fig. 1. Chemical structure of cyclosporin A showing 11 amino acids and four hydrogen bonds.

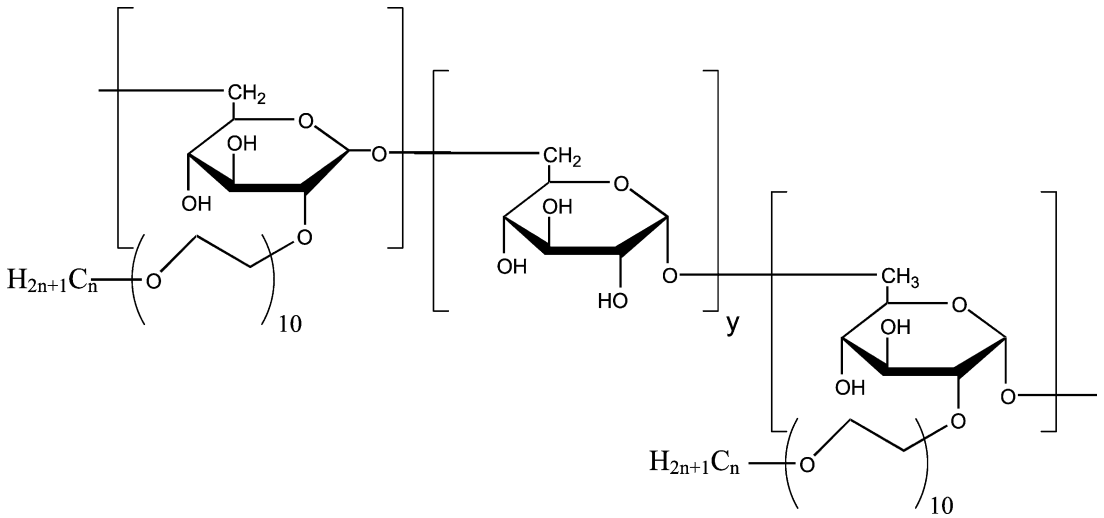

Fig. 2. Chemical structure of DEX-*g*-PEG-C*n* copolymer where $n = 16$ and $n = 18$ for DEX-*g*-PEG-C_{16} and DEX-*g*-PEG-C_{18}, respectively.

surfactants suffer from adverse effects on mucosal integrity [29].

The objectives of the work presented here were (1) to determine the physicochemical properties of the polymeric micelles formed in aqueous solutions of hydrophobically-modified dextrans (DEX-*g*-PEG-C*n*); (2) to assess the solubility of CsA in DEX-*g*-PEG-C*n* micelles; and (3) to evaluate the cytotoxicity of the polymeric micelles. The latter study was of particular importance in view of the known toxicity of the PEG-C*n* surfactants when they are not linked to polymers. The effects of the polymer molecular weight, the level of hydrophobic substitution, and the chemical nature of the hydrophobic substituents on the properties of the polymeric micelles were examined using fluorescence spectroscopy, dynamic light scattering, CsA assay by high-performance liquid chromatography (HPLC) and MTT cytotoxicity assay.

2. Materials and methods

2.1. Materials

Cyclosporin A (CsA), polyethyleneglycolcetyl ether (PEG-C_{16}; $C_{16}EO_{10}$; Brij 56), polyethyleneglycolstearyl ether (PEG-C_{18}; $C_{18}EO_{10}$; Brij 76), sodium chloride (NaCl), monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), sodium dodecyl sulfate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Optical grade pyrene (99%), dichloromethane (DCM) and deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dextran T10 (DEX10; M_w 10 000 Da) and dextran T40 (DEX40; M_w 40 000 Da) were supplied by Pharmacia Fine Chemicals (Uppsala, Sweden). HPLC-grade acetonitrile (ACN) and water were obtained from Anachemia Science (Montreal, PQ, Canada). Ethanol (95%) was obtained from Commercial Alcohols Inc. (Brampton, ON, Canada). The Caco-2 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD) at passage 18. Dulbecco's modified Eagle medium (DMEM), penicillin–streptomycin (10 000 U/ml penicillin G and 10 000 μg/ml streptomycin), fetal bovine serum (FBS), 0.25% (w/v) trypsin–1 mM EDTA·4Na (1 ×) and non-essential amino acids (NEAA) were supplied by Invitrogen Inc. (Burlington, ON, Canada).

2.2. Synthesis of DEX-g-PEG-C_n copolymers

Hydrophobically-modified DEX-*g*-PEG-C*n* graft copolymers were synthesized following a procedure reported by our group [30]. Briefly, the terminal hydroxyl group of PEG-C*n* was tosylated in good yield using the joint action of the amines Et_3N and Me_3N HCl. Subsequently, the tosylated PEG-C*n* was coupled to dextran of various molecular weights (10 000 and 40 000 Da) by a Williamson ether synthesis. The resulting DEX-*g*-PEG-C*n* copolymers were purified by a dichloromethane soxhlet extraction to remove all free PEG-C*n*. They were characterized by ^{1}H NMR spectroscopy of their solution in DMSO-d_6 using a Bruker ARX-400 400 MHz spectrometer (Milton, ON, Canada).

2.3. Critical association concentration (CAC) of DEX-g-PEG-C_n polymeric micelles

The CAC of the DEX-*g*-PEG-C*n* copolymers were estimated by fluorescence spectroscopy using pyrene, a hydrophobic fluorescence probe that preferentially partitions into the hydrophobic core of the micelle. It undergoes changes in its photophysical properties as a result of the change in the micropolarity it experiences upon diffusion from bulk water (hydrophilic environment) into the micelle core (hydrophobic environment) [31,32]. Two methods

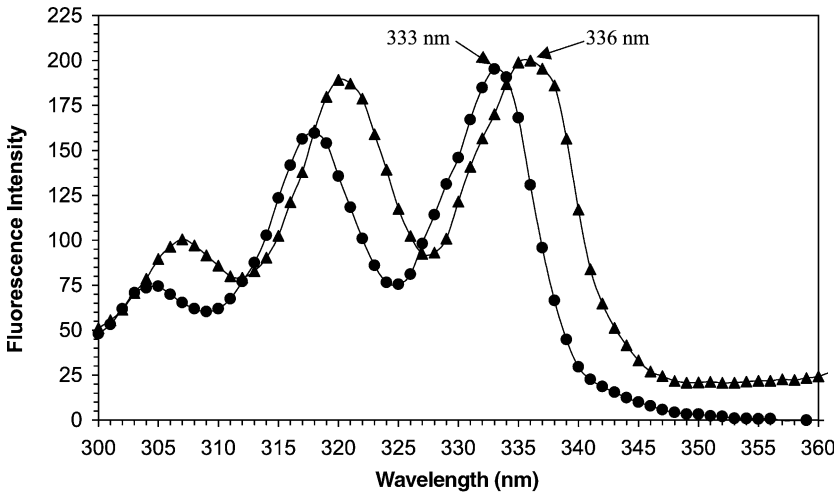

Fig. 3. Excitation spectra of pyrene (2×10^{-7} M aqueous solution) monitored at λ_{em} 390 nm in absence (●) or presence (▲) of PEG-C_{16} at a concentration of 5×10^3 mg/l.

exist for determining the CAC of polymeric micelles with pyrene fluorescence [33]. The original method, proposed by Kalyanasundaram et al. [34], takes advantage of the changes in the vibronic fine structure of the pyrene emission and monitors the changes in the ratio of the intensities I_1 and I_3 of the [0,0] and [0,2] bands, respectively. More recently, it has been suggested that a more accurate determination of the CAC can be obtained by monitoring the changes in the ratio of the pyrene excitation spectra intensities [35] at $\lambda =$ 333 nm for pyrene in water and $\lambda = 336$ nm for pyrene in an hydrophobic medium. The latter method was used here and is exemplified in Fig. 3 where we present excitation spectra of pyrene in water and in the presence of the non ionic surfactant PEG-C_{16} at a concentration higher than its CMC.

Samples for spectroscopic analysis were prepared as follows. Two-milliliter aqueous solutions of increasing polymer concentration ($0.04–5 \times 10^3$ mg/l) were equilibrated overnight with pyrene saturated water ([Py] $\sim$ $6–7 \times 10^{-7}$ M). Excitation spectra were monitored at $\lambda_{em} =$ 390 nm (excitation and emission bandpass: 2 nm). Fluorescence spectra were recorded at 25 °C on a series 2 Aminco Bowman spectrofluorimeter (Spectronic Instruments Inc., Rochester, NY).

2.4. Physical loading of CsA in DEX-g-PEG-C_n polymeric micelles

A dialysis method was employed to prepare CsA-loaded polymeric micelles. A DEX-*g*-PEG-C*n* solution (5 mg/ml) in deionized water (Milli-Q water purification system, Millipore, Billerica, MA) and a CsA solution (5 mg/ml) in ethanol were prepared separately. Each solution was stirred 1 h at room temperature. Subsequently, different mixtures of polymer with varying CsA concentrations (2.5–40% w/w) were prepared by mixing the two solutions to a final volume of 4 ml.

The different mixtures were stirred for 2 h at room temperature. To remove free CsA and form CsA-loaded micelles, the mixtures were transferred into a pre-swollen semi-permeable Spectra/Por 1 dialysis membrane (molecular weight cutoff 6000–8000 g/mol, Spectrum Laboratories Inc., Laguna Hills, CA) and were dialyzed against distilled water for 48 h. During the first 2 h, the water was exchanged two times (every hour) and then six times during the following 46 h. After a total of 48 h of dialysis, each solution was filtered through a 0.22-μm pore-size nylon filter (Whatman Inc., Clifton, NJ) and the filtrate was freeze-dried in the absence of any lyoprotectant.

2.5. Micelle size measurement

The hydrodynamic diameter of CsA-free as well as CsA-loaded DEX-*g*-PEG-C*n* polymeric micelles in aqueous solution was evaluated by dynamic laser light scattering (DLS) using a Malvern system (Autosizer 4700, Malvern Instruments Ltd, Malvern, UK) during 180 s at 25 °C, with a scattering angle of 90°. Samples were passed through 0.22-μm pore-size filter before size measurement to remove dust particles. It was verified that the filtration step did not significantly influence the mean micelle size. The correlation decay functions were analysed by the cumulant method to determine the *Z*-average size. The constrained regularized CONTIN method was used to obtain the particle size distributions. The values in Table 2 represent the average particle diameter together with the polydispersity index obtained for the same sample. All measurements were performed in triplicate; the data presented are the mean ± SD.

2.6. HPLC analysis

The micelle-incorporated CsA was extracted from freeze-dried micelles using ACN. The resulting suspensions

were sonicated for 10 min then agitated for 8 h. They were then filtered through 0.45-μm pore-size Gelman GHP Acrodisc filters (Waters, Milford, MA) and assayed byHPLC [36] using an Agilent Technnologies HP 1100 chromatography system with a quaternary pump, a UV-visible detector, a column thermostat and a HP Vectra computer (Agilent Technologies, Waldbronn, Germany) equipped with HP-Chemstation software. A symmetry octadecyl-silane C_{18} (5 μm, 250 × 4.6 mm i.d.) column and the corresponding guard column of similar characteristics (20 × 3.9 mm i.d.) (Waters) were used. The mobile phase consisted of ACN/water (80:20) with a flow rate of 1.2 ml/min. The column was thermostated at 70 °C. In all cases, the injection volume was 50 μl and the run time was 10 min. The CsA peak, monitored at 210 nm, appeared at a retention time of 6.5 min. A CsA calibration curve was prepared using standard solutions of concentrations ranging from 3.125 to 400 mg/l, with a first-order correlation coefficient (r^2) greater than 0.99. Finally, the extent of drug loading (DL) was calculated using Eq. (1):

$$DL(\%) = 100(W_C/W_M) \quad (1)$$

where W_C is the weight of CsA loaded in micelles calculated using the calibration curve, and W_M is the weight of CsA-loaded micelles before extraction with ACN.

2.7. Cell culture

The human colon adenocarcinoma cells, Caco-2, were grown as described previously [37]. Briefly, Caco-2 cells were routinely maintained in DMEM with 4.5 g/l D-glucose, supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) NEAA and 1% (v/v) penicillin–streptomycin antibiotics solution (100 U/ml penicillin G and 100 μg/ml streptomycin). Cells were allowed to grow in a monolayer culture in 75-cm^2 T-flasks in an incubator at 37 °C with controlled atmosphere containing 5% CO_2 and 90% relative humidity. Culture medium was changed every 48 h and cells were passaged at 80–90% confluency at a split ratio of 1:3 using 0.25% trypsin–1 mM EDTA.

2.8. Colorimetric MTT cytotoxicity assay

The cell viability in presence of dextran T10, PEG-C_{16} or DEX10-*g*-PEG-C_{16} (7 mol%) was evaluated using the MTT colorimetric assay. Caco-2 cells were seeded in triplicate in 96-well culture plates at a density of approximately 5×10^4 cells in 100 μl of cell culture medium per well. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air for 48 h. Subsequently, increasing concentrations of dextran T10, PEG-C_{16} or DEX10-*g*-PEG-C_{16} (0–10 g/l in culture medium) were added to the cells. Caco-2 cells were further incubated at 37 °C for 4 h. Thereafter, cell viability was determined by a MTT test according to the procedure described by Mosmann [38]. The test is based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. Ten microliters of 5 mg/ml MTT solution in phosphate-buffered saline (PBS; 75 mM NaCl, 53 mM Na_2HPO_4, 13 mM NaH_2PO_4, pH 7.2) were added to each well. After 4 h of additional incubation at 37 °C, 100 μl of 10% SDS in 0.01 N HCl solution were added to each well to stop the reaction and to ensure solubilization of formazan crystals. The plates were incubated overnight at 37 °C, after which the optical density values were measured at 570 nm using a multiwell-scanning spectrophotometer (PowerWave; Biotek Instruments, Winooski, VT).

3. Results and discussion

3.1. Characterization of the modified dextrans

Various modified dextrans were prepared. They differed in three molecular aspects: (i) the molecular weight of dextran (10 000 and 40 000 Da or ca. 62 and 247 glucose units per chain, respectively); (ii) the level of grafting, i.e. the number of hydrophobic substituents linked to the chain; and (iii) the size of the hydrophobic group (hexadecyl or octadecyl). The association properties, summarized in Table 1, were gathered from fluorescence probe experiments that yield the critical association concentration, and from DLS measurements that give the average size of the polymeric micelles (Table 2). The ^{1}H-NMR spectroscopy data, summarized in Table 1, showed that DEX-g-PEG-C*n* copolymers with different molar content in PEG-C*n* (2–7 mol%) were synthesized. The degree of substitution was calculated as $I_{Me} \times 100/I_a$, where I_{Me} is the average integral of the signal due to the terminal methyl protons of the PEG-C*n* groups (~0.85 ppm) and I_a is the integral of the signal due to the anomeric protons of dextran (~4.7 ppm).

3.1.1. Critical association concentration (CAC) of DEX-g-PEG-C_n micelles

A fluorescence assay described in detail in Section 2 was used to determine the polymer concentration at which micellization first takes place. The hydrophobic pyrene probe was added to polymer solutions of increasing concentration and pyrene excitation spectra were measured for all solutions. The excitation spectrum undergoes a small shift to longer wavelengths as the probe passes from a hydrophilic to a hydrophobic environment (Fig. 3). This shift is quantified in terms of the ratio, I_{336}/I_{333}, of the fluorescence intensities at 336 and 333 nm.

Plots of the I_{336}/I_{333} ratios versus the logarithm of the concentration of the aqueous solutions of DEX-*g*-PEG-C*n* of varying compositions are shown in Fig. 4, together with those of solutions of the surfactants PEG-C_{16} and PEG-C_{18}. Sigmoidal curves were obtained for all the DEX-*g*-PEG-C*n* copolymers. The CAC value was determined for each polymer solution from the intersection of two straight lines (the horizontal line with an almost constant value of the ratio I_{336}/I_{333} and the vertical line with a steady increase in

Table 1
Characteristics of DEX-*g*-PEG-C*n* copolymers with various compositions

Polymer	Grafted PEG-C*n*[a] (mol %)	Average number of PEG-C*n* units per dextran chain	Maximum CsA loading[b] (w/w%)	CAC	
				Polymer concentration[c] (mg/l)	PEG-C*n* concentration (nmol/l)
PEG-C_{16}	100	–	17.5 ± 0.5	2.5	3600
PEG-C_{18}	100	–	13.1 ± 0.9	3.0	4200
Dextran T10	0	0	0.6 ± 0.1	–	–
DEX10-*g*-PEG-C_{16}	3.0	2	4.0 ± 0.1	7.5	23
DEX10-*g*-PEG-C_{16}	7.0	4	4.8 ± 0.4	6.5	49
DEX10-*g*-PEG-C_{18}	3.9	2	3.0 ± 0.2	12.5	50
Dextran T40	0	0	1.0 ± 0.02	–	–
DEX40-*g*-PEG-C_{16}	2.3	6	1.2 ± 0.1	110.0	65
DEX40-*g*- PEG-C_{16}	3.5	9	1.5 ± 0.1	18.0	16

[a] Determined by ^{1}H NMR measurement in DMSO-d_6 [30].
[b] Determined by HPLC analysis with UV detection at 210 nm.
[c] Determined by change in $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence with log polymer concentration at 25 °C.

the ratio value). The estimated CAC values are presented in Table 1. The CAC values decrease with increasing molar content of PEG-C*n* residues linked to the polymer backbone, for each hydrophobic group and each dextran molecular weight. The CAC values of DEX40-*g*-PEG-C*n* are larger than those of the corresponding DEX10-*g*-PEG-C*n* samples, when determined in terms of weight of polymer. It is more insightful to compare the CAC in terms of the alkyl group concentration (Fig. 4B and Table 1). These results are consistent with recent evidence suggesting that the increase in the length of a hydrophobic residue at a given length of a hydrophilic polymer chain causes noticeable decrease in CAC value and increase in micelle stability [39].

3.1.2. Size of the DEX-g-PEG-C_n micelles

The hydrodynamic diameters of polymeric micelles, determined by DLS, ranged from 10 to 30 nm (Table 2). In all cases the size distributions were unimodal, indicative of the absence of free polymer chains. We note that the micelles formed by DEX40-*g*-PEG-C*n* are larger than those formed by DEX10-*g*-PEG-C*n*, independently of the size of the hydrophobic substituent and of the level of modification. This observation can be taken as an indication of the steric hindrances induced by carbohydrate chains, which are expected to take place over a larger volume for the polymer of higher molecular weight [25,40]. It has been shown that the uptake of particles within the intestine and the extent of drug absorption increase with decreasing particle size and increasing specific surface area [41]. Thus, the small size exhibited by all the polymeric micelles studied here show a favorable trend towards oral drug delivery.

3.2. Characterization of the CsA-loaded polymeric micelles

Cyclosporin A was incorporated into the polymeric micelles by a dialysis method which involved treatment of an aqueous polymer solution with a solution of CsA in ethanol, followed by extensive dialysis of the mixed solution against water. The amount of CsA released upon dissolution of the CsA-loaded polymeric micelles was then determined by an HPLC assay and plotted against the CsA concentration added initially for each preparation. Both free and micelle-entrapped CsA were measured with this assay. The amount of free CsA is expected to be low since the undissolved fraction of the drug was removed by filtration after the dialysis procedure. We evaluated first the ability of the surfactant micelles to solubilize CsA (Fig. 5). Both PEG-C_{16} and PEG-C_{18} micelles were able to incorporate

Table 2
Size measurements of CsA-free and CsA-loaded polymeric micelles with different compositions, determined by DLS measurements of 5 mg/ml aqueous solution at 25 °C with a scattering angle of 90°

Polymer	Grafted PEG-C*n* (mol %)	CsA-free polymeric micelles		CsA-loaded polymeric micelles	
		Mean diameter ± SD (nm)	Polydispersity ± SD	Mean diameter ± SD (nm)	Polydispersity ± SD
DEX10-*g*-PEG-C_{16}	3.0	18 ± 2	0.3 ± 0.08	20 ± 1	0.4 ± 0.01
DEX10-*g*-PEG-C_{16}	7.0	9 ± 0.3	0.2 ± 0.07	10 ± 0.3	0.3 ± 0.02
DEX10-*g*-PEG-C_{18}	3.9	21 ± 1	0.4 ± 0.04	22 ± 0.5	0.4 ± 0.03
DEX40-*g*-PEG-C_{16}	2.3	23 ± 1	0.3 ± 0.02	25 ± 1	0.5 ± 0.04
DEX40-*g*-PEG-C_{16}	3.5	30 ± 1	0.5 ± 0.03	35 ± 0.5	0.4 ± 0.03

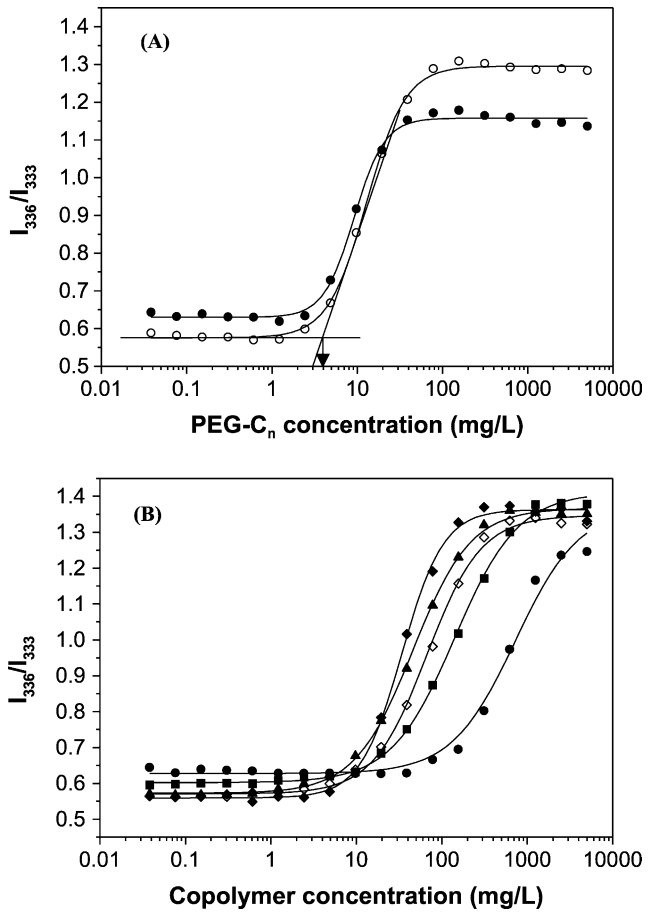

Fig. 4. Change in the $I_{336\ nm}/I_{333\ nm}$ ratio of pyrene fluorescence intensity with the different concentrations ($0.04–5 \times 10^3$ mg/l) of (A) (●) PEG-C_{16} and (○) PEG-C_{18}; and (B) (◆) DEX10-*g*-PEG-C_{16} (3 mol%); (▲) DEX10-*g*-PEG-C_{16} (7 mol%); (●) DEX40-*g*-PEG-C_{16} (2.3 mol%); (■) DEX40-*g*-PEG-C_{16} (3.5 mol%); and (◇) DEX10-*g*-PEG-C_{18} (3.9 mol%) copolymers. Each value is the mean of two independent measurements.

relatively high levels of CsA, 17.5% (w/w) and 13% (w/w), respectively.

Next, we assessed the incorporation of CsA within polymeric micelles. In the case of polymers prepared with

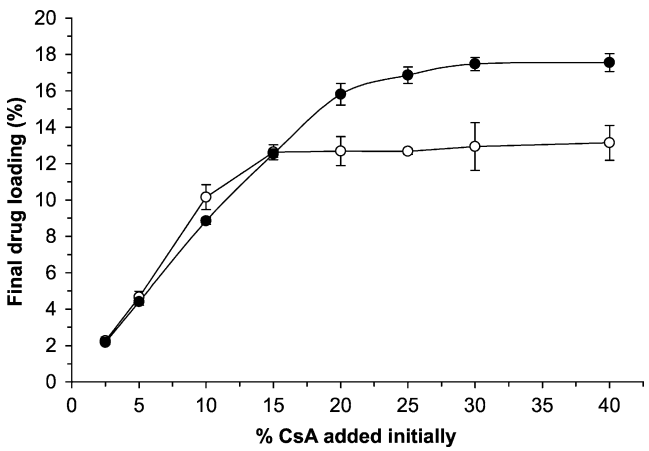

Fig. 5. CsA loading (w/w%) in micelles of PEG-C_{16} (●) and PEG-C_{18} (○) at 2.5–40 (w/w%) of initially added CsA. Mean ± SD ($n = 3$).

DEX10, the maximum amount of CsA loaded within the micelles was 4 and 4.8% (w/w), for DEX10-*g*-PEG-C_{16} (3 mol%) and DEX10-*g*-PEG-C_{16} (7 mol%), respectively, while DEX10-*g*-PEG-C_{18} (3.9 mol%) resulted in a maximum CsA loading of 3% (Fig. 6A). The micelles formed by the polymers of higher molecular weights were not as effective CsA carriers (Fig. 6B). Nonetheless, in all cases the amount of incorporated CsA was larger in the case of polymeric micelles than in the case of unmodified dextrans, which have a very low affinity for CsA (Fig. 6). The entrapment efficiency of polymeric micelles remained low (<35%) and further work will be aimed at improving the drug loading procedure.

Several trends are apparent if one compares the highest CsA loading percentages determined for the various polymers (Table 1). The percentage of CsA loading increases with increasing number of PEG-C*n* units grafted per dextran chain. For a constant number of PEG-C*n* units (~2 units/dextran chain), the CsA loading achieved with DEX10-*g*-PEG-C_{18} (3.9 mol%) is lower than that determined for DEX10-*g*-PEG-C_{16} (3 mol%). These results are consistent with the fact that PEG-C_{16} micelles are more effective in solubilizing CsA than PEG-C_{18} micelles, 17.5 and 13%, respectively (Fig. 5). They may be taken as an indication that the micropolarity of the hexadecyl-PEG chains core might present a better solubilizing core than that offered by the octadecyl-PEG moieties.

DLS measurements carried out on CsA-loaded micelles confirmed that the integrity of the micelles was preserved during the loading process and that the size of the micelles was not altered substantially, although a slight increase in micelle diameter was noted in all cases (Table 2).

3.3. In vitro cytotoxicity study

The cytotoxicity of DEX10-*g*-PEG-C_{16} (7 mol%), DEX10, and PEG-C_{16} was examined using a MTT assay performed with Caco-2 cells. The dose-dependent viability of Caco-2 cells treated with the various materials for 4 h is presented in Fig. 7. As anticipated, free PEG-C_{16} surfactant inhibited cell growth even at concentrations below 1 g/l. Such deleterious effect is consistent with the propensity of polyethyleneglycol alkyl ether surfactants to affect membrane integrity [29]. Also, the polysaccharide DEX10 showed no toxicity up to a concentration of 10 g/l, confirming the reported inertness of dextran. Turning now to the effect of DEX10-*g*-PEG-C_{16} (7 mol%), we determined that it exhibited no significant toxicity towards Caco-2 cells, up to concentrations of 10 g/l or concentration with PEG-C_{16} content (0.05 g/l) equivalent to that showing cytotoxic effect (80% cell viability) when present free in direct contact with the cell. This important result indicates that by grafting the PEG-C*n* residues on dextran, we succeeded in minimizing their toxic effect on cells. The non-toxic hydrophilic dextran chains forming the polymeric micelle outer shell may be in contact with the cells and

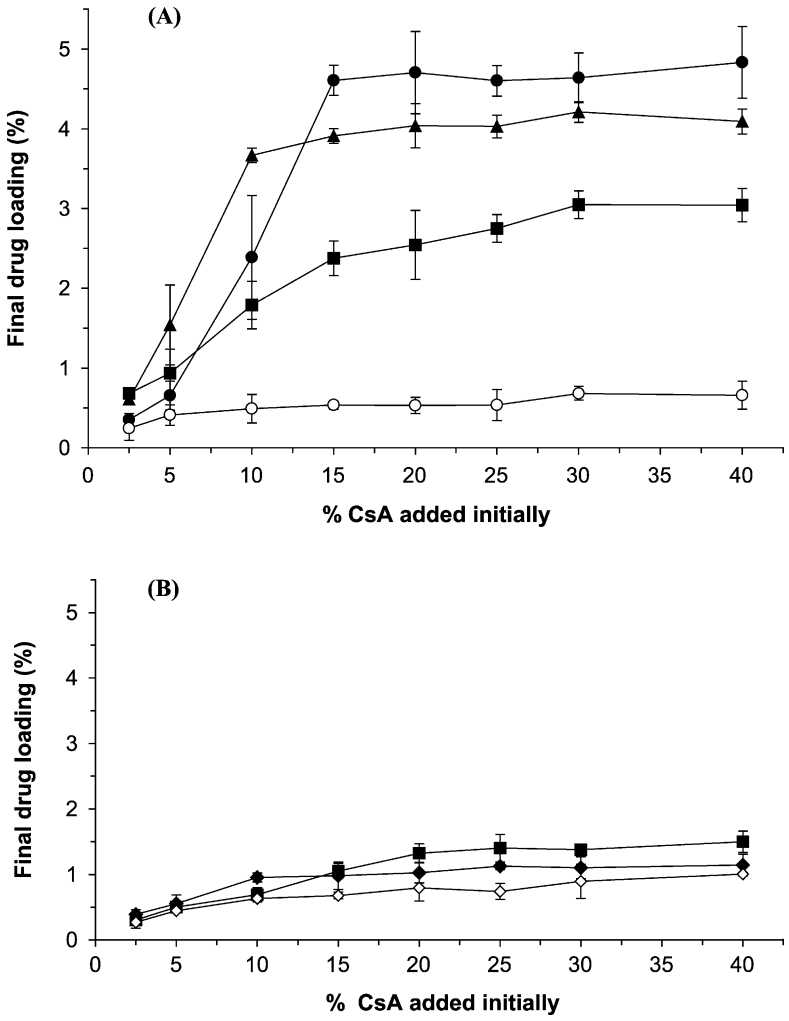

Fig. 6. CsA loading (w/w%) in micelles of (A) (▲) DEX10-*g*-PEG-C_{16} (3 mol%); (●) DEX10-*g*-PEG-C_{16} (7 mol%) and (■) DEX10-*g*-PEG-C_{18} (3.9 mol%) copolymers, or micelles of (B) (◆) DEX40-*g*-PEG-C_{16} (2.3 mol%) and (■) DEX40-*g*-PEG-C_{16} (3.5 mol%) copolymers at 2.5–40 (w/w%) of initially added CsA. For comparison, CsA was incorporated in (A) unmodified dextran T10 (○) or (B) unmodified dextran T40 (◇) polymer. Mean ± SD ($n = 3$).

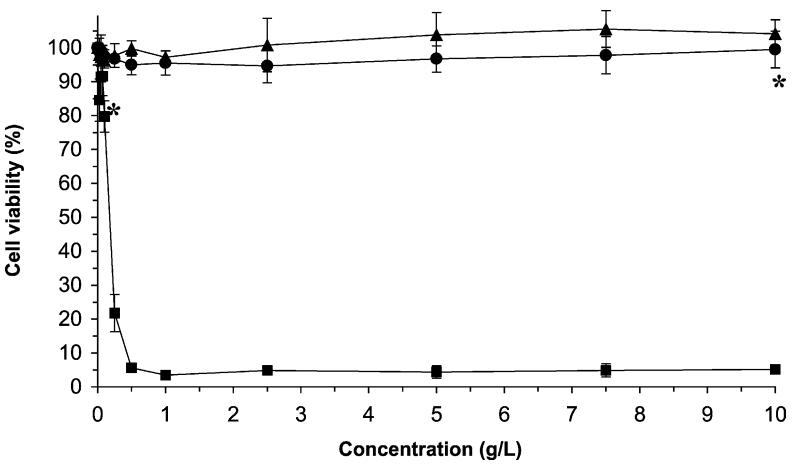

Fig. 7. Effect of unmodified dextran T10 (▲), PEG-C_{16} (■) and DEX10-*g*-PEG-C_{16} (7 mol%) (●) concentration (0–10 g/l) on Caco-2 cell viability measured by MTT assay following 4 h incubation at 37 °C/5% CO_2. Equivalent concentrations of free PEG-C_{16} and PEG-C_{16} grafted to dextran T10 backbone are indicated in the figure by stars. Mean ± SD ($n = 3$).

effectively insulate them from the surfactant residues assembled in the inner core.

4. Conclusion

In the present study, the solubilization potential of different DEX-*g*-PEG-C*n* copolymers to poorly-water soluble drugs has been studied. We showed that the ability of hydrophilic, non-toxic dextran polymers to encapsulate lipophilic drugs, such as cyclosporin A, can be increased by grafting hydrophobic PEG-C*n* domains on the dextran main chain. In aqueous solution, polymeric micelles are formed with low CAC values and relatively small micelle mean diameter. On the cellular level, they presented no significant cytotoxicity. Therefore, this new macromolecular system exhibits promising characteristics for the development of a novel polymeric drug carrier for the oral delivery of poorly water-soluble drugs.

Acknowledgements

This work was financially supported by the Natural Sciences and Engineering Research Council of Canada under its strategic grants program. M.F.F. acknowledges a scholarship from the Rx&D Health Research Foundation. We would like to thank Dr. Sébastien Gouin for his help in development of earlier versions of copolymer synthesis procedure.

References

[1] H. Chen, R. Langer, Oral particulate delivery: status and future trends, Adv. Drug Deliv. Rev. 34 (1998) 339–350.
[2] A.T. Florence, P.U. Jani, Particulate delivery: the challenge of the oral route, in: A. Rolland (Ed.), Pharmaceutical Particulate Carriers: Therapeutic Applications, Marcel Dekker, New York, 1993, pp. 65–107.
[3] T. Gershanik, S. Benita, Self-dispersing lipid formulations for improving oral absorption of lipophilic drugs, Eur. J. Pharm. Biopharm. 50 (2000) 179–188.
[4] D. Horter, J.B. Dressman, Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract, Adv. Drug Deliv. Rev. 46 (2001) 75–87.
[5] W.N. Charman, V.J. Stella, Transport of lipophilic molecules by the intestinal lymphatic system, Adv. Drug Deliv. Rev. 7 (1991) 1–14.
[6] T.S. Wiedmann, L. Kamel, Examination of the solubilization of drugs by bile salt micelles, J. Pharm. Sci. 91 (2002) 1743–1764.
[7] D.D. Lasic, Mixed micelles in drug delivery, Nature 355 (1992) 279–280.
[8] G.S. Kwon, T. Okano, Polymeric micelles as new drug carriers, Adv. Drug Deliv. Rev. 21 (1996) 107–116.
[9] 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.
[10] A. Laupacis, P. Keown, R. Ulan, N. McKenzie, C. Stiller, Cyclosporin A: A powerful immunosuppressant, Can. Med. Assoc. J. 126 (1982) 1041–1046.
[11] R. Wenger, Synthesis of cyclosporin and analogues: structure, activity, relationships of new cyclosporin derivatives, Transplant. Proc. 15 (1983) 2230–2241.
[12] W. Vine, L. Bowers, Cyclosporine: structure, pharmacokinetics, and therapeutic drug monitoring, Crit. Rev. Clin. Lab. Sci. 25 (1987) 275–311.
[13] J.F. Tjia, I.R. Webber, D.J. Back, Cyclosporin metabolism by the gastrointestinal mucosa, Br. J. Clin. Pharmacol. 31 (1991) 344–346.
[14] A. Lindholm, S. Henricsson, M. Lind, R. Dahlqvist, Intraindividual variability in the relative systemic availability of cyclosporin after oral dosing, Eur. J. Clin. Pharmacol. 34 (1988) 461–464.
[15] K. Takada, N. Shibata, H. Yoshimura, Y. Masuda, H. Yoshikawa, S. Muranishi, T. Oka, Promotion of the selective lymphatic delivery of cyclosporin A by lipid-surfactant mixed micelles, J. Pharmacobio-Dyn. 8 (1985) 320–323.
[16] M.H. El-Shabouri, Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A, Int. J. Pharm. 249 (2002) 101–108.
[17] M. Al-Meshal, S.H. Khidr, M.A. Bayomi, A.A. Al-Angary, Oral administration of liposomes containing cyclosporine: a pharmacokinetic study, Int. J. Pharm. 168 (1998) 163–168.
[18] C.T. Ueda, M. Lemaire, G. Gsell, K. Nussbaumer, Intestinal lymphatic absorption of cyclosporin following oral administration in an olive oil solution in rats, Biopharm. Drug Dispos. 4 (1983) 113–124.
[19] T. Chang, L.Z. Benet, M.F. Hebert, The effect of water-soluble vitamin E on cyclosporine pharmacokinetics in healthy volunteers, Clin. Pharmacol. Ther. 59 (1996) 297–303.
[20] S.J. Kim, H.K. Choi, S.P. Suh, Y.B. Lee, Pharmacokinetic and pharmacodynamic evaluation of cyclosporin A O/W-emulsion and microsphere formulations in rabbits, Eur. J. Pharm. Sci. 15 (2002) 497–502.
[21] W.A. Ritschel, S. Adolph, G.B. Ritschel, T. Schroeder, Improvement of peroral absorption of CyA by microemulsions, Methods Find. Exp. Clin. Pharmacol. 12 (1990) 127–134.
[22] C.P. Clagett, F.A.J. Anderson, W. Geerts, J.A. Heit, M. Knudson, J.R. Lieberman, G.J. Merli, H.B. Wheeler, Prevention of venous thromboembolism, Chest 114 (1998) 531S–560S.
[23] N.P. Couch, The clinical status of low molecular weight dextran: a critical review, Clin. Pharmacol. Ther. 6 (1965) 656–665.
[24] K. Moriyama, N. Yui, Regulated insulin release from biodegradable dextran hydrogels containing poly(ethylene glycol), J. Controlled Release 42 (1996) 237–248.
[25] A.V. Maksimenko, Y.V. Schechilina, E.G. Tischenko, Resistance of dextran-modified hyaluronidase to inhibition by heparin, Biochemistry (Mosc) 66 (2001) 456–463.
[26] B. Sayer, J. Lu, C. Green, J.D. Soderholm, M. Akhtar, D.M. McKay, Dextran sodium sulphate-induced colitis perturbs muscarinic cholinergic control of colonic epithelial ion transport, Br. J. Pharmacol. 135 (2002) 1794–1800.
[27] K. Sakai, T.M. Kutsuma, T. Nishino, Y. Fujihara, N. Yata, Contribution of calcium ion sequestration by polyoxyethylated nonionic surfactants to the enhanced colonic absorption of *p*-aminobenzoic acid, J. Pharm. Sci. 75 (1986) 387–390.
[28] I.A. Siegel, H.P. Gordon, Effects of surfactants on the permeability of canine oral mucosa in vitro, Toxicol. Lett. 26 (1985) 153–157.
[29] D. Dimitrijevic, A.J. Shaw, A.T. Florence, Effects of some non-ionic surfactants on transepithelial permeability in Caco-2 cells, J. Pharm. Pharmacol. 52 (2000) 157–162.
[30] M. Cristea, M. Francis, F.M. Winnik, Synthesis of hydrophobically-modified dextrans, Macromolecules (2003) submitted.
[31] D.C. Dong, M.A. Winnik, The Py scale of solvent polarities, Can. J. Chem. 62 (1984) 2560–2565.
[32] C.L. Zhao, M.A. Winnik, G. Riess, M.D. Croucher, Fluorescence probe techniques used to study micelle formation in water-soluble block copolymers, Langmuir 6 (1990) 514–516.

[33] F.M. Winnik, S.T.A. Regismond, Fluorescence methods in the study of polymer-surfactant systems, in: I.C.T. Kwak (Ed.), Polymer-Surfactant Systems, Marcel Dekker, New York, 1998, pp. 267–315.
[34] K. Kalyanasundaram, J.K. Thomas, Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar systems, J. Am. Chem. Soc. 99 (1977) 2039–2044.
[35] M. Wilhelm, C.L. Zhao, Y. Wang, R. Xu, M.A. Winnik, J.L. Mura, G. Riess, M.D. Croucher, Poly(styrene-ethylene oxide) block copolymer micelle formation in water: a fluorescence probe study, Macromolecules 24 (1991) 1033–1040.
[36] E. Ugazio, R. Cavalli, M.R. Gasco, Incorporation of cyclosporin A in solid lipid nanoparticles (SLN), Int. J. Pharm. 241 (2002) 341–344.
[37] I.J. Hidalgo, T.J. Raub, R.T. Borchardt, Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability, Gastroenterology 96 (1989) 736–749.
[38] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[39] R. Nagarajan, K. Ganesh, Block copolymer self-assembly in selective solvents: theory of solubilization in spherical micelles, Macromolecules 22 (1989) 4312–4325.
[40] A.L. Baldwin, S. Chien, Effect of dextran 40 on endothelial binding and vesicle loading of ferritin in rabbit aorta, Arteriosclerosis 8 (1988) 140–146.
[41] A.T. Florence, N. Hussain, Trancytosis of nanoparticle and dendrimer delivery systems: evolving vistas, Adv. Drug Deliv. Rev. 50 (2001) S69–S89.